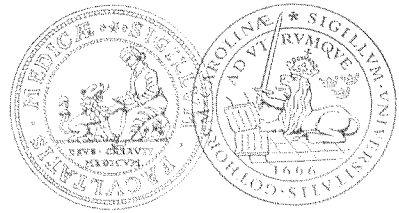
Retinal Transplants

Growth Differentiation Integration Organization and Survival

Rajesh Kumar Sharma



Retinal transplants

Growth, Differentiation, Integration, Organization and Survival

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1999

University of Lund, Sweden

ISBN 91-628-3430-4

This work was carried out at the Department of Ophthalmology, University Hospital of Lund, S-221 85, Sweden. Part of the work was also carried out at Schepens Eye Research Institute, Harvard Medical School, Boston, MA, USA

ISBN 91-628-3430-4

ISRN LUMEDW/MEVL—1020—SE

Published by Rajesh Kumar Sharma under the provisions of Medical Faculty, University of Lund, Sweden

Printed by Wallin & Dalholm Boktryckeri AB, Lund

Cover page designed by Sven Jakobsson and Rajesh Kumar Sharma

Book layout: Sven Jakobsson and Johan Wassélius

Cover page legend: The background of the cover page, taken from paper I, shows MIB-1 immunoreactivity in embryonic day 25 rabbit retina. The picture has been modified and composed by using Photoshop® and PageMaker® for aesthetic considerations. Although the cover page was not intended to convey any information, while processing the mirror images of a picture to cover the entire spread of the page, patterns at the junction of mirror images became obvious. This conveyed one of the most amazing qualities of human mind, ability to see patterns.

This quality is the hallmark of evolution of primate vision. It is suggested that there are three stages in the evolution of vision (Sir Stewart Duke-Elder in 'System of Ophthalmology'). Motor taxis, appeared in simplest unicellular organisms as an automatic response and reached its highs in homing birds. This form of vision need not enter consciousness. From this evolved the 'perceptual vision' a passive registration of the outside world serving the needs of hunger, fear and sex. This started in worms and upon reaching its pinnacle became a major determinant of conduct in man. This type of vision depended upon the organization of the nervous system to create symbol-association. Later, owing to the explosive development of the frontal brain in the highest primates during the arboreal adventures of the ape-man, developed the 'imaginative vision' with all its aesthetic and creative qualities. This type of vision was well established about 20,000 years ago, when following the melting of the ice modern man, moved northwards to replace the Neanderthals and established the first cave civilizations. It reaches its highest in the form of cognitive vision in humans whose minds are relieved of inhibitions with the help of chemicals known as hallucinogenic.

Pattern recognition has played a major role in many human endeavors. From being a mere means of capturing the outside word in portraits and landscapes, it developed into a way of 'synthesizing' images in the minds in later styles such as impressionism, expressionism, cubism, surrealism and symbolism. In the journey of art through its history pattern recognition, and its associations with the brain centers responsible for emotions, feelings and intellect, has played a pivotal role. Be it architecture or music, pattern recognition is at the core. Basically, science is no different; it is recognition of patterns under a microscope (or some other system), and building a story around it.

Indira Vani

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INTRODUCTION

Hereditary retinal degenerations

CLINICAL FEATURES

In hereditary retinal degenerations the photoreceptors progressively degenerate, which results in visual impairment. The disease affects one out of three to four thousand people.[10,51,63,159,176,185,329] Worldwide, about 1.5 million people are expected to be affected, which makes it one of the most common causes of blindness among adults in the Western world. Partly due to lack of understanding of the nature of the disease, various terminologies have been used in the past, including hereditary retinal degeneration, hereditary retinal dystrophy, tapetoretinal degenerations, and retinitis pigmentosa.✎

There is considerable heterogeneity in the clinical presentation of hereditary retinal degenerations, which has raised speculations that the disease may constitute a variety of diseases at the molecular level. This indeed is true. The earliest symptoms to appear in patients with retinitis pigmentosa are usually nyctalopia (night blindness), and difficulties with dark adaptation. The mid-peripheral field of the vision is affected first, and as the degeneration spreads from equatorial to central retina, insidiously and progressively the far peripheral and eventually the central fields are also lost. Central vision is affected late in the disease, which to some extent also depends upon the disease type. The central vision in RP may be affected for a variety of other reasons such as cystoid macular edema, diffuse retinal vascular leakage, wrinkling of the internal limiting membrane, preretinal fibrosis[164] and macular or foveal RPE defects.[122,123] Occasionally patients complain of flashes of light or photopsias.[168]

In the early stages, fundus examination may reveal no abnormality. In later stages, retinal vessels become attenuated, and in advanced cases blood vessels may become invisible in the peripheral retina. Intraretinal pigmentation first appears in the equatorial retina and later in both more central and peripheral parts. The pigmentations, which arise from retinal pigment epithelium, often look like bone corpuscles. At times, they appear along the vessels. In some patients, pigmentation may be

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✎There have been vast advances in the understanding of the disease since Donders coined the term retinitis pigmentosa in 1855. The term is not quite appropriate, because there is no true inflammation involved in it, as suggested by the term "retinitis". Therefore, the disease has also been called tapetoretinal degeneration, but humans do not have any true tapetum. In this presentation, the terms retinitis pigmentosa and hereditary retinal degeneration will therefore be used, and the terms are defined as descriptors for a set of congenital progressive degenerative disorders which primarily, but not exclusively, affect the photoreceptors or the retinal pigment epithelium.

present only in one sector of the fundus and in others in central rather than peripheral retina.

Macular changes are commonly seen,[122] often in form of increased luster or wrinkling, suggesting edema.[120] As the disease advances, the macula appears drier and smoother. The prevalence and type of macular involvement in different inheritance types of RP is variable, and this variability can be seen even within single families.[122] The optic disc develops a characteristic waxy pale appearance, as a result of consecutive optic atrophy and gliosis.[164] Vitreous abnormalities may appear.[101,268,297] In due time, a characteristic posterior sub-capsular cataract appears in most cases.✎

Electroretinographic changes are pronounced already in early stages of the disease, well before fundus changes are apparent.[13-15,37,309] The amplitudes of 'a' and 'b' waves are reduced, usually by more than 90%[40,41] and there is also a delay in the b-wave implicit time.[37-41] This can very effectively be used to predict which of the relatives of a retinitis pigmentosa patient will develop the disease clinically. ERG analyses performed on siblings in families with dominant or recessive forms of retinitis pigmentosa correspond well with what would be expected by Mendelian laws.[36] In families with retinitis pigmentosa, a patient older than 5 years and with a normal ERG is not likely to develop the disease.[36,38]

Etiology and pathogenesis

Hereditary retinal degeneration results from mutations in certain genes. In recent years many genes and numerous chromosome locations that contain retinitis pigmentosa causing mutated genes have been found. This confirms the speculation that retinitis pigmentosa is a group of disorders at the molecular level. Some of the genes have been identified, and the list is continuously growing.[365] ✎✎

Retinal degeneration is not a direct result of these mutations.[177] The death of cones resulting from mutations in the rod photopigment, and the death of photoreceptors in RCS rats where the primary defect lies in pigment epithelium[279] are some examples that illustrate the point. In experiments with chimeric mice where embryos from normal and transgenic mice (with a mutant rhodopsin gene) were aggregated together, one could expect patchy degeneration, and only in photoreceptors having

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✎Other ocular abnormalities that have been reported to appear with a higher frequency in RP are keratoconus and glaucoma; only half of these being open angle glaucoma. One form of sector RP is associated with hypermetropia and closed-angle glaucoma.[295] In many forms of RP, especially X-linked retinitis pigmentosa (XLRP), patients have high myopia and astigmatism.[43] Hypermetropia is also associated with a type of Leber's congenital amaurosis[86,128] and preserved para-arteriolar pigmented epithelium-RP.[167,310]

✎✎Updated lists of the genes and/or chromosomal locations involved in retinal degenerations can be accessed on the Internet (http://utsph.sph.uth.tmc.edu/www/utsph/RetNet/genes.htm and http://www3.ncbi. nlm.nih.gov/Omim/).

abnormal rhodopsin. However, the degeneration was uniform and independent of the genotype of the cells.[177] These results indicate that it is not the mutation *per se* but subsequent (mostly unidentified) cellular events triggered by the mutation that result in eventual photoreceptor cell degeneration. The details of the cellular interactions are not known, but they may involve mechanisms like light damage, oxidative stress, and excitotoxicity. However, it is clear that in various types of retinal degenerations ultimately the cells die by apoptosis.[71,311,400] In certain specific type of hereditary retinal degenerations such as the Refsum disease, gyrate atrophy of the choroid and retina, and abetalipoproteinemia, the biochemical defect is precisely known, and in others it is speculated. In most it remains a mystery.

In general, the mutations result in the formation of an abnormal protein, which causes a structural or functional instability. Sometimes different mutations can present with the same phenotype, a phenomenon called non-allelic or locus heterogeneity. This can be viewed as the limited response of the eye to a variety of genetic insults. In another pattern, called gene sharing, different mutations in the same gene may have different clinical manifestations. In yet other cases, the same mutation may present itself as clinically distinct diseases in different individuals suggesting a profound role of other factors (especially environmental) in the genesis of the phenotype. There are many possible mechanisms that can explain this. The expression of one gene can be influenced by another gene in what is called a compound heterozygous state, where a phenotypic presentation of a mutation on one gene is affected by a different mutation on the allelic gene.[280] Two different mutations may produce an apparently dominant disease with incomplete penetrance or a recessive disease.[200] In some cases inheritance of a mutation from father or mother may produce different diseases[251,284] or different phenotypic characteristics by the mechanism of genomic imprinting.[276,425] The disease may be more severe if inherited from the mother than from the father.[165,232] Moreover, in some inherited disorders like myotonic dystrophy the disease becomes more severe over generations because of a repeating nucleotide sequence becomes longer by repeated insertions.[178]

Finally, the disease is the result of a complex interplay between the genes and the environment. Environmental factors such as light may contribute to the photoreceptor damage in retinitis pigmentosa. Transgenic mice with P23H rhodopsin mutation have a slower rate of photoreceptor degeneration in darkness or at low light levels than in light.[281,421]

Possibilities of treating retinal degenerations

It is only in the recent years that a rational approach to treat hereditary retinal degenerations has been possible[360,363,365]. In those hereditary retinal degenerations where the biochemical defect is known, it is possible to influence the course of the disease by specific treatment aimed at correcting the abnormalities caused by the mutation. Examples include controlling serum phytanic acid levels in the Refsum disease by modifying the diet, controlling the serum ornithine levels in gyrate atrophy of the

choroid and retina, and supplementing fat-soluble vitamins in abetalipoproteinemia. However, the number of patients that suffer from these disorders is small, and thus only a small percentage of patients with retinal degenerations actually benefit from these treatments.

In other instances, research is focused on the possibility of evolving a non-specific "anti-degenerative" intervention in the hitherto largely unknown pathways that link mutations to the final degeneration of the photoreceptors. These investigations include the use of growth factors and attempts to control apoptosis.

ESTABLISHED METHODS

- Dietary control of phytanic acid helps patients with the Refsum disease.[38,322]
- Control of serum ornithine levels helps patients with gyrate atrophy of choroid and retina.[198,199]
- Co-enzyme Q 10 helps respiratory distress in patients with the Kearns-Sayre syndrome.[376]
- In some patients with retinitis pigmentosa the rate of progression of the disease is retarded by vitamin A therapy.[42]
- Sorsby fundus dystrophy patients benefit from Vitamin A treatment.[183]
- High doses of vitamin A and E help abetalipoproteinemia patients.[45,143,332]

EXPERIMENTAL APPROACHES

- Certain growth factors have been found to protect the retinal neurons from degeneration in animal models of retinal degeneration.[116,230,231]
- Antioxidants have been suggested to play a role in retinal degenerations, but the clinical trials to establish their role in treating retinal degeneration have been inconclusive.[42,169]
- It might be possible to treat retinal degenerations by manipulating the pathways in apoptosis.[72,184,254,331,378,396]
- It may be possible to introduce a healthy gene in to the genome to supplement the function of the defective one.[30,233,238,398]
- It might be possible to control the expression of the defective gene and prevent photoreceptor degeneration.[124]
- Retinal pigment epithelium transplants have been shown to rescue photoreceptors from degenerating in animal models of retinal degeneration.[360,363]
- Neuroretinal transplantation

Rationale behind neuroretinal transplantation

The logic behind retinal cell transplantation is obvious. It attempts to reconstruct the degenerated photoreceptor layer with healthy photoreceptors. Furthermore, it is possible that the retinal grafts may act as a source of trophic factors that may rescue the photoreceptors from death. ✎ It is clear that the photoreceptor degeneration is

the final manifestation of a large number of genetic insults. If the treatment is to be aimed at correcting the basic cause of the disease, the mutation, as is hoped in most types of gene therapy, the treatment will have to be specific for each genotype of the disease. Also if the metabolic defect is to be aimed for therapy, each type of degeneration will have to have its own treatment. The way out of this problem could be to find a general 'anti-degenerative' treatment or to deal with the final manifestation of the disease processes; the retinal degeneration. Even though patient selection criteria will have to be defined, retinal transplantation will likely work for most types of retinal degenerations, independent of the mutation causing it.

Progress in retinal transplantation research

RETINAL PIGMENT EPITHELIUM TRANSPLANTS

Since 1983, when retinal pigment epithelium transplantation was first reported,[148] the technique has shown its potential as a possible therapeutic measure. Retinal pigment epithelium grows well in culture medium, forming sheets that can be transplanted, and recently patients with macular degenerations and subretinal membranes have received pigment epithelium transplants.[7]

Cultured pigmented epithelium maintains most properties of normal RPE

Human retinal pigment epithelium removed from the adult donor eye up to 12 to 24 hours after death can be maintained in tissue culture for months, where it forms monolayers of epitheloid cells.[52,104,126,149,175] These cells can be further subcultured, and thus a large number of cells can be obtained from one and the same donor. The cells show the normal apical-basal polarity and junctional complexes. In cultures, both human[52,175] and non-human[118] retinal pigment epithelium cells have been shown to maintain their ability to phagocytize photoreceptor outer segments. A lack of such activity is known to cause photoreceptor degeneration in Royal College of Surgeons (RCS) rat.[223]

Cultured cells are able to take up retinol and to synthesize retinyl esters since they have enzymes for esterifying retinol[125] and hydrolyzing retinyl esters.[49] These enzymes are necessary for the handling of vitamin A in the visual pigment cycle. The cells also synthesize cytoskeletal proteins in culture[160,161] as well as glucosaminoglycans.[104] However, the cultured cells quickly loose their vitamin A stores and subsequent subcultures synthesize more of oleate than palmitate esters.[125]

✎It is also possible that these trophic factors may help recover the reversible damage in the retina such as regeneration of the photoreceptor outer segments and may result in physically more widespread visual recovery than expected from the size of a neuroretinal transplantation.

The retinal pigment epithelial cells divide in the cultures which makes them susceptible to labeling with tritiated thymidine for identification purposes.[149,150] Excellent survival of the pigment epithelium cells in cultures suggests it may be possible to produce autologous donor tissues or donor tissue suitably modified by recombinant DNA technology.

Various techniques are available to transplant RPE

Various techniques have been used to transplant retinal pigment epithelium, that include 'open sky' procedure, as well as transvitreal and transchoroidal approaches.[149,235,243]

In 'open sky' methods, the anterior chamber is opened, and a retinal flap is created. Before the transplantation, the retinal pigment epithelium is scraped off with the aid of 0.5% trypsin. This is a vital step for the attachment of the transplanted cells on to the Bruch's membrane. The donor cells are then transplanted using a microsurgical cannula. It is often difficult to appose the retinal flap at the transplantation site, which is one of the difficulties with this technique.

In the transvitreal approach, a *pars plana* incision is made. This is a much less traumatic procedure than the open sky method because it maintains the hydrodynamics of the eye and allows for a much faster reattachment of the retina, an important aspect of retinal epithelium function. In the transchoroidal approach, a scleral incision is made between the superior vorticose veins. Through this incision a suspension of pigment epithelium cells is injected into the subretinal space.

In both the transvitreal and the transchoroidal approach, it is difficult to remove the host pigment epithelium by scraping it off, because the retinal detachment that has to be created tends to obstruct the view of the retinal pigment epithelium. Instead, the host retinal pigment epithelium is dislodged by a jet stream of fluid from a micropipette.[149,235]

Transplanted RPE cells survive

After transplantation, cultured retinal pigment epithelium cells undergo rapid organization in the host subretinal space. Autoradiographic studies of cells labeled with tritiated thymidine have shown that the transplanted human retinal pigment epithelium cells attach firmly to the Bruch's membrane of the owl monkey [149] within two hours after the transplantation. Within 6 to 24 hours these cells form epithelial-type monolayers and continue to divide for days. Transplanted cells regain their apical-basal polarity in relation to the Bruch's membrane. Whether these cells attach to this membrane with the right polarity, or the cell plasma membranes modify themselves is not known. There is evidence that the plasma membranes are plastic enough to be influenced by the microenvironment.[345]

Transplanted RPE rescues degenerating photoreceptors

Retinal pigment cell transplantation has been shown to rescue the degenerating photoreceptors in the RCS strain of rats.[235,244] RCS rats were first described in 1938 [53], and their photoreceptors undergo a rapid photoreceptor degeneration beginning 3 weeks after birth, and spreading centrifugally to the peripheral retina within 2 months.[96] The cells of the inner retina are relatively preserved.[371,372] One of the important functions of the retinal pigment epithelium is to phagocytize the ends of photoreceptor outer segments.

Many ocular anomalies have been reported in RCS rats,[66,115,170,171,222,428] but in terms of photoreceptor degeneration, the retinal pigment epithelium is the most important site affected by the mutation.[279,431] Because of the loss of phagocytosis, outer segment debris accumulates at the pigment epithelium cells[96,222] and eventually the photoreceptors disappear. Transplanted healthy retinal pigment epithelium restores its phagocytic function soon after transplantation, and rescues the photoreceptors. Since the rescuing effect is not limited to the transplantation site, it is possible that the effect may be mediated by some diffusible factor, possibly bFGF. Injections of bFGF have been shown to rescue the photoreceptors in RCS rat.[116]

Sham operations also rescue the photoreceptors,[382] possibly due to release of some factors during the surgery, but this effect lasts for a much shorter period as compared to rescuing effects of up to one year by RPE transplants.[152,225,236,237] Rescued cells maintain normal opsin and S-antigen gene expression, allowing normal transcription and translation in them, and rendering them capable of participating in the visual transduction cascade.[135] With immunohistochemistry it has been shown that the rescued photoreceptors contain membrane bound Na^+,K^+-ATPase and opsin at their normal sites.[237,375] These substances are essential for the normal function of the photoreceptors. Since the rescuing effect is not limited to the transplantation site it is possible that the transplanted cells or the shed outer segments migrate subretinally, or the effect is mediated by some diffusible factor. Retinal pigment epithelium transplantation also prevents neovascularization in the RCS rats.[346] Transplantation of retinal pigment epithelium in rat eyes also appears to delay age related changes in various retinal layers.[427]

Up until recently there was no human hereditary tapetoretinal degeneration that had been shown to reside primarily in the retinal pigment epithelium, but neither has it been excluded that some forms of retinitis pigmentosa, choroideremia, Leber's amaurosis, macular degenerations, dominant drusen, or gyrate atrophy could be the diseases of the retinal pigment epithelium. Most recently, defects in the pigment epithelium involving vitamin A associated proteins called cellular retinaldehyde-binding protein (CRALBP), and RPE 65 have been described.[157,256] The gene coding for bestrophin may also reside in the pigment epithelium, and mutations in it cause macular degeneration of the Best disease type.[299,304] (and Wadelius, personal comm. 1998) Any disease residing primarily in the retinal pigment epithelium may

be amenable to pigment epithelium transplantation therapy. However, besides the need for further improving the surgical technique, attention should also be paid to potential problems such as transplant induced proliferative vitreoretinopathy. It should be noted that proliferating retinal pigment epithelium is at least partly responsible for certain vitreoretinopathies,[250,333] especially when the neural retina is detached[11] as happens in ocular tumors[402,418] or following trauma.[221,253,401,419]

NEURORETINAL TRANSPLANTS

Surgical techniques

Retinas can be transplanted to various intraocular sites

Royo and Quay[330] used a corneal incision for transplanting the embryonic rat retina to the anterior chamber of adult rat, and a very similar procedure was later used by del Cerro.[129] ✎ The anterior chamber was entered with a microsurgical knife at the 12 o'clock position at the periphery of the cornea. About 2 µl of a suitable medium containing the retinal tissue was taken up in a fine pipette, connected to a 10 µl precision syringe. The tip was advanced about 2 mm into the anterior chamber and the contents were deposited. The corneal incision was self-sealing and did not require suturing. The procedure is safe and has minimal complications.

In 1986, Turner and Blair transplanted embryonic rat retina to the epiretinal vitreous space of adult rats,[408] and with some modifications, the same method was used to transplant embryonic mouse and human retinas into rats.[19,21,106] In this method, an incision was made through the sclera, choroid and retina and the donor tissue was slowly deposited. An uncontrolled deposition sometimes resulted in transplants floating in the vitreous rather than being attached to the retina, especially when the method was used in rabbits.

To overcome this problem in rabbits, a different procedure was developed. A thin, flexible polyethylene capillary containing the donor tissue is brought into the eye transvitreally with the help of a cannula, which is mounted on a specially designed instrument.[32] With this method, fragmented pieces of embryonic rabbit retina could

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✎History: The earliest attempt to use the anterior chamber of the eye as a transplantation site was in 1873 when Doodermaal placed cells from human labial mucosa and a variety of other tissues in the anterior chamber of rabbits.[412] Subsequently, this technique served as the method of choice for studying tissue growth in an isolated milieu until improved tissue culture methods were developed. A variety of tissues grew well *in oculo* because of the immune privilege of the eye. Retinas were transplanted to the anterior chamber only in 1959 when Royo and Quay transplanted the fetal retinas of the rats into the mothers' eyes.[330] Turner and Blair[408] did the first transplantations of retinal neurons to the posterior segment.

successfully be placed between the photoreceptors and the pigment epithelium in adult rabbit eyes. ✎ Using this method, large transplants could be obtained and the misplacement of the transplants into the vitreous became more infrequent. Transplants matured, differentiated and formed approximations of the retinal layering, but anomalies like rosettes were always seen.

During this procedure, the polyethylene capillary can pierce the Bruch's membrane occasionally and thus the transplant may end up in the choroid instead of in the subretinal space. In rabbits, the choroidal transplants also survive and develop [31]. This is not what one would expect, because the capillaries in the choroid are fenestrated, with almost free flow of large molecular substances between the blood and the choroid [46]. Therefore, the choroid is usually not considered to be protected by the blood-retinal barrier.[409] This was also shown to be the case immediately after the transplantation, but after a few days, transplants to the rabbit choroid develop a barrier similar to the blood-retina barrier.[31] This seems at least in part to explain their unexpectedly good survival.
In another method a whole sheet of outer retina is inserted through an incision at the corneoscleral junction.[380] Retina from 8 days old rats are flattened with the help of radial cuts and embedded in gelatin, photoreceptors facing downwards. The inner layers of the embedded retina are cut in 20-50 µm thick sections on a vibratome until the photoreceptors are reached. A 200-300 µm thick section with the photoreceptor layer is then taken and transplanted into the rat eye, using a 2.5 mm wide acrylic carrier to guide the tissue. The carrier is introduced into the eye through a transverse corneal incision and advanced through the pupil that has previously been dilated with topical atropine. On reaching the *ora serrata*, the carrier is guided to the subretinal space, detaching the retina there. The carrier is then removed, leaving the gelatin containing the transplant in place. Silverman and Hughes[380] report consistently good positioning and survival with this technique. A further refinement of the technique has been described in which a photoreceptor sheet embedded in gelatin can be scrolled and inserted into the subretinal space with a thin cannula.[384] With a microsurgical manipulation the sheet can then be unscrolled and adjusted.✎✎

✎For convenience, these transplants are called subretinal, even though the space they occupy is actually intraretinal from an ontogenetic point of view.

✎✎Extraocular transplantation sites: To study the development, retinas or entire eyes have been implanted at various other sites in the body. Survival of peripheral retina in the subcutaneous space of guinea pigs was studied by Wyburn and Bacsich in 1952[426]. Grafts survived in the absence of direct blood supply. The pathogenesis of anophthalmia has been studied by obtaining the optic cup from E10 fetuses of a ZRDCT/an mouse strain that develops anophthalmia, and transplanting it into the subcutaneous space of normal mice fetuses.[339] The study showed an improved development of the optic cup when removed from the mutant environment. Research involving transplantation of the retina to various locations in the central nervous system[130,248,255,261-263,265] has given invaluable knowledge relevant to retinal transplantation. It has shown not only that retinas can continue to grow in CNS but also that they are also able to form functioning connections.[80,212]

Donor retinas can be transplanted in various physical forms

In retinal degenerations, photoreceptors are mainly involved. It is therefore not always necessary to aim for replacing all retinal layers. The concept of specific cell transplantation has come a long way in other forms of neural transplantation. Replacement of dopaminergic cells for Parkinson's disease is now under clinical trial.[240] It appears reasonable to think that under certain circumstances transplantation of a specific cell population or a retinal layer may be a better approach than transplanting the whole retina. Moreover, the physical form of donor tissue may have profound effects on the eventual organization[195] and cellular connections in the transplant.

In the past, many types of retina to retina transplantations have been performed and studied both in rats and rabbits[32,88,352,408]. It has been shown that tissue fragments,[408] cell suspensions,[93,195] and enzymatically isolated photoreceptors,[144,145] and whole sheets of photoreceptors[380] can survive transplantation into adult rats.

Transplanting fragments of donor retina is a technically simple procedure. Large pieces of the embryonic retina get fragmented when they are taken up into the capillary for transplantation. Long term results in terms of overall morphology have been good with fragments of embryonic retina transplanted to rats or rabbits.[18,19,32] It is an excellent method for studying the development of transplants.

Transplantation of dissociated cells would allow a better integration of the transplanted cells with the host retina. Such a strategy has earlier been tried in neural transplantations.[47] Based on the same procedure, del Cerro's group[87,93] dissociated retinal cells from post-natal 0-2 day pups of Lewis and Fisher 344 rat strains by treating them with 0.1% trypsin for 15 minutes. The tissue was then turned into a single cell suspension by pulling it through a fine pipette This suspension was transplanted, and transplants were reported to survive, to integrate with the host, and to display many features similar to results seen with tissue fragment transplants.

Enrichment of the cell suspension by cell sorting with a flow cytometer[292] is an interesting technique and has been used successfully in brain cell transplantation.[245] Work prefatory to retinal cell transplantation has been published,[292] but no actual transplantation results.

The organization of the cells in different types of retinal transplant has been found to vary significantly. Juliusson et al.[195] transplanted E17 to E19 Sprague Dawley rat retina in suspended and fragmented forms and studied the cellular organization 28 days later by immunohistochemistry. Rhodopsin staining revealed photoreceptors arranged in rosettes in tissue fragment transplants, whereas in cell suspension transplants, photoreceptors were heterogeneously distributed. In tissue fragment trans-

plants, the photoreceptors had well developed inner and outer segments, pointing to the center of the rosettes, whereas only rudimentary inner and outer segments were seen in the suspension transplants. Müller cells (demonstrated with vimentin staining) were nicely arranged in rosettes in the tissue fragment transplants, whereas they were randomly oriented in the cell suspension transplants. In other studies of rat retinal cell transplantation, the same type of Müller cell organization has been reported, using GFAP (glial fibrillary acidic protein) as and S-100 staining as a marker for the Müller cells.[353] The amacrine cell staining was very similar in both types of transplants. The results show that cell suspension transplants and tissue fragment transplants give morphologically different results. Similarly, suspensions of photoreceptor cells were found to form clusters in the region of the outer nuclear layer when transplanted to the adult RCS rats.[144] These studies together show that fragment transplants develop some degree of organization (into rosettes) whereas cell suspensions show much less organization of the cells, and photoreceptor cell outer segments tend not to develop when the organization gets too disturbed.

Survival

When grafted into eyes, immature retinas survive and continue to grow. Several studies have been conducted to find optimum conditions for the growth of these transplants. However, many important questions regarding the survival of the transplanted cells are still unanswered. Long-term survival was one of these questions that the following study attempts to answer (Paper VI). Parameters used to asses the survival of the cells in most studies are inexact, and applying more precise quantitative methods have met with difficulties such as the need for a more reliable and persistent marker for donor cells than what is currently available. There have been no studies directly counting the percentage of surviving cells of different types, either in standard procedures in different laboratories or as a systemic study on the efficacy of the surgical procedure. Such studies are now needed. In transplants of dopaminergic brain cells, the survival rate has been estimated to be in the order of one cell in a thousand.[61] Various factors influencing the survival of the retinal transplants are further discussed below.

Donor age influences the survival of the transplant

Evidence from neural transplantation suggests that young fetal donor material does better than more mature tissue. In brain transplantation, donor tissue must ideally be collected before neuron precursor cells have undergone their terminal mitosis (rat[58]; human[59]). Human grafts taken from substantia nigra at 9 weeks of gestation contain dopaminergic cells that survive transplantation in immunosuppressed rats, whereas rat tissue grafted with human nigral tissue from 11 to 19 weeks old embryos contain few if any dopaminergic cells.[59] However, in the central nervous system, it has been difficult to establish unequivocally the most suitable transplant ages due to variations in the method of collecting and handling the material. In case of retinal transplants, the methods of handling the tissue are enough standardized to

allow a comparison.

Previous studies suggest that there is a wide range of donor ages at which the fetal retina can be used as a viable tissue for transplantation. Aramant et al.[20] found that rat retina can be successfully transplanted within a large time span extending up to two weeks into postnatal life. Embryonic day 15 retinas are useful because of their consistent lamination and comparatively good integration with the host. Retinas taken up to two days after birth may also be successfully transplanted, but in terms of organization and survival, the success rate starts to fall when using tissue from between postnatal day 2 to day 4. There is a loss of neuroepithelial layers at this stage in the developing rat retina[55] that may be responsible for the drop in the survival rate. Grafts with postnatal day 21 tissue degenerated within 1-2 days after transplantation.[20] The mechanism behind the differences in survival rates as the tissue matures is not clear. It remains to be seen whether it is due to an increased immunological activity of the astrocytes in a more mature retina[121] or some other factor.

Transplants of fetal E60 and E90 monkey retinas have shown good survival, growth and differentiation in rat eyes.[91] In these experiments, the host rats were immunosuppressed.

Homografts need no immunosuppression but xenografts do

Before retinal transplantation becomes a possibility in humans it is essential that the human retinal tissue be studied as a donor material under the conditions of the transplantation. Permeability barriers around the central nervous system diminish the ability of the host immune system to recognize foreign tissues and cells within the eye. It is known for a long time that many tissues that will not survive transplantation in other parts of the body will readily grow in the eye. ✎

Embryonic mouse retinas have been transplanted to the adult rat retina,[21] sheets of adult human photoreceptor cells have been transplanted into normal and light damaged rats,[381] and fetal monkey retina has been transplanted into adult rats[91] in various studies. In order to study the ability of the retina to interact with the host, mouse retina has been transplanted into the CNS of newborn rats whose immune system is not yet fully developed.[212,249,308,348] Studies on the development of human retinal cell transplants and their connections have been performed on human embryonic or fetal tissue obtained from elective abortions at the post conceptional age of 3-12 weeks and xenotransplanted to immunosuppressed rats.[106] Xenografting can serve as a model for studying the transplantation of the human retina for clinical purposes.[19,106] The studies show that human embryonic and fetal retinas can be used as

✎As early as 1914, it was found that mucous carcinoma grafts survived in brain but not in subcutaneous tissue [102], and many subsequent studies on the development of human embryonic brain tissue have been conducted in different xenograft models.[60,73,154,203,287,321]

donor material.

Cryopreserved retinal can be transplanted

Foreseeing the future needs of transplantation surgery, attention has been paid to the effects of cryopreservation on the survival of retinal transplants. A retina bank of frozen tissue would free the research from dependence on availability of fresh donor tissue. Aramant *et al.*[16] studied the success rate of cryopreserved and fresh donor tissue transplanted to rat retinas. Rat embryonic retinal tissue was cryopreserved in liquid nitrogen up to eight months in a medium containing 10% dimethyl sulfoxide. Transplants with this donor material was compared with age matched fresh tissue. The comparison criteria were modified from the protocol described by Blair and Turner[48] and Aramant *et al.*,[20] and included volume, absence of degenerating cells, degree of development of retinal layers, and degree of integration of the transplant with the host. Transplants survived in all age groups and at all different sites, but cryopreserved grafts were significantly less well laminated. The best lamination in cryopreserved grafts was seen with donor age E16 transplants. Epiretinal cryopreserved grafts contained more degenerated cells and vacuoles than subretinal ones, suggesting they were less viable. No differences were seen in the degree of integration with the host retina.

Other studies have shown that cryopreserved nerve tissue is more fragile than the normal and tends to fragment.[77,188,395] There could be several reasons for this. Freezing and thawing may change the membrane proteins and thus the adhesion between the cells. DMSO (used for cryoprotection) may also be toxic to the cells, and these and other factors may be responsible for the relatively poor lamination seen in cryopreserved transplants. Thus, cryopreserved tissue is not as good as fresh donor tissue. If used, it survives better in the subretinal space than epiretinally.

Transplanted retinas survive in the degenerated host

Keeping in mind the ultimate clinical application of retinal transplants, donor retinas have been placed in hosts with damaged retinas to study interactions of the transplant and the damaged retina.

In light damaged retinas of rats showing extensive neural and vascular changes, transplanted photoreceptor cell sheets[380] and suspended neuroretinal cells[92] have been shown to survive, to develop inner and rudimentary outer photoreceptor segments, and to develop synapses of both the ribbon type and the conventional type. The transplants also develop a common vascularization with the host[92] and show immunoreactivity to opsin.[380] Thus the transplants in these cases perform like in a normal host.

Strips of developing neural retina of normal newborn mouse transplanted into the subretinal space of a *rd* mouse showed differentiation into photoreceptors and ex-

presses S-antigen. The mutant *rd* host had lost its photoreceptors as a result of the *rd* mutation. However, histological analysis showed a significant fall in graft survival with increasing time after the transplantation, indicating the adverse effect of the mutant environment on the transplant.[189] This could also mean that the site of affection in the *rd* mutation might not be the rod cells alone.

Unlike rats and mice[222,229] there are at present no known models of hereditary retinal degeneration in rabbits, but experimental photoreceptor degeneration can be produced in these animals in other ways. Exposure of these animals to high concentration of oxygen damages photoreceptors in the central part of the retina.[56,288-290] Embryonic retinas transplanted to such eyes showed good transplant survival, and by light microscopy, the morphology of the transplants were not different from transplants to a normal host retina (unpublished observations).

Structure

Grafts form rosettes if the donor tissue is traumatized

When embryonic tissue is transplanted in fragmented form, it continues to differentiate and organizes itself to form a graft. In this process, the differentiating cells form all the cell types and the layers that are found in normal retinas. Most of the normal cell types also appear. However, the layering is often organized around a lumen surrounded by an outer limiting membrane, photoreceptors and other retinal layers, forming small spherical structures called rosettes. Rosettes have also been reported in other types of transplants like cell suspensions[94,146,195] and subretinal transplants of sheets of outer retina.[380]✎

The rosettes develop early in the transplants. The reason for the formation of rosettes in the retinal transplants is not clear. It is possible that mechanical factors and separation of the retinal cells from their native neighbors in the process of trans-

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✎Rosette formation is nothing specific to retinal transplantation, but typically appears when a disturbed retina develops. Rosettes have been described in a variety of conditions such as trauma[219] and retinitis pigmentosa[271]. Retinal explants cultured in vivo also develop rosettes.[64] In the Flexner-Wintersteiner rosettes found in retinoblastoma, relatively well differentiated photoreceptor cells arrange themselves around a central lumen containing hyaluronidase-resistant acid mucopolysaccharides similar to that found in normal photoreceptors and pigmented epithelium.[336] Even though the Flexner-Wintersteiner rosettes have a structure that is similar to the outer limiting membrane, they lack photoreceptor outer segments. In the Homer-Wright type rosettes of neuroblastomas and medulloblastomas the cells are arranged around a central tangle of fibrils.[336] Since the photoreceptors in transplant rosettes have a well formed inner segment and, to a large extent, also an outer segment; it would be better to use the word fleurettes, which is a term suggested for the corresponding structures in retinoblastomas, where well differentiated photoreceptors can be seen.[399] Nevertheless, the term rosettes is widely used in connection with retinal cell transplantation.

plantation could be among the reasons.[356]

The extent of rosette formation has been different in various studies. In some models it is a prominent feature [94,261] and in others it is less and sometimes totally absent, particularly in cell suspension transplants.[146,195] In the latter case, the distorted and small photoreceptor outer segments[94,98,144,145] suggest the transplants remained relatively undeveloped. Further, there was hardly any organization at all, which most likely was the reason why no rosettes were seen.

Formation of rosettes in the transplants will no doubt interfere with the resolution of the image, but not necessarily with the perception and even localization of light. Retinas with rosettes have been shown to respond to light in vitro[3] and to send the signals to the higher centers.[211,347] For the clinically acceptable retinal transplants it is desirable to have rosette free grafts. In paper V possibility of obtaining such grafts is investigated.

Electron microscopically most of the retinal cells are found in the transplants

Ehinger *et al.*[106] transplanted 6-12 week old (postconceptional age) human fetal retinas to immunosuppressed rats and studied the ultrastructure of these transplants shortly after the transplantation, as well as when the postconceptional age of the transplants was 40-41 weeks. This age was selected to allow near full term maturation of the transplanted cells. Electron microscopic examination could directly identify the photoreceptors by the localization of their nuclei and the appearance of the synaptic pedicles and spherules. Photoreceptor outer segments usually faced the lumen of the rosette. In well-developed transplants, cones could be distinguished from the rods. Cones were situated in the innermost layers of the rosettes and had a larger and paler nuclei than the rods. In transplants where photoreceptors had not developed outer segments and synaptic terminals, their identification is difficult.[151] Zonulae adherentes were seen in the transplants soon after the transplantation.[34]

Horizontal cell perikarya are not easily distinguishable in neither normal nor transplanted retinas because they largely lack distinguishing features. However, on the basis of their location and lack of postsynaptic membrane specialization and vesicles, horizontal cell processes could be identified in photoreceptor triads in the transplants. Such triads have been seen in human xenografts to rat retina[106,112] and in rabbit and rat homografts.[34,436]

Like horizontal cells, bipolar cells also lack distinctive cellular features that can identify them directly at the electron microscopic level. Again, bipolar cell processes could in the transplants be easily identified in the photoreceptor triads, where they faced the synaptic ribbon. Further, bipolar cell dyads with their characteristic ribbon synapses are common in the inner plexiform layer, and are easily identified in the region corresponding to the inner plexiform layer in retinal cell transplants.[34,106,112,408,436]

Amacrine cells can be recognized more confidently on the basis of their so-called conventional synapses than on the features of the cell body, and in transplants, such synapses can be easily recognized in regions corresponding to the inner plexiform layer.[34,87,106,112,408,436] The perikarya of the amacrine cells were more difficult to identify, but some cells in the region corresponding to the inner nuclear layer had deep invaginations in their lightly stained nuclei, similar to that often seen in amacrine cell perikarya.[106]

It is difficult to identify ganglion cells or their processes already in normal adult retina. Conventionally, a postsynaptic process in the bipolar cell dyad that is devoid of vesicles or organelles is presumed to be a ganglion cell process. This was suspected in many places in the transplants, but their presence in the graft is doubtful.[18,34,436] In the scanning electron microscope, Turner and Blair[408] saw many fibers from the retinal grafts extending into the optic nerve fiber layer of the host retina. However, the nature of these fibers was never unequivocally determined. Before the presence or absence of ganglion cells can be established in retinal cell transplants, more direct methods will have to be developed for their identification. ✎

Under congenial conditions transplant photoreceptors develop outer segments

The development of photoreceptor outer segments in retinal cell transplants has been described at the electron microscopic level in human[106] and monkey[91] embryonic xenografts, in mouse to mouse, rabbit to rabbit, and rat to rat homografts.[34,146,436] From these studies it seems that the development of the photoreceptor outer segments varies with the organization of the transplants. With tissue fragmentation techniques, transplants develop rosettes that show reasonably developed outer segments whereas in cell suspension transplantation techniques outer segments are reported to be small and distorted,[94,98,109,144-146,195] or absent. Such photoreceptors are still capable of phototransduction[314] as has been shown in the retinas transplanted to the optic tectum.[211,213] These results suggest that photoreceptors depend on each other for their proper development. Consequences of this are further discussed (elsewhere) in the text.

Many authors believe that lack of apposition of photoreceptors to the pigment epithelium results in maldevelopment of outer segments.[224] Photoreceptor layers transplanted along with the retinal pigment epithelium,[350] and studies on retinal reattachment[12] show similar defects even in the presence of appropriate retinal pigment epithelium in apposition with the photoreceptor cells. It appears that in retinal transplants microglial cells take up the phagocytic function of the pigmented epithelium.[25,302] The presence of microglial cells has not been confirmed in the retinal

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✎One of the ways to demonstrate the ganglion cells in the transplants that have formed connections with the host is to retrogradely label them with DiI. This technique has been used to demonstrate projections from the transplant to the host.[22]

transplants in the eye, but other types of phagocytic cells have been observed in the center of the rosettes.[146]

Transplants develop all the synapses type found in normal

Short-term grafts show only few or poorly developed synapses, whereas in grafts that have been allowed to grow for long enough, all the synapses found in the normal adult retina develop.[34,106,436] The formation of synapses in the graft clearly shows that the graft is growing in its new environment. The number of synapses seems to be less than normal.[106] No precise counts have been made.

Both rod and cone type terminals can be found in retinal cell transplants, and the transplanted photoreceptor cells contain their normal complement of synaptic organelles.[34,106,112,146] Rod-spherules can be seen, containing abundant synaptic vesicles and ribbons as well as presynaptic and postsynaptic membrane densities. The transplanted photoreceptor cells display synaptic triads of ribbon synapses and invagination of horizontal and bipolar cells. Certain regions of human to rat and epiretinal rat to rat transplants may be less developed with poorly defined structures in the photoreceptor terminals.[111] Occasionally, synaptic ribbons were observed with no direct association with any membrane or postsynaptic process.[34,106]

Regions corresponding to the inner plexiform layer consistently showed a high density of synapses. Most terminals were filled with conventional small synaptic vesicles (350-450 nm), but occasionally large (>800 nm) dense-cored vesicles were also seen.[34,106] Conventional synapses, presumed to be made by amacrine cells, were common and were found to make contacts with bipolar cell processes, and with other amacrine processes including some whose origin could not be identifies. Infrequently, amacrine cells were been seen to make synapses with small spines or thin intervaricose processes.[106]

In the regions of the transplant that corresponded to the inner plexiform layer, processes that contain numerous synaptic vesicles and ribbon synapses were seen. The postsynaptic elements were then arranged in dyads, which are characteristic for bipolar cell processes. Ribbon synapses with only one postsynaptic process (the monad arrangement) were also common.[111] A few gap junctions between amacrine cell processes were seen in rat to rat[111,436] and rabbit to rabbit transplants.[34] In most places, the postsynaptic elements of the dyad could be identified to be of amacrine cell origin. When the identifying morphological features were not prominent, the origin of the processes remained obscure and it is possible (but far from certain) that such processes originated from ganglion cells.[34,436] More advanced types of connections were also seen, such as reciprocal synapses made by a postsynaptic amacrine cells back to the bipolar cell or serial synapses between amacrine cell processes.

Synapses have also been observed in retinal tissue transplanted in the anterior cham-

ber.[88,255] When normal donor tissue was transplanted to an eye that has a dystrophic retina, the transplant showed more abundant synapses than the host retina.[144] A similar result have been presented in abstract form, where a transplant of a photoreceptor cell sheet also resulted in a greater number of synapses between photoreceptor cells and bipolar cells.[278]

Differentiation

Transplants develop most of the retinal cell types

At the time of transplantation, the fetal retinal tissue contains undifferentiated neuroblastic cells. Depending upon the technique, the tissue becomes fragmented at the time of transplantation. During the process of development the neuroblastic cells reorient themselves in rosettes.[366] They also develop and differentiate towards the various cell types of the normal adult retina. Many histochemical and electron microscopic studies have been conducted on the development of various cell types in the transplants.✎

Middle and short wavelength sensitive cones can be identified by their different opsins. In most parts of the normal rabbit retina, the middle wavelength sensitive cones dominate. However, in transplants, it has been found that short wavelength sensitive cones dominate.[394] It has been proposed that cones in some non-primate mammals may during their development start as short wavelength sensitive cells and then at some later point switch to middle wavelength photopigments.[394] The

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✎Labeling the transplant cells: Under certain circumstances, especially when cell suspensions are transplanted, or the synaptic connections are being studied, it may be difficult to say if a particular cell belongs to the donor or the host. Therefore, labeling the donor tissue may sometimes be important. Fluoro-Gold, a substituted stilbene, is a cytoplasmic stain[341] and has been used to mark the dissociated donor retinas.[93] Intravitreal injections of Fluoro-Gold have also been shown to stain the neural retina of rats in vivo.[90] Fast Blue has been used to mark suspensions of CNS and retinal cells for transplantation.[93,259] These substances work well with suspended cells, but not with large pieces of retina. Another drawback with these stains is that if the stain leaks out (as may happen if the donor cells degenerate), the stain may be incorporated by the host cells. A different approach to identify the donor tissue is by using tritiated thymidine, which is incorporated into the nuclei of dividing cells. When transplanted, these can be identified by autoradiographic methods for a long time, because the isotope has a long half-life. Even if the donor cells degenerate, the released isotope will not be incorporated into the host cells because they are not dividing. However, since it is a nuclear marker it can not be used to determine the origin of fibers or synapses. A more recent approach to this problem has come from the use of certain transgenic strains of mice.[145,146,432] These mice had a bovine rhodopsin gene transcription promoter in tandem with an *E. coli* LacZ gene inserted into their genome. This results in the expression of the enzyme, b-galactosidase, in about 30 to 40 % of the rods. A simple histochemical reaction can then be used to produce discrete particles that can be detected with the electron microscope. The particles appear throughout the cell, and are specific for the rods of this transgenic mouse strain.

observations in transplants suggest that this switch is disturbed in them, making them a possible tool for analyzing factors that might influence the change.

Depending upon their degree of maturation, rods in human, rat or rabbit retinal grafts stain with different intensities with antisera against S-antigen, rhodopsin, or rod alpha transducin (a membrane bound G-protein). The staining intensity of the graft photoreceptors varied in different parts of the transplant, and tended to be stronger and more often prevalent in the rosettes closest to the host retina.[18,354] Possibly then, the host retina influences the development of transplanted photoreceptors.

In human embryonic retinal tissue transplants, the round cell bodies of the developing cones can be found near the outer limiting membrane as early as 13 weeks after the conception. The S-antigen and other photoreceptor markers like alpha transducin and rhodopsin express themselves in the photoreceptors at 20 weeks post conception.[18,354] Some cones stain with neuron specific enolase (NSE) and synaptophysin (SYN), one or two weeks earlier.[354] Areas of different maturation often appear in one and the same transplant, possibly because central and peripheral regions of the donor retina were mixed together during transplantation, and they may develop at different rates. The development of the central retina precedes the peripheral by about 6 weeks in humans.[192,313]

In the normal retina, the interphotoreceptor matrix (IPM) links the photoreceptors to the pigment epithelium and serves purposes such as molecule transport,[241] photoreceptor isolation,[270] retinal adhesion,[430] and regulation of phagocytosis of photoreceptor outer segments by the retinal pigment epithelium.[1] The interphotoreceptor matrix comprises insoluble and soluble components such as proteins, glycoproteins, acid hydrolases, glucosaminoglycans and proteoglycans [2]. Immunohistochemical staining of four different components of interphotoreceptor matrix, namely chondroitin-6-sulfate, the F22 antigen, peanut agglutinin (PNA) binding protein, and interphotoreceptor retinol binding protein (IRBP) in rat retinal transplants has demonstrated that chondroitin-6 sulfate and the F22 antigen have the expected distribution both in the host and in the corresponding structures in the transplant rosettes.[196] The PNA lectin was found to bind to many more photoreceptors in the transplant than in the host. This lectin has a high binding affinity for D-galactose-ß(1-3)N-acetyl-D-galactosamine disaccharide linkages and binds to interphotoreceptor matrix surrounding the cones and not the rods.[196] IRBP could not be demonstrated in standard transplants, even though the inner and outer segments of the transplant photoreceptors were well developed and contained opsin. The photoreceptor cells synthesize IRBP. This protein is found in abundance in the interphotoreceptor matrix.[305] It is a glycoprotein with a molecular weight of approximately 140 kDa,[70] and it helps in transporting retinoids between the neural retina and the retinal pigment epithelium. These observations show that physically and chemically stable photoreceptor outer segments had formed in the rosettes, even though the interphotoreceptor matrix in retinal cell transplant does not seem to be entirely nor-

mal.[196]

In rabbits, horizontal cells processes in the outer plexiform layer are immunoreactive to 160 and 200 kD neurofilaments and vimentin, whereas HPC-1 and MAP 1A antibodies stain horizontal cells. In rats, horizontal cells could be seen with a 160 kD neurofilament antibody at two but not one week after the transplantation.[18] This may mean a delay of one week in the maturation of these cells as compared to their normal development.[374]

The development of different other retinal neurons in the transplants has been studied by immunocytochemistry of the specific neurotransmitters or neuron specific substances. The HPC-1 antibody marks a special synapse related protein, syntaxin, predominantly present in amacrine cells,[28,180,181] and appropriate numbers of such cells have been seen in transplants. Amacrine cells can also be identified according to the different type of neurotransmitter(s) they contain.[103,108,283,334,335,403,414-416] Rarely, amacrine cells in the inner nuclear and the ganglion cell layers are immunoreactive to somatostatin.[119]

In rats, the first differentiating amacrine cells appear in the graft at a time corresponding to the first postnatal day. They were identified by their immunoreactivity to choline acetylase (ChAT) and tyrosine hydroxylase (TH).[18] In the normal embryonic rat retina, measurable levels of ChAT have been found,[315] but possibly because of difference in sensitivity of the methods, Mitrofanis *et al.*[273] could find ChAT immunoreactive cells only at postnatal day 15. Tyrosine hydroxylase immunoreactive fibers appeared on postnatal day 3 in the normal rat retinal development.[273,283] HPC-1 (syntaxin) and glutamic acid decarboxylase (GAD) staining showed that the inner plexiform layer of the graft starts differentiating at the 8th postnatal day. Further, at this age, the outer plexiform layer of the graft contained neurofilament immunoreactive horizontal cells.[18] In grafts with an age corresponding to 4 and 6 weeks postnatally, the staining intensity was same as in the host cells. Immunoreactive fibers to somatostatin-28 were found mostly at the border of the inner nuclear layer and the inner plexiform layer in the host retina, but in retinal fragment transplants, these fibers were randomly distributed in the inner plexiform layer and the inner nuclear layer, revealing an abnormal lamination pattern. Marking human retinal transplants with the HPC-1 antibody (syntaxin) showed a beginning differentiation of the plexiform layer at the 15-16th week after the conception, seen as a faint staining. It became distinct at 25 weeks.[351]

Although different types of amacrine cells develop in the transplants, no ganglion cells have been observed with certainty by neurofilament staining or by using other markers. Antibodies against a glycoprotein on the neuronal surface, Thy 1.1,[27,301] against the microtubular associated protein,[294] against the 200 kD neurofilament[374] and against neuron specific enolase (NSE)[424] can all be used to mark ganglion cells. One antibody (OX-7) against Thy 1.1 faintly labeled rat retinal grafts with an age corresponding to postnatal day 1, but no cell bodies were stained.[17] Some small

cells in the ganglion cell layer of rabbit grafts have been found to be immunoreactive to an antibody against MAP 1A.[352]

It is possible that some ganglion cells may develop in the graft but fail to contain immunohistochemically detectable amounts of the markers mentioned. On the other hand, it has been shown that the retinal ganglion cells for at least a limited time depend on target derived substances for their development, both *in vivo*[265,303,348,349] and *in vitro*.[23,193,258,293,406,407] When grafted to ectopic sites in the brain (away from the target tissue), the embryonic retinal grafts show no ganglion cells 5 weeks after the grafting.[255,265] It therefore remains unsettled as to what extent ganglion cells develop and survive in retinal cell transplants.

Glial cells in transplants have been marked with antibodies against the S-100 antigen (a calcium binding protein), and glial fibrillary acidic protein (GFAP).[352,353] In rat transplants, Müller cells turned reactive to GFAP at an age corresponding to postnatal day 14. Two days after the transplantation, host derived glial cells appeared to invade the transplant. Morphologically, the Müller cells appeared to be normal in the outer nuclear layer and at the outer limiting membrane, but they did not develop endfeet at the inner limiting membrane at the vitreal surface. These findings have been confirmed in human xenografts. Vimentin immunoreactivity appeared early (14-16 weeks were the earliest transplants studied) and became comparable with that of the host at 19 weeks. GFAP labeled processes that were present at the lesion site all the times invades the graft, apparently from the host at 20 weeks. At 25 weeks, graft Müller cells became faintly stained, indicating gliosis with in the graft. CRALBP immunoreactivity begun to appear at 19-20 weeks and was intense at 25 weeks, mostly in the regions near the host.[354]

Retinal transplants may serve as a source of neurotrophic factors

A number of agents (neurotrophic factors, also known as neurotrophins or cytokines) known to participate in nerve cell differentiation and growth have been found to play a role also in the maintenance and survival of certain nerve cell populations, even in the mature CNS. In the retina, particular cell types have been shown to be responsive to such agents. For instance, an intravitreal injection of the brain derived neurotrophic factor (BDNF) has been seen to retard the degeneration of adult rat retinal ganglion cells after optic nerve transection[252,269] and to significantly reduce degeneration of rat photoreceptors following exposure to constant illumination.[230] More recently, genetically engineered astrocytes that synthesize and secrete bioactive BDNF have been shown to promote ganglion cell survival in cell cultures,[69] but also in transplants to the brain.[298] These observations suggest that growth factors and cytokines might be used to improve retinal cell survival and differentiation in transplants.

The *rd* (retinal degeneration) mouse is a model for retinitis pigmentosa, with a genetic defect in the rod cGMP phosphodiesterase. This enzyme is expressed in the

rod cells,[54] leading to a rapid loss of them. Even though cones do not express this defect, in due course of time, the *rd* mouse loses most of its cone cells also, implying that cones are dependent on the presence of rods for their survival. It has been suggested that transplantation of normal photoreceptor cells (mainly rods) to the *rd* mouse retina prevents and reverses the degenerative changes otherwise seen in the cones.[275,384] Such a rescuing effect should be mediated by diffusible factors and should therefore not require synaptic contacts between the transplant and the host.

Retinal transplants function

In order to function properly, it is important that retinal transplants develop the fundamental retinal cell types, and that they contain their functionally important components like neurotransmitters, their receptors, and other specific substances. It is also essential that they form appropriate and functional synaptic contacts.

S-antigen, opsin and alpha-transducin are examples of substances needed for the phototransduction process, and they have all been seen in histochemical analyses of the photoreceptor outer segments in retinal cell transplants.[18,19,33,354,380] In this respect, the photoreceptors thus appear to be reasonably normal. However, IRBP is known to be synthesized by the photoreceptors,[127,413] and the failure of this substance to appear in retinal cell transplants[196] might be due to malfunctioning of the photoreceptors. IRBP is also essential for the transfer of retinoids from the retinal pigment epithelium to the neural retina, and it appears likely that the phototransduction will be compromised if IRBP is lacking around the photoreceptors. It appears plausible that the lack of contacts between the rosette photoreceptor cells and the retinal pigment epithelium cause the absence of IRBP in the transplants. IRBP develops in transplants where the photoreceptors are well apposed to the host retinal pigment epithelium.[139]

There is good evidence that the transplants contain the various neurotransmitters present in the normal retina. Immunohistochemical staining for choline acetyltransferase indicates the presence of cholinergic neurons, tyrosine hydroxylase suggests the presence dopaminergic neurons, and glutamic acid decarboxylase points to the presence of GABA neurons.[18] The presence of their receptors has not been demonstrated in the transplants. Is an important prerequisite for at least some function(s) in the transplants that horizontal cells, bipolar cells as well as various types of amacrine cells and their synaptic connections are all present. The observations suggest that the transplant might process the light signal at the level of both the inner and outer plexiform layers. However, note that the presence of the various substances is only a necessary prerequisite and not an infallible sign of transplant function.

Direct evidence of the retinal cell transplant function has come from electrophysiological experiments. With a single electrode on the surface of isolated grafts, transient 'ON' or 'ON-OFF' spike-like responses and local electroretinograms could

be recorded.[4] The local electroretinograms are reminiscent of the proximal negative response or M wave seen in normal retinas, which reflect light induced amacrine cell activity. In these experiments, the presence of transient responses at the onset and at the end of short light flashes, as well as signs of a center-surround organization suggest that the transplants have nerve cell activities similar to that found in the inner and outer plexiform layers of the normal retina.[95] In retinal transplants grafted to the CNS of unilaterally enucleated host, cells with 'on' and with 'on-off' responses have also been found.[385] These light evoked responses were similar to the normal ERG.

Many of the donor tissue cells die after transplantation

All the efforts in retinal transplantation have so far been concentrated on studying the development of the cells. There is almost no knowledge regarding the death of transplant or host cells. In most neuronal tissues each cell type undergoes differentiation, progressing up to the formation of synaptic connections. Certain cells then go through a naturally occurring programmed cell death called apoptosis.[81,246,312] The cycle is unique for each cell type. Optimum time for harvesting cells for transplantation appears to lie within the time window starting at the time when cells begin to differentiate, and ending at the time when apoptosis begins. Generally, ganglion cells are the first cells to differentiate[277] and to die,[81,312] and in rats the time for apoptotic ganglion cell death lies in the first postnatal week. At the time of transplantation, the ganglion cells of the donor tissue are thus closer to the onset of their apoptosis than the photoreceptors, and this may be one of the factors explaining why photoreceptors become more numerous in the transplants than ganglion cells. Cell death has been reported early after the transplantation. It sharply declines in a few weeks time.[32,87] After one month, transplants seem stable, with some amount of debris. The causes of this early cell death in transplants are not known, but judging from general knowledge about the normal histogenesis it can be guessed that these dead cells must largely represent apoptosis, and to some extent, cell death due to trauma.

In the CNS, programmed cell death eliminates supernumerary neurons[74,113,296] that are not able to grow axons and form synapses, thus making appropriate connections between neurons necessary for their survival. In some situations, one of the determining factors in this relationship may be the specific growth factors produced by target cells. During the critical period of synapse formation, neurotrophins like NGF and BDNF are essential for neuron survival.[26,172,234,405] Later, the same neurons often become independent of them. Recent developments have shown that neuronal damage may lead to molecular and cellular events that culminate either in death or regeneration of cells. In mice, the *fos* gene has been found to be associated with neuronal death and the *jun* gene with regeneration.[186,187] Many of the molecules that are responsible for programmed cell death and axon growth, for example neurotrophins and their receptors, may reappear in adults under certain circumstances[99,100,114,133,239,337,338,433] suggesting that the genetic programming used during

development may be reutilized in adults.

HOST

Retinal transplants integrate with the host retina

Neural transplants to the brain often become encapsulated by a glial barrier.[24] Retinal transplants have been seen to fuse with the cut edges of the host. Immunohistochemical studies[352,353] using glial cell specific antibodies like that against glial fibrillary acidic protein (GFAP), S-100 (a calcium binding protein) or vimentin have demonstrated that already two days after the transplantation, host glial cells may start invading the transplant to give it some architectural support.[353] Sometimes a vitreal membrane of mesenchymal elements and some vessels can be observed in the host. The lack of astroglial cells in these membranes and the observation that these developed when the donor tissue contained retinal pigment epithelium suggests that these membranes may have developed from choroidal mesenchymal cells that contaminated the retinal pigment epithelium in the transplant tissue. Since astroglial cells are important elements of the retinal histogenesis,[34] their presence may be important for the development of the transplants.

Connections

Retinal ganglion cells have the potential for growing axons

It is known that the mammalian visual system does not regenerate even though abortive sprouting occurs following injury to the optic nerve.[9,65,140,327] ✎ Taking the lead from abortive sprouting, many authors studied the regeneration of the optic nerve.[5,6,9,35,85,307,389] Further, peripheral nerve implants were found to have possible trophic interactions that could enhance ganglion cell survival and axon fasciculation. Following observations in the spinal cord,[85] autologous sciatic nerve grafts were used as bridges for the growth of ganglion cell axons.[387] After 4-12 weeks, sciatic nerve grafts contained ganglion cell axons from the cells that had lost projections during the insertion of the graft. Horseradish peroxidase and fluorescent dye double labeling confirmed this finding. Some ganglion cells axons in the graft showed light induced responses.[209] This and a subsequent study[35] support the find-

✎The capacity of neural retina to regenerate from retinal pigment epithelium persists throughout life in some salamanders and until metamorphosis in some tailless amphibians.[323] In adult mammalian vertebrates, this can happen only in early embryonic life, with the exception of regeneration of photoreceptor outer segments under certain pathological conditions.[50] Very recently, pluripotent, dividing cells have been described in mammalian brains. In paper I, dividing cells were found where the pigment epithelium transits in to the neural retina for the longest examined time. If the retina also contains pluripotent cells, this could be the place to look for them

ing that ganglion cells have an inherent potential to regenerate if transected intraretinally.

The extraretinal regenerating potential of ganglion cells has been reviewed earlier already by Ramón y Cajal.[65] Later, Politis and Spencer[307] and Berry[35] studied the regeneration of partial and complete lesions of the optic nerve into a grafted peripheral nerve. Both models showed regenerating axons and ganglion cells with horseradish peroxidase retrograde staining. Axonal regeneration has also been shown in hamsters into peripheral nerve segments in the optic tract.[389]

Because of trophic or substrate influences of the permissive peripheral nervous system environment of the nerve bridges, ganglion cells can regenerate axons for distances which sometimes can be greater than their normal projections.[387] However, their entry into the CNS has remained limited to a few millimeters.[5,85] Some studies have indicated that few regenerating axons reach the target tissue through the bridges and form cone-like dilatations suggesting synaptogenesis.[306] These findings suggest that ganglion cells have the potential to regenerate axons through the peripheral nerve bridges and to reach the target tissue in the CNS.

Retinal ganglion cells can form connections with host target tissue

Much of the evidence concerning the formation of connections between the host and the graft has come from studies in which retinas have been transplanted into the brain, or have been co-grafted with brain tissue. The capability of the host nervous system to synaptically integrate with the transplanted cells was first shown by Beebe *et al.*[29] and subsequently by many other investigators.

Retinas transplanted close to the target tissue[255,264,265] in the brain show highly specific axonal outgrowth to a number of subcortical visual centers[163,316] such as the superior colliculus, the pretectal region, the accessory optic nucleus,[263,316] and the surface of the dorsal lateral geniculate nucleus. Grafts do not tend to project to non-visual nuclei. The pattern of innervation is not normal even to the superior colliculus, being more on the surface and lacking any topographic organization.[134]

The extent of the neurite outgrowth from the transplants depends upon many factors. It seems likely that the presence of a target area is important for the survival of the ganglion cells and thus, if the grafts are placed too far away from such an area, they do not project. BDNF-producing fibroblasts co-grafted with embryonic mouse retinal cells in the cerebral cortex have been seen to promote the outgrowth and survival of axons from the transplants.[298] In older grafts, mature ganglion cells are more prone to damage by axotomy caused during grafting and, consequently, they project poorly.[262]

The extent of the neurite outgrowth also depends upon the available synaptic space.[263] Retinas were transplanted to a site adjacent to the superior colliculus of neonatal

hosts, where retinocollicular projections had not yet developed. The contralateral eye was enucleated at the time of grafting. The projections from the graft to the host superior colliculus appeared to be much denser than the projections from the normal control eye to the contralateral tectum. Without the enucleation, only a few fibers extended from the graft to the superior colliculus of the host.[262] Also, when the embryonic retinas were grafted close to the superior colliculus of the adult hosts, projections penetrated only up to about 2 mm into the host superior colliculus.[264] On the other hand, in the neonatal host, the projections were seen to terminate over the whole extent of the superior colliculus. The dissociation and reaggregation of donor tissue prior to grafting did not affect projections from the graft.[261]

Freed and Wyatt[130] transplanted whole eyes to a location near the dorsal lateral geniculate nucleus of blind adult rats. On stimulation of the transplants with a light flashes, 'short latency negative waves' (NI) were recorded. They were sometimes followed by excitatory positive responses. The predominant NI wave was suggested to represent an erroneous coupling of photoreceptors to bipolar cells. Some 'long latency responses' indirectly suggest connections between host and graft.

On light or electrical stimulation of embryonic rat retinal transplants in the tectum of newborn rats, light evoked slow wave potentials could be recorded from the transplants and the tectum,[385] closely conforming to observations on normal rodent colliculus [132]. Responses could also be recorded from cortical area 18a, and they were most likely mediated by circuitry involving intermediate relays.[80] Stimulation of the transplant activated regions several intermediate synapses away as seen from results with *c-fos* activation.[79]

It may thus at least theoretically be possible for ganglion cells in transplants to survive, to project, and to form connections with the target area in the brain. However, integration of the transplant with the neurons in the host retina seems like a much more feasible strategy, as will be argued in the following.

Synaptic connections between transplanted neurons and host inner retina could suffice

In hereditary retinal degenerations the inner retinal layers are relatively well preserved,[371,372] and it is therefore not necessary for successful retinal grafts to have surviving ganglion cells. If the transplanted photoreceptors or the cells of the inner nuclear layer can form functional connections with the host, the graft should be able to convey information to the host. It is with this logic photoreceptor sheet transplants are being tried. Paper (IV) of the work presented here investigates if the transplants are capable of forming connections with the host. In this respect it is also important to determine if the host inner retinal layers remain capable of receiving such connections after transplantation.

Transplants convey information to the host

The capability of transplants to mediate pupilloconstrictor responses in the host was investigated by exploiting the consensual pupillary reflexes. Retinal transplants in the brain were able to induce pupillary constriction in the host.[211,212] The transplant mediated pupillary responses could be elicited also from long-term transplants where the photoreceptor layer was no more evident suggesting that these reflexes may be an indicator of already minimal functional capability.

More recently, Silverman *et al.*[383] have summarily reported on the pupillary responses and visually evoked cortical potentials in light blinded rats that had received transplants. However, in this case, there are not yet enough details published to exclude that the responses were due to the residual visual function that can be seen in the animal models of retinal degeneration .[97,208,228,282]

Hosts 'see'

To address the question of whether animals can see with retinal transplants, the effects of retinal illumination on the suppression response,[75] alerting behavior[247] and photophobic behavior[76] were studied in rats with retinal transplants to the brain. The results of the conditioned suppression experiments showed that the animals could detect an illumination of the transplant.[75] Experiments with photophobic behavior of the rats suggested that the hosts experienced the illumination, but not in the same fashion as normal eyes.[76]

If transplanted retinal cells survive, grow, differentiate and form synapses with the host neuroepithelial cells, then they may provide useful input to a visually handicapped host. del Cerro *et al.*[89] analyzed the inhibition of the acoustic startle reflex in rats with light-damaged retinas with or without transplants. Light-blinded rats showed a phase of exaggerated reflex followed by an aberrant phase of delayed inhibition. Grafting fetal retinal cells into the light damaged rat retinas restored a modest degree of properly timed reflex inhibition indicating that *in oculo* transplants may have provided useful visual information to the blinded host.

IMMUNITY

The eye is an immune privileged site

It has been known for many years that histoincompatible tissues survive unexpectedly well at certain sites,[191,205,266] and the interior of the eye is one of them. It was originally thought that this was so because the interior of the eye was sequestered from the systemic circulation and thus hidden from the immune system. More recently, it has been shown that antigenic material from the eye is able to reach the

systemic circulation[204,206,285] and may elicit an immune response. However, the immunity thus generated differs from the conventional, and lacks some of the standard cellular and molecular effects. The response is called *anterior chamber associated immune deviation (ACAID)*, and it is characterized by a grossly impaired expression of antigen specific delayed hypersensitivity,[286] preserved humoral immunity and primed cytotoxic T cell responses,[391] all achieved by an active down-regulation of the immune responses. Induction of ACAID depends upon certain unique features of the spleen and upon the immunosuppressive properties of the intraocular microenvironment.[78] They are all mediated by cytokines, especially transforming growth factor β (TGFβ).

It has been shown that neural retinal grafts are immunogenic and that the immunity generated is directed against both the graft and the retina restricted autoantigen.[190] Histocompatibility antigens are scant in the neural retina, but their expression is upregulated after transplantation.[317] Depending on the site of transplantation, the grafts may induce delayed hypersensitivity (in the subconjunctival space, for instance) or ACAID (in the anterior chamber of the eye). Even though most antigens are reported to be capable of inducing ACAID (including the major and minor histocompatibility antigens of the transplant,[392] not all of them evoke a permanent state of ACAID. For example, some tumor cells transplanted to the anterior chamber of the mice induce only a transient ACAID.[216] Allogenic retinal grafts placed in the anterior chamber are eventually destroyed, indicating that conventional immunity can overcome the ACAID.[190]✎
Studies of the subretinal space and vitreal cavity as immune privileged sites have indicated that they enjoy the same privilege as the anterior chamber not only in the normal eye but also in disease.[194] The study implies that both the vitreal and subretinal spaces have an immunosuppressive microenvironment. It is interesting to note that transforming growth factor β is produced by the retinal pigment epithelium[397] and that its production by the astrocytes is upregulated in pathological conditions.[417] The Müller cells of the retina suppress T-cell proliferation by a direct contact mechanism.[68] These facts indicate that retinal cells may be contributing to the immunosuppressive microenvironment of the subretinal and vitreal spaces. Strengthened by the blood retinal barrier formed by the pigment epithelium, Bruch's membrane, and the endothelium of the retinal vessels, the subretinal space has all the features of an immune privileged site. The results with cell transplantation show that the immune privilege of this site is able to withstand the disruption of the blood retinal barrier induced by the surgery.

✎The immune privilege of the eye can be regarded as a physiological adaptation to an evolutionary need, ensuring that the immune protection against the intraocular antigens is delivered with minimal immunogenic inflammation. This avoids damage to the 'innocent bystander' tissues and relevant to our context, the retinal transplant.[393]

Influence of host age

There has been little research regarding effect of host age on retinal transplants. The immunological maturity and hence also the host age should directly affect the transplants. Immunologically matched grafts can survive in adult hosts but an immunologically disparate donor tissue is more likely to survive if the host is immature and thus immunologically incompetent.[423] Neural xeno-transplants into adult rat brains showed a high degree of rejection,[248] whereas 70% of similar grafts survived in the brain of neonatal rats for at least 90 days.[347] Grafts survived well in rat hosts aged up to about seven days *post partum*, but in rat hosts older than 11 days at the time of transplantation, grafts got infiltrated with lymphocytes within a few days and showed advanced destruction by day 15.[248] This indicates the time of development of immunological competence of rats. Other studies on neural transplants including retinal grafts in CNS[264] and the growth of fetal brain explants in the anterior chamber of the eye[105] indicate that host age can influence the outcome.

Host immunity can be altered for better survival of the grafts

Cyclosporin-A is a commonly employed drug that suppresses the immunity of the host, and it has been shown to enhance the survival of for instance kidney, bone marrow and liver transplants,[141,201,202] and is now the standard treatment for all types of organ transplants. Mouse retinas grafted into rat eyes survive and differentiate for up to 30 days with Cyclosporin-A treatment.[21] Postnatal day 21 rat retinas are more mature and survive when grafted to the subretinal space. However, they do not survive well if transplanted to a site where the retina has previously been lesioned, but the survival improves if the animal is treated with Cyclosporin-A.[21] Similarly, immunosuppression with FK 506 has also been shown to prolong cross-species graft survival.[429]

Transplant antigenicity can be altered for better survival of the grafts

Cells lining the anterior chamber do not express any class II histocompatibility antigen complex, but this is not true for the pigment epithelial and neuroretinal cells. Cultured pigment epithelium can be made to express such antigens by lymphokines and γ-interferons,[242] but the histocompatibility antigens in retinal cell transplants do not necessarily lead to a rejection of the graft because of the immune privilege of the eye. Nevertheless, the immune privilege of the eye is far from absolute, and under certain conditions ACAID fails to develop so that the eye can become the target of immunogenic inflammation.[393]

Theoretically it is possible to eliminate the major histocompatibility complex gene by recombinant DNA methods in tissues that are capable of considerable proliferation in the cultures.[57] These methods are at present in their infancy, but theoretically it should be possible to develop universal donor cells that do not evoke an immune response.

Hurdles

There is hope that retinal cell transplantation could prove a practical way to treat retinal degenerations, which are hitherto untreatable. The history of retina to retina transplantation is short, but the achievements have been considerable. Pigment epithelial cells and retinal neurons have very recently been transplanted in humans,[7,8,84,207] with published observation times up to about a year.

There are certain areas in the field of retinal transplantation where more detailed studies are needed. It is generally known that even when retinal transplants are allowed to survive and to attain postnatal ages, their sizes remain limited even though they often contain donor tissue from more than one embryonic retina. Technical difficulties have precluded more precise studies of transplant growth by either measuring the size, the volume or in other ways. It is therefore important to study the growth of the transplanted cells, and the factors affecting it.

There is also a need for detailed studies on the physiology and development of retinal transplants, as well as on what is normal and what is abnormal in them. Such knowledge will be useful to develop remedies for the function limitations that the transplants may have.

Even though fibers have been seen connecting the transplant with the host, they do not appear regularly and their number is small. For transplants to convey information to the brain, they must integrate well with the host and form sufficient synaptic connections. More work is needed to find factors that influence the formation of connections between the host and the graft.

In most of the transplant modalities being used at present, the transplanted cells organize themselves into rosettes.[88,144,146,380,408] Rosette-free transplants are highly desirable. Attention is needed for identifying the factors that are responsible for rosette formation in transplants, and to develop surgical procedures by which rosette-free transplants can be obtained. Paper V deals with the development of such a technique. Taking the lead from this, further modifications in the technique have resulted in reasonably well laminated grafts.[110,138,366]

Transplanting cells that produce some trophic factor that will prevent neurons from degenerating is an attractive idea that is just beginning to get explored. It is known that at least some diseases in experimental animals are caused by a lack of some trophic factors, and a number of candidates have been found.[230] However, cell-systems able to deliver the required factors are not available. It is worth exploring the possibility of using retinal transplants, peripheral nerve transplant, or some other cell line as a source for trophic factors.

In certain clinical situations, retinal transplant will have to survive *in oculo* for a

long period of time. There are reasons to believe that the long-term survival of retinal transplants may not be as good as desirable. It has been shown that neural retinal grafts are immunogenic,[190] and the immune privilege of the eye is not perfect.[190,216] Also, soon after the transplantation the grafted cells and the host retina degenerate at the host graft interface.[83] It is therefore important to assess the fate of retinal transplants after long survival times.

GOAL AND AIMS OF THE STUDY

The general goal of the study was to get a better understanding regarding the growth, differentiation, integration, organization and survival of the retinal transplants, with a view of developing retinal transplantation as a clinical procedure for treating degenerative retinal disorders such as retinitis pigmentosa.

The present study attempts to provide clues to the answers of some questions raised in the introduction of this work. The specific aims of the present study were:

1. To reach a better understanding regarding the rosette formation in the retinal transplants, and to develop a surgical procedure for producing better organized retinal transplants in rabbits.
2. To examine the development of different cell types in the retinal cell transplants and to compare their development with that of normal retina.
3. To study the proliferation of cells in normal developing rabbit retinas
4. To study the proliferation of cells in rabbit retinal transplants.
5. To study the connections between the host and the graft.
6. To study the fate of long-term retinal transplants in the eye.

MATERIALS

Ethics

All the experiments in the study were conducted according the rules set forth in the ARVO resolution on animal experimentation. Appropriate permits for the study were obtained from the Swedish Government Animal Experimentation Ethics Committee at the University of Lund and appropriate authorities at Harvard University in USA.

Tissue

Developing rabbit retinas for comparative studies

Rabbit retinas in various stages of development were obtained from rabbit embryos (Papers I and III). The ages of the embryonic retinas ranged from post-conception day (E) 15, to the adults. . The age of the animals was determined by mating the rabbits during a 1-hour period on a known date.

Donor tissue for retinal transplantation

Ordinary mixed strain pigmented rabbit retinas from stage E 15 were used as donors (Papers II, III, IV, V, and VI). Embryos were obtained by cesarean section after euthanizing the pregnant female rabbits. They were kept at +4° C in Ames' solution, which has the following composition (mM): NaCl (120), KCl (3.6), $MgSO_4$ (1.2), $CaCl_2$ (1.2), $NaHCO_3$ (23), NaH_2PO_4 (0.1), Na_2HPO_4 (0.4) and glucose (10). Eyes from these embryos were enucleated, and the neural retina was then carefully dissected from the posterior eyecup under an operating microscope. These retinas were kept in the same solution until transplanted.

Recipients for retinal transplantation

Adult rabbits of the same strain as the donors, weighing between 2.5 and 3 kgs, were used as recipients (Papers II, III, IV, V, and VI). Fifteen minutes before surgery, the right pupil of the recipient was dilated with 1% cyclopentolate and, if necessary, also with 10% phenylephrine HCl. For surgery, animals were anaesthetized with 1 ml/kg Hypnorm® (fluanison 10 mg/ml and fentanyl 0.2 mg/ml).

Transplantation instrument

FOR TISSUE FRAGMENT TECHNIQUE

For transplanting fragmented pieces of the donor tissue, a previously described instrument was used[32] (Papers II, III, IV, V, and VI). The plastic capillary used in this instrument is narrow (with inner and outer tip diameters of 0.15-0.4 and 0.3-0.5 millimeters), with the result that the embryonic retinas get fragmented when drawn up into it.

FOR LARGE SHEET RETINAL TRANSPLANTS

A special instrument was developed for transplanting large pieces of full thickness embryonic rabbit retinas into the adult eyes (Paper V). The instrument comprises a cannula made from a flattened 18 gauge injection needle, which serves as a jacket around a thin-walled flat polyethylene tube (about 1.8 X 0.6 mm outer and 1.6 X 0.4 mm inner dimensions). This polyethylene tube and the cannula are mounted on an instrument that can push the tube out of the cannula in a controlled fashion. Further, the plastic tube is attached to a precision microsyringe. Donor tissue is sucked into the polyethylene tube with the help of the microsyringe. In this process, the slightly cup-shaped neural retina enters the tube as a single piece. There is inevitably some damage to the transplant tissue at the cut margins, but the central portion remains as an intact sheet.

METHODS

Surgical procedure

The cannula with the polyethylene tube containing 3 to 4 embryonic retinas (fragmented or large whole retinal sheets) was introduced into the eye through a *pars plana* incision (5-6 mm behind the limbus) and advanced transvitreally until it reached the predetermined transplantation site at the posterior pole. The procedure was monitored under an operating microscope, using a standard contact lens. On reaching the retina, the polyethylene tube was pushed out of the cannula, and if required introduced into the subretinal space (for subretinal transplants) by doing a retinotomy with the tube containing the tissue. The donor tissue was then ejected at the appropriate site by pushing the piston of the microsyringe attached to the cannula (Papers II, III, IV, V, and VI).

After transplantation, animals were allowed to wake up and were transferred to their normal cages with a 12/12-hour light/dark cycle. No antibiotics or immunosuppressives were given postoperatively.

Tissue processing

At predecided times after the transplantation (varying from 1 day to 583 days), the animals were sacrificed. Their eyes were enucleated and fixed in 4% formaldehyde for 24 hours (Papers II, III, IV, V, and VI). After the fixation, the eyes were hemisected and the part carrying the transplant was excised. The tissue was washed with Sørensen's phosphate buffer (0.1M pH 7.2) and then cryo-protected, if needed, by washing with the same buffer containing 5%, 10% and 20% sucrose. Twelve-micrometer sections were cut on a cryostat. For paraffin and plastic sections, the fixed tissue was washed in 30, 50 and 80% ethanol and xylol (for paraffin) or aceton (for plastic) in which it was also stored until embedded in paraffin wax or plastic and sectioned (6 µm and 2 µm respectively). Developing retinas were fixed and processed in the same way (Papers I, and III).

Staining for light microscopy

The cryostat sections were stained with hematoxylin and eosin, where as the plastic and paraffin sections were stained with methylene blue and toluidine blue respectively for light microscopic examination.

Immunohistochemistry

Antibodies

MIB-1

The antibody (Vector Lab. Inc. Burlingame, CA., USA) marks a protein called Ki-67 [210] which is found only in the dividing cells.[136,137] Thus MIB-1 immunostaining is a good marker for proliferating cells. The sections were blocked with normal horse serum. Anti-mouse antibody raised in horse (Vector Lab. Inc. Burlingame, CA., USA) was used as the secondary antibody and the slides were developed with the ABC technique (Papers I and II).

Anti-nitric oxide synthase

The NOS antiserum employed was raised in sheep against purified rat recombinant neuronal NOS protein (a gift from Dr. I. Charles and Dr. P. C. Emson, MRC, Cambridge, UK). The antibody recognizes a protein with a molecular mass of 155 kDa on Western blot analysis of rat hypothalamus, and is therefore specific for neuronal NOS. Adsorption of the antiserum with the recombinant neuronal nNOS protein abolished all immunoreactivity. This antibody was used in papers III and IV

R2-15

The antibody (R2-15) marks rhodopsin, which is a protein of phototransduction cascade and is mainly found in the photoreceptor outer segments. Rabbit-anti-mouse immunoglobins (Dakopatts) were used as secondary antibody and the slides were developed with the ABC method (Paper V).

Anti-vimentin

The antibody (Dakopatts) marks vimentin, which is found in the Müller cells. The glial cell architecture of the retina can thus be studied with vimentin antibodies (Paper V). Rhodamine-conjugated rabbit immunoglobulins to mouse immunoglobulins (Dakopatts) were used as secondary antibody.

Method

Mouse monoclonal antibodies against rhodopsin (diluted 1:6000), vimentin (diluted 1:100), the Ki-67 antigen (diluted 1:200) and nitric oxide synthase (1:3500) were used for immunohistochemistry on cryostat sections. The antibodies were diluted in phosphate buffered saline (PBS) containing 0.25% Triton X-100® (ICN Biomedicals Inc.) and 1% bovine serum albumin. Sections were washed for 15

minutes in 0.01 M PBS (pH 7.2) containing 0.25% Triton X-100 (and 1% BSA for the MIB-1 antibody), which was also used for all the washes in the immunohistochemical staining. Sections were then incubated with normal blocking serum (rabbit 1:10, Dakopatts for rhodopsin and vimentin, and 1.5% normal horse serum for MIB-1, and 1.5% normal donkey serum) followed by incubation with primary antisera for 20-25 hours for antibodies against rhodopsin, vimentin and NOS, and 72 hours for antibodies against Ki-67. Sections incubated with antibodies against vimentin and NOS were washed, incubated with secondary antibody (for vimentin rhodamine-conjugated rabbit immunoglobulins to mouse immunoglobulins, Dakopatts, and for NOS Texas Red sulphonyl chloride-conjugated donkey anti sheep IgG, Jackson ImmunoResearch) for 30 minutes, washed again and mounted with Vectashield (Vector Lab. Inc.). Sections incubated with anti-rhodopsin and MIB-1 were incubated in secondary antibody (rabbit-anti-mouse immunoglobins, Dakopatts for rhodopsin, and biotin conjugated horse-anti-mouse secondary antibody, Vector Lab. Inc. Burlingame, CA., USA for MIB-1). To develop the peroxidase reaction, sections were first washed for 15 minutes in 0.05 M Tris buffer (pH 7.4) and then developed for 10 minutes in a substrate solution of 0.05% diaminobenzidine (DAB) and 0.015% H_2O_2 in 0.05 M Tris buffer (pH 7.2). For MIB-1, the HRP reaction was developed with the ABC method (Vector Lab. Inc.).

How do transplanted cells proliferate compared to normal retina?

MIB-1 IMMUNOREACTIVITY IN DEVELOPING RETINA

At embryonic day 15, the earliest stage examined, some non-reactive cells were already present in the innermost third of the neuroblastic cell mass. ✎ Certain intensely stained, round cells (cells in metaphase) were present close to the pigmented epithelium. At embryonic day 25, when the ganglion cell layer has separated following the formation of a inner plexiform layer, certain cells in the ganglion cell layer/ nerve fiber layer remain immunoreactive, and these immunoreactive cells persist even in the oldest examined retina (PN 15) at least in the mylinated streak. At E 25 there is an accumulation of non-reactive cells in the innermost part of the neuroblastic cell mass (close to the IPL), and these cells increase in number at E 29 and corresponds to rounded cells in the hematoxylin and eosin stained sections. At E 29 the outer plexiform layer forms in the central retina and the immunoreactivity ceases in its vicinity.

At the day of birth the central retina was almost non-reactive, but the reactivity persisted in peripheral retina and increased from center towards periphery. The OPL had further spread peripherally decreasing the immunoreactivity along its spread. In the outer nuclear layer, there was some reactivity and most of the reactive cells were accumulated proximally close to the OPL. Cells in the metaphase were close to RPE. Some elongated cells were present in the middle regions of the INL including some cells in the metaphase.

As the retina matures in PN 3, 5 and 7 the central non reactive area enlarges and most of the reactive cells found were elongated and were located in the INL. At PN 11 and 15 immunoreactivity was only seen in the extreme peripheral parts of the retina Immunoreactivity was also seen in the RPE, but at and after E 29 it became difficult to judge the reactivity due to increased pigmentation in these cells (Paper I).

✎Terminology: When describing and discussing retinal development the term "basal" or "proximal" and "apical" or "distal" are used to denote localization in terms of how the tissue develops. In the neuroretina, "basal" thus means closer to the vitreous and "apical" closer to the photoreceptors. For transplants the term "luminal layers" or "inner layers" are used to denote the layers of cells towards the lumen of the rosettes (containing cells found in the outer nuclear layer of normal retina) and "outer layers" to denote layers away from the lumen. The outer layers and the cells lying in between the rosettes containing the cells found in the inner retinal layers of normal retina.

MIB-1 IMMUNOREACTIVITY IN RETINAL TRANSPLANT

One day after transplantation reactive (including cells in metaphase) and non-reactive cells were randomly distributed in the graft. Some cells in the metaphase formed rosette-like clusters. A few patches of small non-reactive (judged to be degenerating) cells were seen close to the host retina.

In later developmental stages in the grafts (E 19, 21, and 22) the rosettes became more and more distinct. The pattern of immunoreactivity changed around the rosettes. Cells in the metaphase were often found arranged in the innermost parts of the rosettes, close to the lumen. At E 21 few cells in the innermost layers of the rosettes became non-immunoreactive. Reactive cells were present in the outer layers of the rosettes. There were clusters of immunoreactive and non-reactive cells in between the rosettes. Small non-reactive cells at the host graft interface increased and these corresponded to the pyknotic cells on hematoxylin and eosin staining. However, a few immunoreactive cells were present in this non-reactive area, and these cells persisted even in the oldest examined transplants. In embryonic age 26 and 29 days, cells in the luminal layers of rosettes progressively became non-reactive; the reactivity was confined to outer layers of the rosettes and in cells located between the rosettes. At postnatal day 2, the immunoreactivity was found only in cells situated between the rosettes. In later stages (PN 5, 11 and 12), very rarely some cells were reactive in between the rosettes (Paper II).

How do the transplanted retinas develop at various transplantation sites?

LIGHT MICROSCOPY AND RHODOPSIN IMMUNOHISTOCHEMISTRY OF EPIRETINAL TRANSPLANTS

In the large sheet retinal transplant in the epiretinal space, cells differentiated in two distinct nuclear layers. The apical layer comprised of dark and oval nuclei of neuroblastic cells. The convex surface of this layer showed no photoreceptor outer segments and the immunoreactivity for rhodopsin was negative. On the basal side, this nuclear layer was followed by a layer with the appearance of the normal inner plexiform layer. A cell layer equivalent to the normal ganglion cell layer was also present, but the type of the cells in this layer remained undetermined (Paper V).

LIGHT MICROSCOPY AND RHODOPSIN IMMUNOHISTOCHEMISTRY OF SUBRETINAL TRANSPLANTS

The transplants done with fragmentation technology developed rosettes, where as those done with modified large-piece technology developed arcuates (further discussed below). Both the arcuate arrays and the rosettes showed two distinct layers of cell bodies. The first comprised comparatively small, oval cell bodies, apparently developing photoreceptor cells. The second consisted of cells with rounder

and more lightly stained nuclei, which resemble cells of the inner nuclear layer. A plexiform layer was also present between the two nuclear layers, as was in places another plexiform layer, most likely equivalent to the inner plexiform layer. Thus the rosettes had two cell-rich layers and two layers resembling the outer and inner nuclear and plexiform layers, respectively. A cell layer resembling the ganglion cell layer was also present, but it was not established whether it contained true ganglion cells or some other cell type, such as displaced amacrine cells.

Both in the arrays of cells and in the rosettes, the photoreceptors most often had well-developed outer segments. This was confirmed by immunohistochemistry for rhodopsin. Particularly in cell arrays, photoreceptors were also associated with a well-developed outer limiting membrane as judged by light microscopy (Paper V).

How do the transplanted retinas differentiate compared to normal retinas?

NOS IMMUNOREACTIVITY IN THE DEVELOPING RETINA

A few weakly stained cells, at times with short stained processes, were found immunoreactive at E 29 in the proximal margin of the neuroblastic layer of the central retina. At PN 0, these cells had long and well-stained dendrites projecting towards the developing inner plexiform layer. At PN 3, the number of reactive cells increased, and these could at times also be seen in the ganglion cell layer. The cells projected towards the inner plexiform layer. At PN 5 and PN 7, these cells formed a distinct immunoreactive band in the middle of the inner plexiform layer. At PN 11, it became possible to distinguish labeled cells of at least two types. One larger cell type exhibiting stronger immunoreactivity and another smaller and weakly labeled. Labeling in the inner plexiform layer was seen in a thick band located in the middle of the layer, and in a weaker and more proximal sublamina. Some lightly stained dots could at times be seen also in the most distal sublamina of the inner plexiform layer. At PN 60, and in the adults in the adult, NOS-immunoreactive cells were seen both in the inner nuclear and ganglion cell layers, and at times even within the inner plexiform layer. These cells formed plexuses in the inner plexiform layer as described above. Rarely, the immunolabeled cells located in the inner nuclear layer were seen to emit processes towards the outer plexiform layer. However, no specific labeling was detected in the latter (Paper III).

NOS IMMUNOREACTIVITY IN TRANSPLANTED RETINA

In transplants corresponding to E 29, a few stained cells were occasionally observed. The labeled cells were found between rosettes, where cells belonging to the inner retina are located. The same observations were made at later stages (transplants corresponding to PN 0 - PN 123). The number of stained cells was however seen to vary between the specimens and at times also between sections. Yet, a rela-

tively large number of distinctly labeled cells could be seen even in older transplants. At least two subtypes of NOS-containing cells appeared to be present in the grafts (Paper III).

Do grafts Integrate with the host retina?

NOS IMMUNOREACTIVITY IN FIBERS BETWEEN THE GRAFT AND THE HOST

Most labeled cells found in the grafts projected to the equivalent of an inner plexiform layer within the transplant, which in many cases was located next to the host retinal pigment epithelium. This layer was at times made of a dense plexus of stained fibers that often ran for considerable distances (Paper III). At times, immunolabeled processes were also found in the grafts close to the graft-host border. In regions where one or more photoreceptor cell rows of the host outer nuclear layer remained, such processes could be seen to run parallel to the border, without entering the host retina.

At other times, labeled fibers originating in the graft could be seen crossing the graft-host border. This was seen in regions where the host photoreceptor layer was absent. Such bridging was observed at all survival times, but not in all specimens examined. Further, it was in some cases possible to follow a labeled process originating in the graft all the way into the host inner plexiform layer (Paper IV).

At places certain immunoreactive cells could be seen to project to plexiform layers both in the transplant and in the host thus connecting the plexiform layer in the transplant to the inner plexiform layer of the host.

Does the transplantation technique influence the organization in the grafts?

LIGHT MICROSCOPY AND VIMENTIN IMMUNOHISTOCHEMISTRY OF FRAGMENT TISSUE RETINAL TRANSPLANTS

To study the overall architectural organization of the transplants, Müller cells were immunostained with vimentin. Regular rosettes dominated in fragmented tissue transplants, but rosette-free areas were infrequently found in places where the host pigment epithelium was disturbed. In these transplants, vimentin immunoreactive cells were radially arranged in the outer nuclear layer of the rosettes. In the areas between them, which corresponds to the inner retinal layers, vimentin immunoreactive cells appeared abnormal. In these areas, an inner limiting membrane was not visible and the Müller cell end feet plates were not regularly visible in the transplants.

LIGHT MICROSCOPY AND VIMENTIN IMMUNOHISTOCHEMISTRY OF LARGE SHEET RETINAL TRANSPLANTS

Six weeks after the transplantation (equivalent to 8 weeks after the conception) the epiretinal transplants had grown into a more or less spherical or cup-shaped laminated sheet. This piece of the donor tissue did not show rosette formation except at the margins of the transplant, where a few small rosettes could be seen. Vimentin staining of these transplants revealed a relative regular palisade-like arrangement of the Müller cells.

Subretinal large sheet retinal transplants in the same eye showed an organization different from that of epiretinal large sheet transplants. Here the cells were predominantly arranged in irregular arcuate arrays. The photoreceptors in these arcuate arrays always faced the host retina, whereas the photoreceptors in the rosettes faced their centers. The vimentin immunoreactive cells were in these transplants regularly arranged in an arcuate manner.

Do transplants survive for long time?

LIGHT MICROSCOPY OF LONG-TERM TRANSPLANTS

In the long-term transplants, graft was identified in most specimens (Paper VI). In some few the glial tissue was found to dominate whereas nerve cells were found in abundance in others. However, even these transplants had more glial cells then seen in the transplants of shorter survival time. In long-term transplants photoreceptors were found to survive only when they were well apposed to the host retinal pigment epithelium. The surviving photoreceptors were well differentiated. In portions of the graft where the transplanted tissue was not in contact with the host retinal pigment epithelium the rosetted layering of the transplant was largely lost. Nevertheless, the outlines of the rosettes could still be seen.

After long survival times, the physical fusion between the transplant and the host was excellent. The thickness of the long-term grafts was much reduced in comparison with the ones that had survived for only short times. In short-term grafts the total thickness of the transplant and the remaining host retina overlying the graft was approximately 3 times the thickness of the intact host retina adjacent to the graft. In long-term transplants the thickness of the graft and the overlying degenerated host retina was almost equal to, or slightly more (<1.5 times) than the thickness of the intact host retina (Paper IV).

How does the host react to transplantation?

LIGHT MICROSCOPY, MIB-1 AND NOS IMMUNOHISTOCHEMISTRY OF HOST RETINA ADJACENT TO THE GRAFT

Soon after the surgery the host retina overlying the graft started degenerating. The degeneration was confined to the photoreceptors and outer nuclear layers. In the transplants of the short survival time cell debris belonging to the degeneration host as well as the transplanted cells could be seen at the host graft interface (Paper II and VI). After long survival time this debris was not visible, and due to the good integration of the host with the graft, the outer plexiform layer of the host overlying the transplant was not easily distinguishable. Both after short and long survival times the cells of the inner nuclear layer, the inner plexiform layer, and the ganglion cell layer were clearly distinguishable. The number of cells in the inner nuclear layer appeared to be less than what was seen in the non-degenerated parts of the host retina. Certain large cells (presumed to be ganglion cells) were visible in the host retina covering the transplants. The deeply stained cells of the outer nuclear layer along with photoreceptor outer segments were seen in the host adjacent to the transplant. In the host retina adjacent to the transplant, the number of cells in the outer nuclear layer and the size of the outer segments tended to taper off. These observations were also noted in the transplants of younger ages.

In the host retina close to the transplant certain cells could be seen to proliferate already 6 days after transplantation and these persisted till PN 12 (Paper II).

NOS-immunolabeled cells were seen in the host retinas at all time points. NOS-positive cells could be seen in the host retina also in the region contacting the graft. These had a normal appearance, and were seen to project towards the inner plexiform layer as seen in intact retinas. There was no indication that the number of NOS-labeled cells was altered in the host retina following transplantation (Paper III and IV).

DISCUSSION

Growth of retinal transplants

TRANSPLANTED RETINAS MAINTAIN THE PATTERN OF PROLIFERATION

Proliferation of cells in developing retina

At E 15, the proliferating cells were seen in the outer two thirds of the neuroblastic cell mass. However, mitotic figures visible as large, rounded and deeply staining cells were found only at the apical border of the neuroblastic cell mass in this[367] (Paper I and[367]) and other[156,319] studies, indicating that the proliferating cells undergo interkinetic migration.

Ganglion cells are the first cells that leave the mitotic cycle,[131,173,182,197,373,377,420] migrate to the proximal part of the neuroblastic cell mass,[435] and start to project axons towards the optic fissure.[162,379] Therefore, the post mitotic cells at E 15 which accumulate in the most proximal part of the neuroblastic cell mass are likely to be differentiating ganglion cells [367](Paper I).

The non-proliferating cells in the innermost part of the neuroblastic cell mass at E 25-29[367] (Paper I) which correspond to the round, lightly stained cells in hematoxylin and eosin sections (in E 29) have been described as differentiating amacrine cells in previous studies.[156]

The timing for the differentiation of bipolar cells is not well documented, but various studies on mammalian retinas have suggested that the early proliferation gives rise to ganglion cells followed by the horizontal cells, some amacrine cells and cones. Late proliferation gives rise to the remaining amacrine cells, Müller cells, bipolar cells and rods.[67,227,324,325,377,435]

Differentiated horizontal cells are apparent already at E 27.[156] It therefore seems likely that already at E 25, some of the postmitotic cells could be differentiating horizontal cells, which visibly accumulate proximal to the outer plexiform layer first visible at E 29 (Paper I).

At stages E 25 and E 29, there was a gradually increasing accumulation of postmitotic cells towards the distal retina at the location of photoreceptors (Paper I). However, even after the formation of the outer plexiform layer, proliferating cells in the outer nuclear layer could be observed accumulated on the proximal part and the distalmost part of the same layer (mitotic figures). This suggests that photoreceptors are born throughout a long period of time. Accumulated evidence suggests that the

cones are born in the early phase of proliferation while the rods are born in the late phase.[325]

At the time of birth, the proliferation ceased in a small region in the central retina. This area of non-proliferation successively enlarged in PN 3, PN 5 and PN 7 retinas where the proliferation was found only in the peripheral part of the retina. Isolated proliferating cells could be seen in the extreme periphery of the retina even in PN 15 retinas[367] (Paper I). This is consistent with the previous reports based on observing mitotic figures.[390]

After the formation of the outer plexiform layer, most of the proliferating cells were situated in the middle region of the inner nuclear layer, suggesting the genesis of Müller cells. In support, mitotic figures immunopositive for Ki-67 can be seen in the inner nuclear layer (Paper I), as has also been reported by others.[166,318-320,328,388] The cells situated in this region undergo proliferation for the longest period of time. Many studies have suggested that the Müller cells are born early in development,[44,218,410] whereas others have shown a late proliferation that gives rise to the Müller cells.[67,257] It seems that Müller cells are born over a long period of time, and in the early stage the cells leaving the mitotic cycle give rise to a variety of neurons as well as Müller cells, while later mostly Müller cells are born. In rats[404] and frogs[174,422] the same progenitor cells have been shown to give rise to both neurons and Müller cells.

The presence of Ki-67 positive cells in the most proximal layer of the retina as well as very few cells in the inner nuclear layer after the first postnatal week indicates a second phase of proliferation, presumably glial[367] (Paper I). Similar late proliferating cells in the inner nuclear layer of retina have been reported in cat.[318,319] The cells in the nerve fiber layer are probably astrocytes[343,344] and were reported up to at least 4 weeks after birth. In our study, the late proliferating cells in the inner nuclear layer had the morphological characteristics of Müller cells. In previous studies,[326] [3H]-thymidine labeled cells in this region have been shown to contain immunoreactive vimentin, which marks Müller cells.[342]

Proliferation of cells in retinal transplants.

Already one day after transplantation the cells in the metaphase had began to organize themselves into rosette-like clusters. It is around this clustering that the proliferating cells in the transplant form the zone of interkinetic migration. E 15 may not be the best time for harvesting the donor tissue, which should ideally be harvested before the terminal mitosis.[58,59]

As the transplanted cells reorganize themselves into rosettes (E 19), the dividing cells also adopt the general proliferation pattern seen in normal retinogenesis. Cells in metaphase appear in the luminal layers of the rosettes which correspond to the ventricular surface of the normal retina, whereas cells in other phases are present in

the surrounding layers (Paper II and also presented elsewhere[369]). This also indicates interkinetic migration within the rosettes, similar to what is seen in the normal retina. Around at E 21, the postmitotic cells in the luminal layers of the rosettes begin to differentiate into photoreceptor cells. The proliferation in the outer layers of the rosettes continues to give rise to more photoreceptors and cells of the proximal retina. The non-proliferating cells in between the rosettes could be the differentiating amacrine and horizontal cells. In advancing stages the proliferation in the luminal layers further decreases (Paper II). The pattern of proliferation thus resembles that during the normal development where at E 25 and E 29 most of the proliferating cells are in a region distal to the postmitotic amacrine cells. In E 29 and PN 2 transplants, the proliferating cells are almost completely confined to the regions in between the rosettes, which correspond to the layers of the inner half of the normal retina (Paper II). This shift is also seen in the normal development of the retina[367] (Paper I). In the postnatal day 4 transplants and later, isolated patches of proliferating cells may be due to the mixing of the central and the peripheral retinas. In peripheral retina cells proliferate for longer period of time. Even though the mitotic activity in the transplant is comparable to the normal development in terms of pattern of proliferation, it seems that the transplanted cells do not proliferate as much as the cells in normal development. Indeed there are other factors involved in the growth of developing retina, which need consideration for comprehending the growth of the transplant in terms of area.

THERE IS GLIOSIS AT THE HOST GRAFT INTERFACE

The presence of non-proliferating and degenerating cells at the host-graft interface shows that a large number of transplanted cells undergo cell death (Paper II and also presented elsewhere[362]). Marked proliferation at the host-graft interface may represent abnormal gliosis. In rat transplants it has been shown that the host-derived Müller cells migrate into the graft already 2 days after the transplantation, along the host-graft interface.[353] In human xenotransplants, cellular retinaldehyde-binding protein (CRALBP), and glial fibrillary acidic protein (GFAP) immunoreactivity was found mostly close to the host retina.[354] Neural transplants often get encapsulated by a glial barrier that interferes with the integration of the graft with the host.[24] The observations in the present study suggest that retinal cell transplants also tend to develop a glial barrier. The gliosis at the host-graft interface is likely to interfere with the connectivity of the transplants with the host like it does in neural transplants to the brain.[24] It is likely that the gliosis is due to excessive cell death at this location. If so, eliminating or reducing the cell death in the transplants may improve the connections between the transplant with the host. Thinner, well-laminated transplants may have better access to nourishment from the host, and thus may have reduced cell death.

TRANSPLANTATION SITE INFLUENCES THE DEVELOPMENT OF THE GRAFT

The results of the study show that the transplantation site influence the growth of the grafts. The donor tissue at the time of transplantation (E15) consists largely of undifferentiated cells[367] (Paper I). The development of different retinal layers shows that the donor tissue matured at both the transplantation sites examined (epi- and subretinal), as reported by many authors in various models.[31,32,151,330,352,408]

In the present study, epiretinal and subretinal transplants were placed in the same eye. Nevertheless, only the subretinal transplants developed outer segments demonstrable by light microscopy and rhodopsin immunostaining. Further, in subretinal transplants, an outer limiting membrane was found, whereas this structure was not seen in epiretinal transplants. This suggests that in subretinal transplants, cells develop and mature more rapidly than in epiretinal ones. It is possible that the proximity of the graft to the host retina influences the development. In human embryonic retinal transplants to the epiretinal space of rat eyes, rosettes close to the host retina stain better for the photoreceptor specific proteins.[19] The same was found for cellular retinaldehyde binding protein, which marks the glial cells.[18,354]

IN THE SUBRETINAL SPACE, TRANSPLANTS DIFFERENTIATE ACCORDING TO THEIR INTRINSIC TIMETABLE

The results in the present study (Paper III and also presented elsewhere[368,370]), namely that NOS immunoreactive cells appear at E 29, that NOS was localized to two types of amacrine cells, and that their number increases in the first two post-natal weeks, all agree with previous results obtained by examining the localization of NADPH-diaphorase.[274] The ability of amacrine cells to express NOS even prenatally cannot be associated with a role of nitric oxide in the processing of visual information, as maturation and synaptogenesis involving these cells are not established until later.[82] It rather suggests that the early onset of NOS expression is genetically determined, and that nitric oxide may participate in the process of retinal development.

In the transplants also the NOS immunoreactivity was detected at a corresponding age of E 29, indicating that the ability to express NOS is maintained and follows the same timetable as in the normal developing retina. Similar to what was seen with normal developing retinas, there were no indications that a certain retinal cell type might express NOS only during development. Based upon the location of the reactive cells, it is reasonable to assume that the NOS immunoreactive cells found in the transplants correspond mainly to amacrine cells, like in normal retinas.

An inducible form of NOS can be expressed in macrophages and astrocytes in response to endotoxins and cytokines.[153] It can thus not be excluded that also the

transplanted cells express the inducible NOS isoform. However, the antibody employed in the present study is specific for the neuronal form of NOS, which excludes the possibility that the detected immunoreactivity was due to the inducible form of NOS.

With the transplantation technique used in this study, the grafts tend to organize themselves with rosettes rather than flat layers. Despite this, NOS labeled cells were often seen to emit processes so that the equivalent of an inner plexiform layer was identifiable. At times, NOS labeled processes were found to project for long distances. The development of NOS immunoreactive cells in the transplants according to their intrinsic timetable suggests that many of the factors required for normal development are preserved after transplantation. If this is true for other substances also, it may indicate that the transplants are capable of developing normal structure and functions.

Integration of retinal transplants

TRANSPLANTED NEURONS PROJECT TO THE TARGET AREA IN THE HOST RETINA

A few NOS immunoreactive processes could be seen to project towards the host retina (Paper III), and at times these processes entered the host retina (Paper IV). Such bridging was more often seen at long survival times. This is as expected considering that in normal developing rabbit retinas, outgrowth of NOS fibers is a relatively slow process.[300] Further, a better graft-host fusion is observed in old transplants (Paper VI). Certain NOS immunoreactive neurons were found to project to both the host inner plexiform layer and to an equivalent region within the transplant. Whatever the origin of these neuron might be, they appear to connect the host inner plexiform layer to that of the graft. If so, information from the graft could be conveyed to the host retina not only by neurons, which directly project to the host, but also indirectly. The number of NOS immunoreactive cells found in the transplants and of fibers crossing over was not very large. However, it may be noted that even in normal retinas, NOS is expressed in only a small population of cells.

It has consistently been difficult to demonstrate graft-host connections.[145,146,278,384] It is possible that the formation of connections may be limited in some way. As mentioned earlier, a rapid loss of the outer layers of the rabbit host retina was normally observed. Numerous dying cells are found also in the transplants, mainly in areas adjacent to the host retina (Paper II and VI). A gliotic response is seen to follow neuronal cell death, and as expected, has been observed in the host retina and with time also in grafts (Paper II).[147,354] Factors associated with reactive glia have been identified which inhibit neurite outgrowth.[260,272,386] Gliosis is therefore likely to influence negatively not only the development and survival of the grafted cells (including NOS-expressing cells), but also the formation of connections between graft and host retinas.

Despite conditions that might limit the formation of connections, these do occur. The connections are established by cells in the graft which maintain their ability to synthesize nitric oxide, one of the recognized retinal neuromodulatory compounds.[62,142,155,158,214,215,217,411] It may be noted that a fiber connection does not necessarily imply that the projecting NOS-containing cells establish synaptic contacts. However, there is evidence that neurites projecting from retinas transplanted to the brain are capable of forming synaptic contacts upon reaching the host target tissue.[79,80,385] NOS-containing processes not only project into the host retina, they were also seen to reach the host IPL. This indicates that subretinally grafted cells are potentially capable of recognizing their partner cells in the host retina.

One of the complexities of the neuroretinal transplants is that these grafts not only need to survive and grow in the host environment, but also need to form correct connections with the appropriate partners in the host. If the other cell types in the graft also retain their capacity to identify their targets in the host, a good basis is then provided for the functional integration of the transplants. Our results hint that this may be the case, although we do not know how effective the mechanism is. The long processes of the NOS immunoreactive cells were able to negotiate through the host retina and reach the host IPL. It is important to further investigate how various functionally defined and morphologically different neurons integrate with the host retina. It seems that sparsely distributed retinal cell types have long processes, perhaps because long processes are needed for these cells to form network of contacts with large number of other cell types. On the other hand, densely distributed cell types are able to form network of contacts even with small processes (unpublished observations). The cells with small processes (for example GLYT1 immunoreactive small field amacrine cells[267]) are able to integrate with the host by mingling with similar cell types in the host (unpublished observations).

Organization in retinal transplants

ARCHITECTURAL ORGANIZATION IN TRANSPLANTS IS INFLUENCED BY THE PHYSICAL STATE OF THE DONOR TISSUE

For clinical purposes, rosette-free, well-laminated retinal transplants are desirable. In the study, lamination of the large sheet transplants was different from that of fragmented tissue transplants (Paper V and also presented elsewhere[359]). The lamination in these transplants was largely rosette-free in the epiretinal space and developed arcuate arrays in the subretinal space, unlike rosettes in the fragmented tissue transplants. The difference in the organization of the transplant was confirmed by vimentin immunostaining of the Müller cells, which are radially arranged in fragmented tissue transplants.[353,354] Rosettes in the fragmented tissue transplants showed radial arrangement of the Müller cells also in the present study, but the arcuate arrays that dominate the large sheet transplants and most parts of the epiretinal transplants showed a palisade-like arrangement of the Müller cells.

Clearly, the transplants done with two different techniques can develop different morphologies. The fragmentation of donor tissue is likely to disturb the cell adhesion molecules. The reaggregation of cells may be important for the eventual cytoarchitecture of the tissue. Large sheet transplant donor tissue architecture was minimally disturbed at the time of transplantation, especially in the epiretinal transplants, which were not even covered by the host retina. After transplantation, no or little reaggregation of the cells took place in the minimally disturbed donor tissue and therefore they developed a largely rosette-free lamination when placed in the epiretinal space. When transplanted to the subretinal location, arcuate arrays appeared, which are structurally different from rosettes, as shown by vimentin staining. However, rosettes developed at places in large sheet transplants where the donor tissue was disturbed and thus needed reaggregation, like at the margins of the epiretinal transplants. The observations are in good agreement with those obtained by modifications of this technique.[107,138,366]

Long term survival of retinal transplants

Retinal transplants can survive for long time

Most clinical situations where retinal transplantation could conceivably be of therapeutic use call for the reconstruction of the photoreceptor layer. As seen in the short-term transplants in these studies, photoreceptor cells are capable of developing and differentiating in the rosettes, where they are not in direct contact with the host retinal pigment epithelium.[32,364] The observations in this study suggest that the photoreceptors require the support of pigment epithelium for long-term survival (Paper VI and also presented elsewhere[361]). This support is apparently not needed for the initial development and differentiation of the photoreceptor cells, as they develop and differentiate well even in the absence of the pigmented epithelium. Studies on Royal College of Surgeons (RCS) rats are consistent with this finding. In RCS rats the photoreceptors are capable of initial differentiation even in the presence of a defective RPE.[96,226,279,431] Nevertheless, RPE is needed for the long-term maintenance of photoreceptors also in retinal transplants.[147,350]

It seems that photoreceptors are particularly vulnerable to degeneration. It should be noted that the large and varied complement of genetic mutations found in retinitis pigmentosa seem to damage predominantly the photoreceptor cells. In the RCS rat retina where the host pigment epithelium is defective, it is also the photoreceptor cells of the neural retina, which are effected most, whereas the cells of the inner retinal layers to a large extent are preserved.[340,371,372]

The precise mechanism of cell death in long-term retinal transplants is not clear. Immunity is one possibility, but the absence of inflammatory cells suggests that mechanisms other than that may be at play. It has been shown that there is massive cell death by apoptosis in the graft and the surrounding host retina soon after trans-

plantation, but the cell death stabilizes after some time. Nevertheless, some apoptotic cell death can still be observed at the longest time (61 days post transplantation) examined in the study.[434] It is possible that slow apoptosis continuing for a long time may be responsible for the death of the photoreceptors in the transplants. The excess of glial tissue noted in certain transplants could result from such slow and prolonged but nevertheless increased cell death in the transplants. Excessive glial cell proliferation and gliosis has been observed in retinal transplants (Paper II)[354] as well as in retinal degenerations.[117]

Our long-term transplants had remarkably good morphological fusion with the host retina. It has been observed that among other factors, absence of the debris of degenerating cells at the host-graft interface helps the graft cells to project to, and form connections with the host retina. Such connections remain to be established in the long-term transplants.

Patients with retinitis pigmentosa and other tapetoretinal degenerations who can potentially benefit from retinal transplantation essentially need photoreceptor cell replacement. For the long-term survival of photoreceptors in the transplants, a proper apposition of the graft photoreceptors with the host retinal pigment epithelium seems to be important. It will therefore be essential to evolve surgical techniques in which large sheets of donor retinas can consistently and predictably be placed in to the subretinal space, well apposed to the host retinal pigment epithelium, or to cotransplant the RPE and the neural retina together. Progress is already underway in this direction.[357,358]

Host retina

One of the factors that will determine the success of retinal transplantation is its ability to form synaptic connections with the host retina. Studies have shown that this indeed happens (Paper IV).[22,106,145,146,278,355,384] In retinal degenerations, the outer nuclear layer is most extensively effected, but the inner retinal layers are relatively well preserved.[340,371,372] The strategy of retinal transplantation is based on the hope that the transplanted retinas will be able to form connections with this layer. It is therefore important to study the changes resulting from transplantation surgery in the host retina overlying the retinal transplant.

THE OUTER NUCLEAR LAYER OF THE HOST RETINA DEGENERATES AFTER TRANSPLANTATION

The outer nuclear layer of the host retina degenerate after transplantation surgery. It is not clear whether the cell death observed in the graft and the host is due to some host-graft interaction or due to lack of nourishment reaching the cells in the transplant and the host retina which are situated away from their source of nourishment, the host choroid. The long-term transplants tend to stabilize in thickness, suggesting that the thickness reached might be the optimum one, perhaps determined by the availability of nourishment reaching it from the host choroid. It has been noted

that subretinal transplants that are small in size undergo minimum degeneration (Paper II). Degeneration of the outer nuclear layer of the host retina seems to facilitate the appearance of nerve fiber connections between the graft and the host. At places where a few cell layers of the photoreceptors were left, grafts failed to form contacts with the host (Paper IV). It seems that the contacts between the graft and the host are very specific (projecting fibers are able to reach the target regions) and preserved host photoreceptors may perhaps act as a barrier interfering with the ability of transplanted cells to recognize their possible targets in the host.

Glial cells in the host retina proliferate after surgery

Four days after surgery, proliferating cells were seen in the host retina overlying the transplant. To some extent, the proliferation in the host retina could be seen even in older transplants (Paper II). These cells are likely to be glial cells because Müller cells are known to proliferate after retinal damage.[179,220,291] Further, there are indications that host Müller cells respond to the transplantation surgery. For instance, they express glial fibrillary acidic protein (GFAP) already 4.5 hours after the surgery, and this reaction spreads out to the whole retina within 1 day. It lasts for at least 7 weeks.[353] Hypertrophy and migration of Müller cells within the host retina has also been noticed adjacent to the lesion site, commencing 2 days after the transplantation.[353] This suggests that the proliferating cells in the host retina are likely to be glial cells. It is possible that gliosis in the host retina may interfere with the connectivity of the transplant (Paper IV). It may be worth investigating ways of controlling gliosis with the hope of improving the connectivity of the graft.

Host inner retinal layers remain capable of receiving connections from transplants

Both the light microscopic as well as the immunohistochemical studies have shown that the host inner retina overlying the graft is relatively well preserved (Papers III, IV, V, VI). Indeed, preservation of this layer is vital for the graft connectivity and its subsequent success. In long-term transplants ganglion cells were observed in the host overlying the graft. This indicates that whatever connections the graft forms with the host in this region have a chance to maintain their functionality for long periods of time. It is equally important to demonstrate the functional capability of the host outer plexiform layer because for instance photoreceptor cells/sheet transplants would require integration with the host at this site. Further investigations are needed to assess this.

CONCLUSIONS

Retinal transplants survive, differentiate, and develop all the retinal layers and most of the normal retinal cell types. However, the growth of the transplants is limited and, at least with the tissue fragment transplant technique, the lamination in the transplants is not entirely normal. So-called rosettes are regularly formed in them. It is also important to investigate to what extent transplanted cells maintain their intrinsic ability to differentiate and what factors influence the development of retinal transplants.

Good integration between host and transplant is vital if transplants are to have any functional use as nerve signal generators and conveyors. Transplanted cells must have enough plasticity to be able to form functioning connections with the host retina, which itself should remain capable of receiving such contacts. In a clinical situation, retinal transplants also need to survive in the eye for extended periods of time. It is therefore important to determine the long-term fate of the retinal transplants. The present study has attempted to answer some of these questions. The observations suggest:

1. After transplantation, the donor tissue continues to proliferate, but not as much as in the normal developing retina. The pattern of proliferation in the transplants resembles that of the normal retina. A layer of cells in the retinal transplants close to the host retina does not proliferate. There may be abnormal gliosis at the host-graft interface of retinal cell transplants.
2. Damage to the donor tissue at the time of transplantation plays an important role in rosette formation. Good architectural organization of retinal transplants can be maintained by gentle and careful handling of the donor tissue at the time of transplantation.
3. The transplantation site influences development of the retinal transplants. Donor tissue does not mature as well in the epiretinal space as in the subretinal.
4. In retinal transplants, the nitric oxide containing cells differentiate with the same intrinsic timetable as in the normal developing retina. This suggests that the differentiation of transplanted retinal cells is largely controlled by intrinsic factors rather then by their environment.
5. Transplanted retinal neurons do have enough plasticity to send processes to the appropriate locations in the host retina.
6. Retinal transplants survive for long time after transplantation. The photoreceptor cells appear to need the support of pigment epithelium cells for their long-term survival.
7. The host retina adjacent to retinal transplants appears to maintain at least some ability to form functioning connections with the graft, and it may maintain it for long times (years) after transplantation.

CONCLUDING REMARKS

Most of the research done on retinal transplantation so far has essentially been demonstrations of what happens spontaneously to donor tissue after transplantation. In a sense this has been an easy task, and the information obtained is very encouraging. Nature has been generous: transplanted fetal cells are spontaneously able to overcome several of the conceivable major hurdles when developing transplantation into a clinically useful tool. These include ability to grow, differentiate, integrate and survive. However, certain aspects of transplantation, the most important being the connectivity between the host and the graft, will need some type of further manipulation. This is where the challenge and the key to clinically successful retinal transplantation rest. The task may be difficult, but not impossible. It is possible that the answers to these challenges may come from very unrelated developments in the biological sciences. Our fast increasing knowledge on the plasticity (and its potentials) of the central nervous system, and our understanding of the principles behind neural development have already opened up new venues for manipulating the growth and integration of transplanted cells with the host, and will most likely continue to do so for many years to come.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Gruppen ärftliga degenerativa näthinnesjukdomar är en viktig orsak till blindhet världen över. Man har uppskattat frekvensen till 1 på 3 - 4000 vilket motsvarar omkring 1° miljoner människor i världen som helhet. Den vanligaste orsaken till en sådan sjukdom är ett fel i en gen för ett funktionellt eller strukturellt protein. Mer än hundra sådana gendefekter är kända idag, och antalet ökar kontinuerligt. I vissa av dessa sjukdomar är det exakta biokemiska felet känt vilket möjliggör en specifik terapi, men endast ett fåtal patienter är drabbade av en sådan sjukdom. För de flesta av sjukdomarna saknas effektiv terapi. Senare tids forskning har emellertid öppnat nya möjligheter, till exempel genom att placera en frisk gen i arvsmassan, genom att kontrollera oönskade effekter av den sjuka genen, eller genom att ersätta de felaktiga cellerna genom transplantation.

En svårighet i utvecklandet av specifika behandlingar av degenerativa näthinnesjukdomar är att varje felaktig gen kräver sin egen unika behandling. Därför försöker man istället utveckla ospecifika "anti-degenerationsbehandlingar". I detta sammanhang har man undersökt tillväxtfaktorer, som är ämnen som nervcellerna själva använder för sin utveckling och överlevnad. En annan forskningslinje är att försöka påverka apoptosen, som är den process som slutligen orsakar nervcellernas död i degenerativa näthinnesjukdomar. En tredje väg är att försöka ersätta de sjuka cellerna genom transplantation av friska näthinneceller, något som redan har prövats på människor. Dessa initiala transplantationsförsök har givit intressanta resultat, men också visat att mera experimentellt arbete återstår innan man kan gå vidare.

Huvudsyftet med denna studie var att bättre förstå tillväxten, utvecklingen, integrationen, organisationen och överlevnaden av näthinnetransplantat, med förhoppningen att så småningom kunna utveckla näthinnetransplantation till en kliniskt användbar behandlingsmetod för degenerativa näthinnesjukdomar såsom retinitis pigmentosa.

Omogen näthinna överlever en transplantation bra men utvecklas inte helt normalt. Till exempel så utvecklas cellerna i små bollar, så kallade rosetter, och även om många givarnäthinnor transplanteras tillsammans så förblir transplantatet litet. Den transplanterade näthinnan utvecklar bara ett fåtal förbindelser med värdnäthinnan, och transplantatet får svårt att förmedla nervsignaler till värden på rätt sätt. På många patienter måste transplantatet överleva i värdögat mycket länge, men näthinnetransplantatets förmåga till långtidsöverlevnad är inte känd.

Denna studie adresserar en del av dessa problem. För att undersöka varför näthinnetransplantatens tillväxt är begränsad jämfördes celldelningen i

näthinnetransplantat med celldelningen i normal näthinna med syfte att se om transplantatcellerna betedde sig onormalt. Med immunohistokemi identifierades ett protein (Ki-67) som finns enbart under celldelningen. Transplantatceller visar därvidlag samma mönster som normal näthinna, men delar sig inte lika frekvent. Dessutom delar sig gliaceller (stödjeceller) kraftigt i gränsen mellan värd och transplantat.

Kväveoxid (NO) spelar en roll i synapsutvecklingen i centrala nervsystemet. För att undersöka om transplanterad näthinna bibehåller sitt normala utvecklingsmönster jämfördes kväveoxidsyntasförekomsten (ett enzym som bildar kväveoxid) i transplantat med normal näthinna. Inga skillnader kunde påvisas. Vidare kunde långa cellutskott innehållande kväveoxidsyntas påvisas i rätt cellager (det inre plexiforma cellagret) i värdnäthinnan, vilket visar att transplanterade näthinneceller kan nå fram till värdnäthinnan och kanske även utveckla cellkontakter på rätt ställe.

För att utveckla en metod för att få rosettfria näthinnetransplantat och för att jämföra dess utveckling på olika transplantationsplatser transplanterades stora bitar embryonal kaninnäthinna epiretinalt och subretinalt på vuxna kaninögon med en ny transplantationsteknik. Detta gav transplantat som var rosettfria utom på platser där givarnäthinnan hade skadats under transplantationen. Vidare utvecklades transplantaten bättre subretinalt än epiretinalt.

I kliniska situationer måste transplantaten överleva under mycket lång tid. Därför studerades långtidsöverlevnaden i transplantaten (upp till 2 år). Nervceller överlever i vissa transplantat även om gliaceller ibland tar överhanden. Fotoreceptorer överlever bara om de får god kontakt med pigmentepitelet.

Som tidigare påpekats så utbildar transplantaten förbindelser med värdnäthinnan. Det är viktigt att åtminstone den inre delen av värdnäthinnan fortsätter att fungera och har kvar förmågan att ta emot förbindelser från transplantatet. Våra observationer visar att även om den yttre delen av värdnäthinnan (som har kontakt med transplantatet) degenererar, så fungerar fortfarande den inre delen och har kvar sin förmåga att ta hand om signaler från transplantatet.

ACKNOWLEDGMENTS

Do not the most moving moments find us without words?

I would like to thank scientific adviser and Guru Berndt Ehinger for introducing me to science, and for sharing his punditry of neurobiology. I will specially like to mention two of the mantras that I learnt from Berndt. Sutra 1, look at the scientific problems through different 'data bases'. Sutra 2, words differently arranged have a different meaning and meanings differently arranged have a different effect. I would also like to thank Berndt for allowing me the liberty of doing things on my own.

Anitha Bruun! I would like to thank you for lending your shoulder for crying, grumbling and complaining.

Katarzyna Said! thanks a lot for being a friend. I do not want to thank you for huge technical help you have given me, because I value your friendship far more then your help. I guess I can ignore it.

Brother Sven! Thank you for teaching me what I did not learn at SFI. Thanks also for allowing me to 'boss around' with you a little. At times I have pressed you more than I would have liked to; but it was a choice between my survival, or yours.

Maria Thereza Rodriguez-Perez! Thank you for material, intellectual and spiritual discussions.

Ingela Larsson! Thanks for walking on the 'lower side' of the road in Fort Lauderdale, and listening to my BJs.

Bodil Israelsson! Thank you for being a patient listener, and for smiling even when you should have been furious.

Karin Arnér! Thank you for Å, Ä, Ö

Karin Warfvinge! Thank you for imbibing social sense into me once in a while.

Majvi Persson! thank you for educating me about 'Skåne'

I would also like to thank others in the laboratory and the eye clinic for their help, specially, Kjell Johansson, Theo van Veen, Elisabet Agardh, Anna Makár, Janina Waga, Anitha Blixt, Bengt Juliusson, Fredrik Ghosh, Johan Wassélius, Jörgen Larsson, Ming Hu, Yuning Qian, Angelika Skarin, Maria Elena Caminos Benito, Madeleine Selvander, and Gunnel Jahnsén.

I would like to thank Jeppe Stridh for keeping my reflexes brisk, and his staff for actual help with animals.

I would like to thank Anders Bergström for clinical discussions, Kennerth Wilke for keeping me as a part of the teaching team, Sten Andréasson and Vesna Ponjavic on discussions on retinal degeneration.

Lillian Andersson! Thank you for making my life easier from time to time.

I would also like to thank the co-authors Charles L. Zucker and Alan R. Adolph at the Harvard Medical School, Boston for their cooperation.

I would like to thank Erik Scherfig, Morten la Cour and Anne Katrine Wiencke at the Rigshospitalet in Copenhagen for the collaboration on RPE transplantation. It has been a pleasure to be in Copenhagen, and to hurriedly slip into some museum.

Once again Katarzyna, Ingela, Bodil, Karin and all my coffee room friends; thank you for bearing with my Swedish. Your friendship has reiterated my faith in God. After all that I have been talking to you, if I am not buried under your 'very high heels'; there must be a 'God' up there some where.

I also wish to thank Mats Hugo-Persson for introducing me to Sweden. I would like to thank Lena for painstakingly teaching me the intricacies of the systems here. Jakobssons, Saids, Warfvinges, Sundströms, Manaridous, Klaus and Eva, for providing me social life here.

I am grateful to Bhishm, Adersh, Meenakshi, Yogesh, Rama, Ranveer, Jatashankar, Shivshankar, Vinod, Guptas and all the well wishers who stood by me in difficult times. I thank some for giving me the memories, and others for the dreams. I remember Rameshwar, Darshan, Shyam, Subadhra, Indira, and Trilok for the roots (and the wings).

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APPENDIX

Paper I

Exp. Eye Res. (1997) 64, 97–106

Mitosis in Developing Rabbit Retina: An Immunohistochemical Study

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(Received Seattle 15 February 1996 and accepted in revised form 1 July 1996)

The proliferation of cells in the embryonic and postnatal rabbit retina was studied with the MIB-1 antibody which demonstrates the Ki-67 antigen. Already at embryonic day 15 there were postmitotic cells (i. e. cells that do not stain with the MIB-1 antibody) in the basal part of the neuroblastic cell mass which are presumably the differentiating ganglion cells. After the formation of an inner plexiform layer at around embryonic day 25, postmitotic cells were seen in the proximal part of neuroblastic cell mass (presumably amacrine cells) as well as in the ganglion cell layer. Proliferating cells accumulated distal to the layer of postmitotic cells and their number gradually decreased towards the pigmented epithelium. At birth, proliferation ceased in the central parts of the retina but in the peripheral parts it continued for 7 days although rare cells could be seen for up to 15 days. After the formation of the outer plexiform layer, the proliferating cells in the outer nuclear layer accumulated close to the outer plexiform layer whereas the postmitotic cells (the differentiating photoreceptors) did so at the distal part of outer nuclear layer. Some cells in the middle of the inner nuclear layer (presumably the Müller cells) and some cells in the ganglion cell layer or nerve fiber layer (presumably the astrocytes) proliferated for the longest period of time.

Key words: Proliferation; mitosis; development; retina; cytogenesis; Ki-67; immunohistochemistry.

1. Introduction

Like in other neural tissues, the events in the developing retina occur in a precise and predetermined order. They can be described as proliferation and migration, formation of the final cells and the tissue shape, differentiation of structures at the cell and the tissue level, differentiation of specific receptor or neuron structures, differentiation of specific retinal or neuronal substances and chemical processes, and finally the differentiation of specific retinal and neuronal activities.

Dividing cells in the neural tube characteristically move between the pial surface and the luminal surface or the ventricular surface of the tube. The cells synthesize DNA while away from the ventricular surface and divide while on it. This movement of the dividing cells, called The 'interkinetic migration' (Berry, 1974; Sauer, 1935), marks the 'ventricular zone' (Angevine, 1970) in the wall of the neural tube. Another zone of mitotic activity in the developing CNS, away from the ventricular surface, has also been identified, and it is suggested that this plays a role in the gliogenesis (Schmechel and Rakic, 1979; Lewis, 1968) and that certain intrinsic neurons may also arise there (Altman and Das, 1966). Interkinetic migration in the retina has been reported in mice (Hinds and Hinds, 1974; Sidman, 1961) and cats (Rapaport, Robinson and Stone, 1984). In the cat retina, three zones of cell division have been recognized. Two of these zones correspond to the ventricular zone and the sub-ventricular (non-ventricular) zone proliferation. A third area of proliferation in the nerve fiber layer or the ganglion cell layer gives rise to the vascular endothelium (Rapaport, Robinson and Stone, 1985). Similarly, in addition to the ventricular phase of cellular proliferation, a non-ventricular late phase has been shown in rabbits (Reichenbach et al., 1991).

Previous proliferation studies on the developing rabbit retina utilized the ornithine decarboxylase activity of the whole retina (Foresman, Cohen and Das, 1985), counted the mitotic figures (Stone, Egan and Rapaport, 1985) or used [^{3}H]-thymidine autoradiography (Reichenbach et al., 1991). To our knowledge this is the first time an immunohistochemical approach has been used to study mitosis in the rabbit retina. The observations corroborate and expand results obtained with other methods (Stone, et al., 1985; Reichenbach et al., 1991).

The MIB-1 antibody used in this experiment detects the native protein, Ki-67, as well as fragments of the Ki-67 molecules (Key et al., 1993). The Ki-67 antigen is a non-histone protein assembled from two polypeptide chains with an apparent molecular weight of 345 and 395 kilodalton (Gerdes et al., 1991). It is a nuclear cell proliferation-associated antigen that is expressed throughout the cell division cycle (phases G1, S, G2 and M). It is absent in resting cells (stage G0; Gerdes et al., 1983; 1984). The function of the Ki-67 antigen is unknown but immuno-staining with this antibody is a reliable tool for detecting proliferating

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0014–4835/97/010097+10 $25.00/0/EY960189

cells (Bilous, McKay and Milliken, 1991; Houmand, Abrahamsen and Tinggaard Pedersen, 1992).

Since MIB-1 labels cells in all the phases of cellular proliferation, it is able to give an accurate representation of the dividing cells in various stages of cell cycle in different layers of the retina.

2. Materials and Methods

Developing Retinas

Rabbit embryonic day (E) 15 ($n = 2$), 25 ($n = 2$), 29 ($n = 2$) eyes were enucleated from embryos obtained by cesarean section after killing the pregnant pigmented rabbits with an overdose of sodium pentobarbital. Eyes were also obtained from postnatal day (PN) 0 ($n = 3$), 3 ($n = 2$), 5 ($n = 2$), 7 ($n = 2$), 11 ($n = 2$), 15 ($n = 2$), 20 ($n = 2$), 46 ($n = 1$), and 60 ($n = 1$) pigmented rabbits. The age of the animals was determined by mating the rabbits during a 1 hr period on a known date.

Tissue Processing

The eyes were briefly fixed in 4% paraformaldehyde in phosphate-buffer saline (PBS; 0·1 M phosphate, 0·85% NaCl; pH 7·4). The anterior segment of the eyes was removed and the posterior segment was fixed in the same fixative for 24 hr. The tissue was rinsed in the same phosphate-buffered saline and then with 5%, 10% and 20% sucrose added. The eyes were stored in PBS with 20% sucrose and 0·1% sodium azide until sectioned. Twelve micron thick sections were cut on a cryostat and mounted on glass slides coated with gelatin. They were subsequently stored at −70°C until used. Sagittal sections were cut from the embryonic and postnatal retinas and the sections passing close to the optic nerve were used for immunohistochemistry.

Immunohistochemistry, Hematoxylin and Eosin Staining

Frozen sections were thawed to room temperature and then rinsed in PBS containing 1% bovine serum albumin (BSA; Sigma Chemical Co.). The sections were blocked with 1·5% normal horse serum prior to their incubation with the MIB-1 monoclonal antibody (Immunotech, Inc., Westbrook, ME, U.S.A.) at a dilution of 1:200 in PBS. Optimum working concentration and incubation time for the antibody was determined earlier in pilot experiments. Slides were incubated for 72 hr at 4°C in a humidified chamber. After 72 hr, the slides were rinsed with PBS and incubated for 1 hr in biotin conjugated horse-anti-mouse secondary antibody (Vector Lab. Inc. Burlingame, CA, U.S.A.) at a dilution of 1:200. Slides were rinsed in PBS and endogenous peroxidases in the tissue were quenched by incubating the slides in 0.3% hydrogen peroxide in PBS for 15 min. The slides were then incubated in avidin and biotinylated horseradish peroxidase complex (Vectastain® Elite ABC Kit; Vector Lab. Inc. Burlingame, CA, U.S.A.) for 1 hr. Slides were again rinsed in PBS and the HRP reaction was developed in a substrate solution of diaminobenzidine (DAB; Peroxidase Substrate Kit; Vector Lab. Inc. Burlingame, CA, U. S.A.). Adjacent sections were stained with hematoxylin and eosin.

In this paper the terms 'basal' or 'proximal' and 'apical' or 'distal' are used to denote localization in terms of how the tissue develops. In the neuroretina, 'basal' thus means closer to the vitreous and 'apical' closer to the photoreceptors.

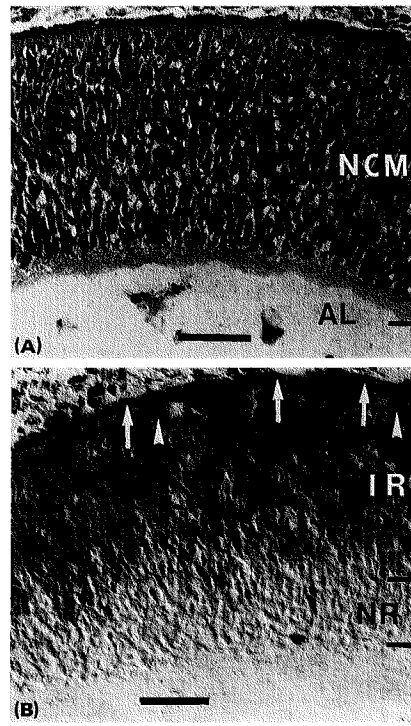

FIG. 1. The central part of an embryonic day 15 rabbit retina. (A) A haematoxylin and eosin stained section showing the multi-layered neuroblastic cell mass (NCM) and a thin anuclear layer (AL). (B) An immunohistochemically stained section with MIB-1 antibody. MIB-1 immunoreactive cells in the apical two third of the neuroblastic cell mass and non-reactive cells in the basal one third. Cells close to the pigmented epithelium are more intensely stained than those in the basal part, especially some large rounded cells in the distal-most rows of the neuroblastic cell mass (arrowheads). Immunoreactive cells are also seen in the pigment epithelium (arrows). Bar = 50 μm.

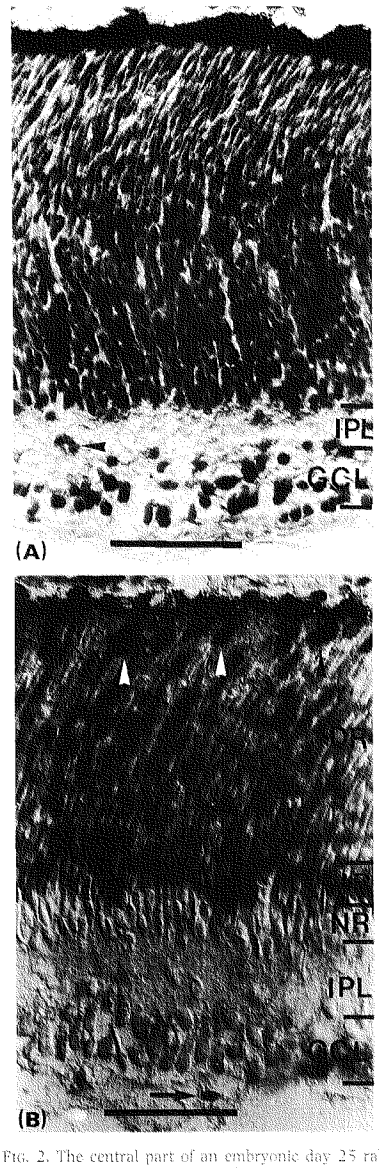

FIG. 2. The central part of an embryonic day 25 rabbit retina. (A) An haematoxylin and eosin stained section showing the formation of inner plexiform layer (IPL) and the ganglion cell layer (GCL). Some cells are observed in the inner plexiform layer (arrowheads). (B) An immunohistochemically stained section with MIB-1 antibody showing a thin non-immunoreactive layer (NR) in the basal part of the neuroblastic cell mass. Immunoreactive cells (IR) are accumulated close to the non-reactive layer, followed distally by the region of diffuse reactivity (DR). Deeply stained cells are accumulated in the apical most part of the retina (arrowheads). Some immunoreactive cells are also seen in the ganglion cell layer or nerve fiber layer (arrows). IPL, inner plexiform layer, GCL, Ganglion cell. Bar = 50 μm.

3. Observations and Results

Embryonic Stages

At stage E15, the sensory retina consists of a thick, multi-layered neuroblastic cell mass and a thin inner anuclear layer with a thickness of about one fifth to one seventh of the total neuroblastic layer [Fig. 1(A)]. MIB-1 immunohistochemistry showed positive cells in the outer two thirds of the neuroblastic cell mass, whereas its innermost third contained mainly non-reactive cells [Fig. 1(B)]. Cells close to the retinal pigment epithelium were more intensely stained than the ones close to the anuclear layer, especially some rounded cells (most likely cells in metaphase) situated in the outermost few cell rows. MIB-1 immunoreactivity appeared in most of the pigment epithelial cells [Fig. 1(B)].

At stage E25, the ganglion cell layer separated from the neuroblastic cell mass [Fig. 2(A)]. As seen in Fig. 2(B), some cells in the ganglion cell layer or nerve fiber layer were MIB-1 immunoreactive. A thin layer of non-immunoreactive cells appeared in the innermost part of the neuroblastic cell mass, close to the inner plexiform layer. There was an accumulation of elongated immunoreactive cells in the part of the neuroblastic cell mass which was adjacent to the non-reactive region. More apically, there was a region with elongated cells showing less immunoreactivity. Finally, there was a population of large, rounded and deeply reactive cells close to retinal pigment epithelium. MIB-1 immunoreactivity was present in most of the pigment epithelium cells.

At stage E29 the developing outer plexiform layer became apparent in the central part of the retina and some cells in the basal part of the neuroblastic cell mass were rounded [Fig. 3(A)]. The distribution of the MIB-1 immunoreactivity was identical to that seen in the E25 retina except that the non-reactive layer in the basal part of the neuroblastic cell mass was thicker, approximately one ninth of the neuroblastic cell mass [Fig. 3(B)]. It corresponded to the rounded cells seen in hematoxylin and eosin stained sections. Apart from the above described non-reactive layer, the rest of the neuroblastic cell mass contained immunoreactive cells of different kinds. Most of them were elongated and appeared in the part of the neuroblastic cell mass which was adjacent to the non-reactive cells, like in E25 retinas. However, their number was smaller (compare Figs 2 and 3). The number of immunoreactive cells gradually decreased towards the distal part of the neuroblastic cell mass. Large, rounded, deeply stained cells were still present in the

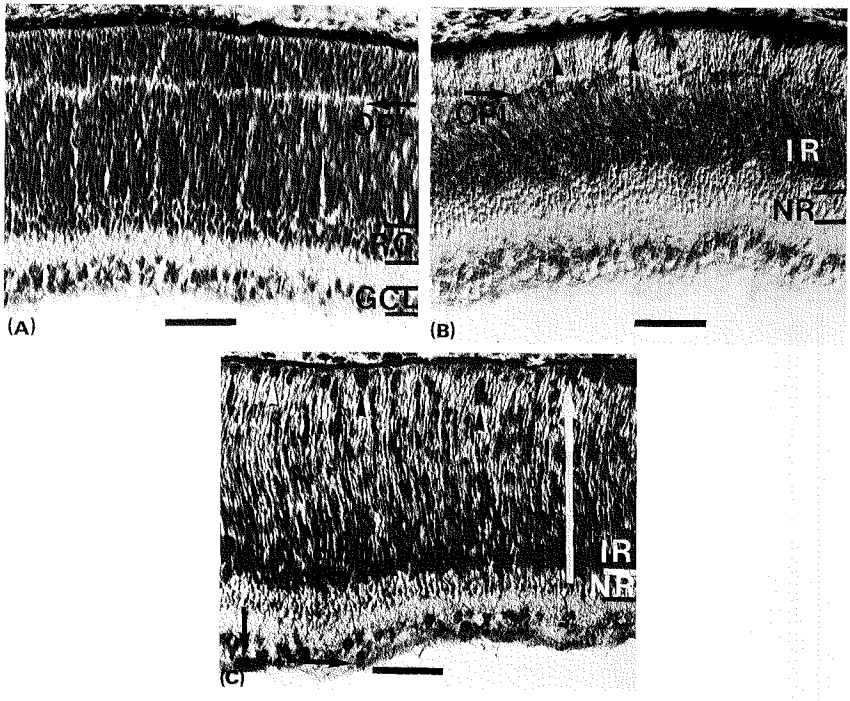

FIG. 3. An embryonic day 29 rabbit retina. (A) An haematoxylin and eosin stained section from the central part of the retina showing the formation of the outer plexiform layer (OPL; arrow). The ganglion cell layer (GCL) is thinner than in the retinas from rabbits at E25. Cells in the proximal (basal) part of the inner nuclear layer are round (RC). (B and C) Immunohistochemically stained sections of the central (B) and the peripheral (C) parts of the retina showing non-immunoreactive layer in the basal part of the neuroblastic cell mass (NR), corresponding to the rounded cells (RC) in 3(A), and an accumulation of reactive cells distal to it (IR). The reactivity gradually decreases [in the direction of the large arrow in 3(C)] towards the distal part. Large deeply stained cells are situated in the distal-most part (arrows heads), and also there are reactive cells in the ganglion cell layer or the nerve fiber layer [small arrows in 3(C)]. Reactivity in the outer nuclear layer is much less than that in the middle of inner nuclear layer in 3(B). Bar = 50 μm.

region close to the pigment epithelium [Figs. 3(B) and (C)]. In the central retina, an outer plexiform layer could be seen, and the number of immunoreactive cells decreased in the vicinity of outer plexiform layer [Fig. 3(B)]. Some cells in the ganglion cell layer or nerve fiber layer were immunoreactive to the MIB-1 antibody. In the embryonic day 29 retina, pigment granules in the pigment epithelium were prominent, making it difficult to judge the MIB-1 immunoreactivity.

Newborn Stage PN0

An area essentially devoid of MIB-1 immunoreactive cells developed in the central retina at PN0 [Fig. 4(A)]. The number of immunoreactive cells increased from this area towards the periphery [Fig. 4(A), (B), (C), (D)]. In the peripheral part of the retina at PN0, the reactivity was generally less than at E29. Especially in the outer nuclear layer, the reactivity decreased in the regions where the outer plexiform layer had developed [Fig. 4(C) and also seen in Fig. 5(B) in PN3]. Some cells were immunoreactive in the outer nuclear layer, but most cells were not or only weakly so. Most reactive cells in the outer nuclear layer were arranged adjacent to the outer plexiform layer and some were scattered through out the thickness of the outer nuclear layer. Large, rounded, deeply stained cells were still present close to retinal pigment epithelium in the outer nuclear layer, and more so in the peripheral retina than the central area of the retina. The inner nuclear layer was more immunoreactive than the outer nuclear layer and some large, rounded and deeply stained cells could be seen in this region also [Fig. 4(C)]. The reactivity in the inner nuclear layer was less in comparison with E29, and was confined to the elongated spindle-shaped

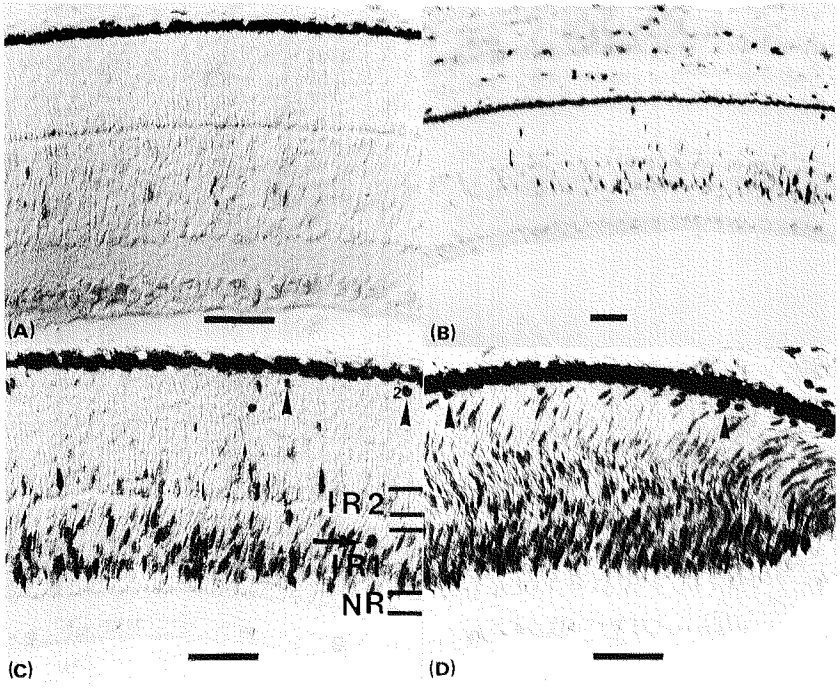

FIG. 4. A postnatal day 0 rabbit retina stained with the MIB-1 antibody at different eccentricities, central (A), mid periphery (B), periphery (C) and retinal edge at the ora serrata (D). The central area shows no immunoreactivity, but it gradually increases towards the periphery. The immunoreactivity is generally less than in comparable areas from 29 day retinas [Fig. 3(C)]. Most of the reactive cells in the outer nuclear layer are accumulated close to the outer plexiform layer (IR2) and large deeply stained cells are situated towards the distal part (C and D, arrowheads). Immunoreactive cells in the inner nuclear layer (IR1) are confined mostly to the middle parts of this layer where some large deeply stained cells can be seen (C, arrow). Cells in the proximal and the distal parts of this layer are non-reactive (C). In 4(D) there are many more large and deeply stained cells (arrowheads) than in the more central retina. Bar = 50 μm.

cells in the middle and the outer parts of this layer. There was a drop in the number of immunoreactive cells in the outer part of the inner nuclear layer, and the inner parts of this layer were not immunoreactive. Most cells in the ganglion cell layer were non-reactive, but in certain regions reactive cells could be seen in the nerve fiber layer. Immunoreactive cells at this location were observed in later stages also.

Postnatal Stages

At stage PN3, more cells became non-reactive in the central retina. Thus, the area with no or few immunoreactive cells enlarged. Most of the immunoreactive cells outside the non-reactive area were found in the middle part of the inner nuclear layer, and were usually elongated [Fig. 5(A)]. However, the number of large, deeply stained cells was less than at previous stages, and these cells were more in the peripheral than in the central retina [Fig. 5(B)]. Only few immunoreactive cells were present in the ganglion cell layer or the nerve fiber layer, especially close to the optic nerve.

At stage PN5 the immunoreactive cells were confined to the peripheral retina, where few cells in the innermost part of the outer nuclear layer were immunoreactive. Most of the cells in the inner nuclear layer were not immunoreactive, and among the cells that were positive, most were situated in the middle part of the inner nuclear layer (Fig. 6). The central area with no or only few immunoreactive cells had enlarged. In the ganglion cell layer or nerve fiber layers, some cells were immunoreactive. In the peripheral parts of the retina, MIB-1 positive cells were occasionally seen in the outer plexiform layer.

At stage PN7 there was a prominent center to periphery gradient in the number of immunoreactive cells, which were essentially absent in the central

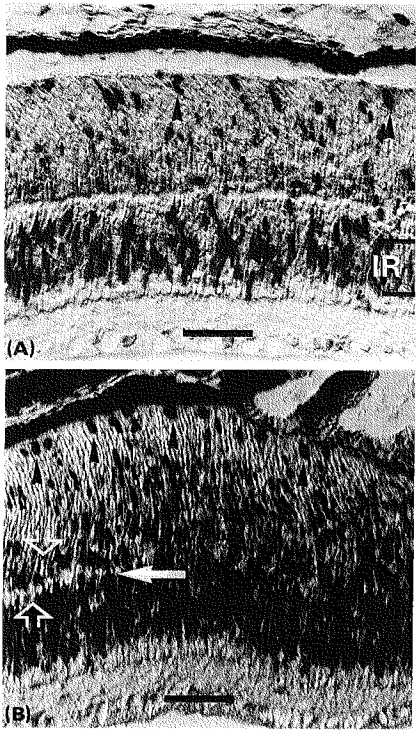

FIG. 5. A postnatal day 3 rabbit retina stained with the MIB-1 antibody. (A) Immunoreactivity in the retina slightly peripheral to the non-reactive central zone. Most of the reactivity is confined to the middle part of the inner nuclear layer (IR). The cells in the inner and outer parts of this layer are largely non-reactive. In the outer nuclear layer most of the immunoreactive cells are situated close to outer plexiform layer. Deeply stained cells appear towards the distal parts of the outer nuclear layer (arrowheads). (B) Immunoreactivity in the extreme periphery of the retina. There are many more reactive cells than in the more central retina as seen in 5(A). The outer plexiform layer (arrow) has not reached the most peripheral part. The immunoreactivity (hollow arrows) both in the distal part of the inner nuclear layer and the outer nuclear layer decreases with the spread of outer plexiform layer. Bar = 50 μm.

area. Towards the periphery, the distribution of immunoreactive cells progressively resembled the more immature stages described earlier [Fig. 7(A) and (B)]. Most of the immunoreactive cells were in the inner nuclear layer. In the outer nuclear layer of the peripheral retina, only few cells were immunopositive, and were situated close to the outer plexiform layer, but large deeply stained cells were still visible [Fig. 7(A) and (B)]. Some cells in the ganglion cell or nerve fiber layers were also positive. They were particularly numerous in the nerve fiber layer in the myelinated

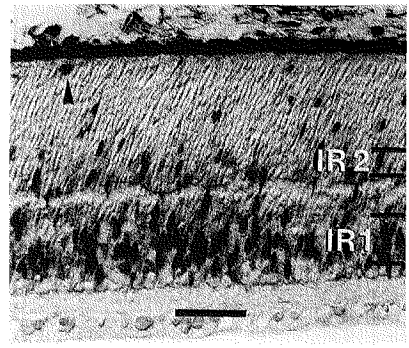

FIG. 6. A postnatal day 5 rabbit retina showing the peripheral region where most of the immunoreactive cells in the outer nuclear layer are situated close to the outer plexiform layer (IR2). Most cells in the distal part of the outer nuclear layer are non-reactive except the some large deeply stained cells (arrow head). The immunoreactivity in the inner nuclear layer is mostly in the middle regions (IR1). MIB-1 immunohistochemistry. Bar = 50 μm.

streak, with a gradient that increased towards the optic nerve [Fig. 7(C)].

At postnatal day 11 and 15, only a few immunoreactive cells were still visible in the extreme periphery of the retina, mostly in the inner nuclear layer. Some cells in the nerve fiber layer of the myelinated streak in the central retina were still also immunoreactive (Fig. 8). The optic nerve also showed immunoreactive cells (Fig. 8), which decreased in number in PN46 retina, but occasional immunoreactive cells could still be seen in PN60 retina (data not shown).

4. Discussion

Developmental studies, using [^{3}H]-thymidine to autoradiographically demonstrate DNA synthesis, have shown that the proliferation of retinal cells passes through different phases. In rabbit, the first proliferative phase (ventricular) occurs in the neuroblastic cell mass. In the central retina, it ceases shortly after birth. A late proliferative phase (non-ventricular) is found in the nerve fiber layer, and it lasts as late as in the third postnatal week (Reichenbach et al., 1991). The late proliferative phase has also been demonstrated in other animals (Rapaport et al., 1985, 1984).

Mitosis in the Ventricular Zone

At E15, MIB-1 reactivity was seen in the outer two thirds of the neuroblastic cell mass. The large, rounded and deeply stained cells, found only at the apical border of the neuroblastic cell mass, are likely to be the cells in metaphase, as cells in this phase are large

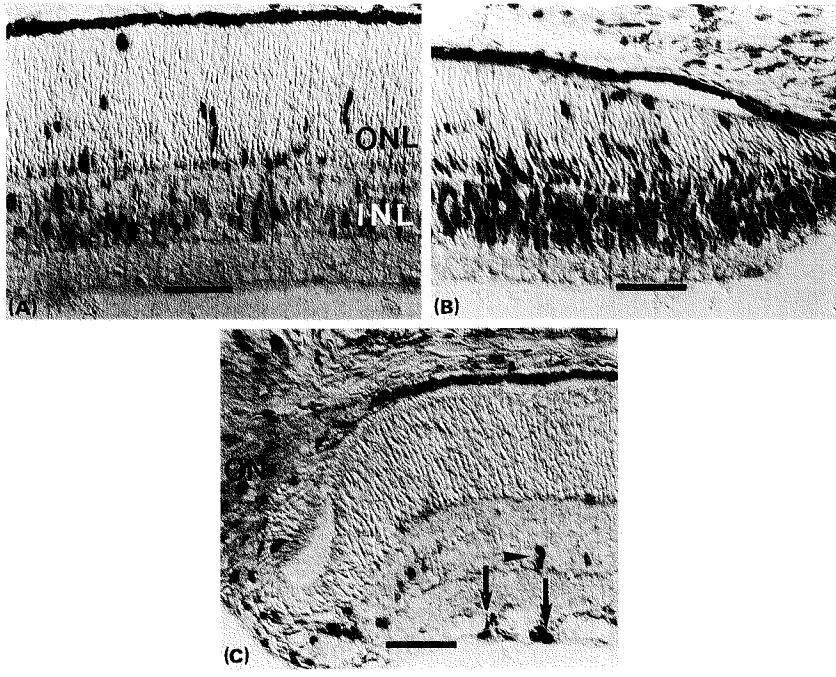

FIG. 7. A postnatal day 7 rabbit retina. (A) Immunoreactivity in the retina taken slightly peripherally of the non-reactive zone. Most of the immunoreactive cells are present in the middle of the inner nuclear layer (INL). Some cells in the outer nuclear layer (ONL) are reactive. (B) Immunoreactivity in the extreme periphery if the retina. (C) A section close to the optic nerve showing immunoreactive cells in the nerve fiber layer or the ganglion cell layer (arrows) close to the optic nerve (ON). Occasional cells in the inner nuclear layer are also reactive (arrowheads). MIB-1 immunohistochemistry. Bar = 50 μm.

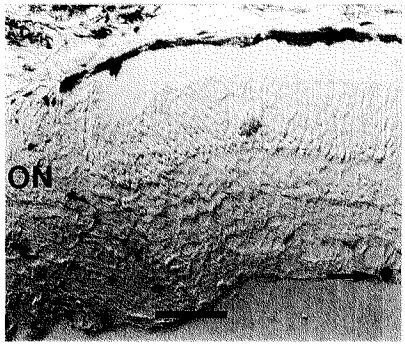

FIG. 8. A postnatal day 15 rabbit retina showing immunoreactive cell in the nerve fiber layer (arrow) close to the optic nerve (ON). MIB-1 immunohistochemistry. Bar = 50 μm.

rounded and have a large amount of chromatin. Other studies have also described mitotic figures in this region (Greiner and Weidman, 1982). Dividing cells synthesize their DNA away from the ventricular surface (towards the anuclear layer or the developing inner plexiform layer) and then migrate through the whole thickness of the neuroblastic cell mass to the ventricular surface for the completion of their division where the mitotic figures can be seen (Rapaport et al., 1985). Since MIB-1 marks cells in all the phases of cell division, immunopositive cells are found throughout the proliferating part of the neuroblastic cell mass. The cells in metaphase (visible as large, rounded, and deeply stained cells) were found in the distal parts of the retina, close to the retinal pigment epithelium. The pattern is expected as a result of the interkinetic migration.

Ganglion cells are the first cells to leave the mitotic cycle and to begin to differentiate in both mammals (Walsh et al., 1983; Sidman, 1961) and non-

mammalian vertebrates (Sharma and Ungar, 1980; Kahn, 1973; Fujita and Horii, 1963; Jacobson, 1976; Hollyfield, 1971). The first group of post mitotic cells, as seen in this study, accumulate in the most proximal part of the neuroblastic cell mass, and are therefore likely to be cells that had started to differentiate into ganglion cells. Sometime near the last cell division, ganglion cells migrate to the proximal part of the neuroblastic cell mass (Zimmerman, 1988) and start to project axons towards the optic fissure (Halfter, Deiss and Schwarz, 1985; Silver and Robb, 1979). It therefore also appears likely that they are the structures forming the most proximal, anuclear layer at this stage.

With the MIB-1 antibody, proliferation was also apparent in the retinal pigment epithelium at E15. This is at variance with Greiner and Weidman (1982) which reported no mitotic figures in the retinal pigment epithelium at E16, as seen in sections stained with hematoxylin and eosin. The reason for this difference is not immediately apparent, but perhaps the mitotic figures were masked in the previous study by the pigmentation of the cells.

In the innermost part of the neuroblastic cell mass (distal to the inner plexiform layer), there was a layer of non-proliferating cells, both at E25 and E29. These non-proliferating cells could not be discerned in hematoxylin and eosin stained sections from E25 tissue, but at E29 they were seen as round, lightly stained cells. Such cells have been described as differentiating amacrine cells in previous studies (Greiner and Weidman, 1982). Electron microscopic studies on E27 retinas (Greiner and Weidman, 1982) have demonstrated that they at this stage contain the conventional synaptic complexes, typical of amacrine cells (Witkovsky and Dowling, 1969; Raviola and Raviola, 1967; Dowling and Boycott, 1966) or interplexiform cells (Kolb and West, 1977; Dowling, Ehinger and Hedden, 1976). These cells are thus likely to have evolved at this stage. Observations on both ganglion cells (E15) and amacrine cells (E25 and E29) thus show that the cells stop proliferating a few days before they are differentiated enough to be identified by standard light microscopy.

The timing for the differentiation of bipolar cells is not well documented, but various studies on the mammalian retinas have suggested that the early proliferation gives rise to ganglion cells followed by the horizontal cells, some amacrine cells and cones. Late proliferation gives rise to remaining amacrine cells, Müller cells, bipolar cells and rods (Reichenbach et al., 1994; Zimmerman et al., 1988; LaVail, Rapaport and Rakic, 1991; Carter-Dawson and LaVail, 1979; Sidman, 1961; for review see Reichenbach and Robinson, 1995). Differentiated horizontal cells are apparent already at E27 (Greiner and Weidman, 1982). It therefore seems likely that already at E25, some of the postmitotic cells are differentiating horizontal cells, which visibly accumulate proximal to the outer plexiform layer, as it begins to form at E29 and spreads peripherally.

Both at E25 and E29, maximum proliferative activity was found in the middle of the neuroblastic cell mass, close to the postmitotic cells. The number of proliferating cells decreased towards the distal retina, distinctly observable from E29 and onwards. Thus, already at this stage there are postmitotic cells in the distal-most retina where photoreceptors are found. However, even after the formation of the outer plexiform layer, proliferating cells in the outer nuclear layer can be observed accumulated in the proximal part (close to the outer plexiform layer) and the distal-most part of the same layer (cells in metaphase). This suggests that photoreceptors are born throughout a long period of time. Accumulated evidence suggests that the cones are born in an early phase of proliferation while the rods are born in a late phase (for review see Reichenbach and Robinson, 1995).

At the time of birth, proliferation ceased in a small region in the central retina. This area of non-proliferation successively enlarged in PN3, PN5 and PN7 retinas where proliferation was found only in the peripheral part of the retina. Isolated proliferating cells could be seen in the extreme periphery of the retina even in PN15 retinas. Previous studies identifying mitotic figures reported the cessation of cytogenesis in a horizontally oriented zone between the optic disc and the temporal margin at the time of birth (Stone et al., 1985). This zone initially spreads preferentially along the visual streak and then into the superior and inferior parts of the retina until by post-natal day 7, cell division is restricted to the extreme periphery of the retina. It has been suggested in cats (Rapaport et al., 1985) that the ventricular proliferation ceases with the development of the outer plexiform layer which was thought to act as a mechanical barrier (Rapaport et al., 1984, 1985), preventing the proliferating cells migrating to the inner retinal layers. A delay between the formation of the outer plexiform layer and the cessation of proliferation has been noted in the rabbit (Stone et al., 1985) and was thought to be due to the interkinetic migration across the discontinuities in the outer plexiform layer. It was noted that the proliferation of cells decreased more in the outer nuclear layer as the outer plexiform layer formed, but the proliferative activity did not completely cease. In the outer nuclear layer, proliferating cells accumulated close to the outer plexiform layer and the pigment epithelium (cells in metaphase) suggesting that these proliferating cells had defined a new zone for interkinetic migration within the outer nuclear layer, and were adding more photoreceptors.

After the formation of the outer plexiform layer, most of the proliferating cells were situated in the middle region of the inner nuclear layer, suggesting the genesis of Müller cells. In support, mitotic figures immunopositive for KI-67 can be seen in the inner nuclear layer, as is also reported by others in other

species (Harman and Beazley, 1987; Spira and Hollenberg, 1973; Robinson, Rapaport and Stone, 1985; Rapaport et al., 1985, 1984; Rapaport and Vietri, 1991). The cells situated in this region undergo proliferation for the longest period of time. Many studies have suggested that the Müller cells are born early in development (Kuwabara and Weidman, 1974; Bhattacharjee and Sanyal, 1975; Uga and Smelser, 1973), whereas others have shown a late proliferation that gives rise to the Müller cells (Blanks and Bok, 1977; McArdle, Dowling and Masland, 1977). We suggest both these views are correct. Electron microscopic studies have shown that the Müller cell nuclei migrate to their final position in the center of the inner nuclear layer from a more sclerad or vitread position throughout the whole period from E14 to PN14 (Uga and Smelser, 1973). Presumably, Müller cells are born over a long stretch of time, and at a certain stage the cells leaving the mitotic cycle gives rise to a variety of neurons as well as Müller cells, while later mostly Müller cells are born. In rats (Turner and Cepko, 1987) and frogs (Wetts and Fraser, 1988; Holt et al., 1988) the same progenitor cells have been shown to give rise to both neurons and Müller cells. This is reminiscent of the development of the multipotent precursor cells in the brain that produce both neurons and glial cells (Korr, 1982).

Mitosis in the Sub-ventricular Zone

The presence of MIB-1 positive cells in the most proximal layer of the retina as well as some very few cells in the inner nuclear layer after the first postnatal week indicate a second phase of proliferation, presumably glial. Similar late proliferating cells in the inner nuclear layer of retina have been reported in cat (Rapaport et al., 1985, 1984). The cells in the nerve fiber layer are probably astrocytes (Schnitzer 1990, 1988) and were reported up to at least 4 weeks after birth. In the present study, the late proliferative cells in the inner nuclear layer had the morphological characteristics of Müller cells. In previous studies (Reichenbach et al., 1991), [^{3}H]-thymidine labeled cells in this region have been shown to contain immunoreactive vimentin, which marks Müller cells (Schnitzer, 1985). Immature neuroepithelial stem cells have also been shown to be immunoreactive to the vimentin (Bennett, 1987; Lemmon and Reiser, 1983), but persisting retinal neuroepithelial stem cells are known only in cold blooded animals.

Acknowledgments

This work was supported by the Swedish Medical Research Council (project 14X-2321), the Faculty of Medicine at the University of Lund, Kronprinsessan Margaretas Arbetsnämnd för synskadade, Riksbankens Jubileumsfond, the Crafoord Foundation, the Segerfalk Foundations, the H and L Nilssons Stiftelse, Stiftelsen Clas Groschinskys Minnesfond, and the T and R Söderberg Foundation.

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Paper II

Cell Transplantation, Vol. 6, No. 2, 141–148, 1997

0963-6897/97 $17.00 + .00

ELSEVIER

PII S0963-6897(96)00256-4

Original Contribution

CELL PROLIFERATION IN RETINAL TRANSPLANTS

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☐ **Abstract — The MIB-1 antibody against a nuclear protein Ki-67 was used to study the proliferation of cells in the rabbit retinal transplants. Fragmented pieces of embryonic day 15 rabbit retinas were transplanted into the subretinal space of adult rabbits and allowed to survive for different times. Fragmented donor tissue starts organizing in rosettes 1 day after transplantation. The transplanted cells continue to proliferate in the host eye and their pattern of proliferation resembles that of normal developing retina, suggesting that the factors responsible for the proliferation pattern are preserved after transplantation. The dividing cells in metaphase line up in the luminal layers of the rosettes. Certain cells become postmitotic in the regions corresponding to the inner retina first, followed by the cells in the luminal layers of rosettes. Cells in the regions between the rosettes, corresponding to the inner nuclear layer, presumably the Müller cells, proliferate significantly for the equivalent age of postnatal day 2. Few cells in these regions proliferate for at least the equivalent age of postnatal day 11 in transplants. There is a layer of nonproliferating, degenerating cells in the transplant situated close to the host retina. However, some cells in this layer, situated at the host–graft interface, proliferate. These cells proliferate for a long time possibly indicating gliosis. © 1997 Elsevier Science Inc.**

☐ **Keywords — Proliferation; Retinal transplants; Ki-67; Development.**

INTRODUCTION

As in other neural tissues, the events in the developing retina occur in a precise and predetermined order. In retinal transplants, survival and differentiation have been studied in considerable detail (27). Transplants survive at various transplantation sites (5,28,29), and they develop all the retinal layers and most of the cell types of the normal retina (7,11,12,25). Many of the essential proteins (17,18,29) and the neurotransmitters (2,7) have also been found in the transplants. However, the first event in the process of development, proliferation, has not been studied so far. It is generally known that even when retinal transplants, often containing donor tissue from more than one embryonic retina, are allowed to survive and to attain postnatal ages, their sizes remain limited. Technical difficulties have precluded more precise studies of transplant growth done by measuring the size, the volume or in other ways.

Studying the appearance and distribution of cell proliferation markers is a way to partially circumvent the problems of measuring size or volume, when assessing the growth of a developing transplant. Such studies can also give information on the influence of host environment on the proliferation of grafted cells. Embryonic donor tissue, depending upon the stage at which it is harvested, contains neuroblastic cells that are still undergoing mitosis at the time of transplantation (30,31). Suitable markers for proliferating cells such as 5-bromo-2-deoxyuridine, ^{3}H-thymidine, antibody against proliferating cell nuclear antigen, and the MIB-1 antibody are now available. To have a better understanding of the growth and development of the retinal transplants, we studied the proliferation of the transplanted retinal cells by using the MIB-1 antibody. This antibody recognizes an antigen, Ki-67, which is present in all dividing cells. It has been widely used to study the proliferating cells in different tissues (8,14). We know of no other study of the proliferative activity and the pattern of cell birth in retinal cell transplants. In a previous paper, we analyzed the appearance of the Ki-67 antigen in the normal developing rabbit retina (31), and we now report the expression of the Ki-67 protein in transplanted retinal cells. In this study we did not attempt to count the number of proliferating cells in various transplant ages, as this is feasible

ACCEPTED 10/29/96.
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only when cell suspensions of the donor tissue is transplanted. The cell suspension transplants do not develop the retinal layers (17), which are essential for studying the pattern of cell proliferation.

MATERIALS AND MEDHODS

Retinal Transplants

Neural retinas from embryonic day 15 outbred pigmented rabbits were used as donor tissue. Embryos were obtained by sacrificing the rabbits dams with an overdose of sodium pentobarbital and kept in cool (+4 degrees) Ames' solution containing (mM) NaCl [120], KCl [3.6], $MgSO_4$ [1.2], $CaCl_2$ [1.2], $NaHCO_3$ [23], NaH_2PO_4 [0.1], Na_2HPO_4 [0.4] and Glucose [10] (1). The eyes were removed and the neural retinas dissected under an operating microscope. They were stored in the same solution until transplanted.

Adult rabbits of the same strain weighing 1–2.5 kg, were used as recipients. The donor tissue was drawn up in a thin polyethylene capillary mounted on a special instrument and connected to a precision microsyringe. The capillary then contained fragmented pieces of both peripheral and central donor tissue mixed together. The recipient eye was entered through a small scleral incision. The capillary was advanced through the vitreous to the posterior pole of the eye and its contents were deposited in the subretinal space. Details of the procedure have been published (6). No antibiotics or immunosuppressive drugs were used on the animals. Appropriate permits for the study were obtained from the Swedish Government Animal Experimentation Ethics Committee at the University of Lund. The experiments were conducted according the rules set by the ARVO resolution on animal experimentation.

The rabbits were sacrificed 1 (n = 2), 4 (n = 2), 6 (n = 2), 7 (n = 2), 11 (n = 2), 14 (n = 2), 18 (n = 1), 20 (n = 4), 21 (n = 2), 27 (n = 2), 28 (n = 1), and 123 (n = 1) days after the surgery. Thus, the transplants attained ages equivalent to embryonic (E) day 16, 19, 21, 22, 26, and 29 and postnatal (PN) day 2, 4, 5, 11, 12, and 107 (donor age + survival time after surgery). The eyes were enucleated and processed as described below.

Tissue Processing

The eyes were briefly fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M phosphate, 0.85% NaCl; pH 7.4). The anterior segments of the eyes were removed and the posterior segments were fixed in the same fixative for 24 h. The tissue was rinsed in the same phosphate-buffered saline and then with 5, 10, and 20% sucrose added. The area containing the transplant was cut out and stored in PBS with 20% sucrose and 0.1% sodium azide until sectioned. Sections were cut on a cryostat (12 microns), mounted on glass slides coated with gelatin, and stored at 70°C until used.

Immunohistochemistry and Hematoxylin & Eosin Staining

After thawing the frozen sections to room temperature, they were rinsed in PBS containing 1% bovine serum albumin (BSA; Sigma Chemical Co.). This solution was subsequently used for all the washes. Nonspecific antibody binding was blocked by incubating the slides in 1.5% normal horse serum prior to their incubation with the MIB-1 monoclonal antibody (Immunotech, Inc., Westbrook, ME) in a dilution of 1:200 in PBS. Optimum working concentrations and incubation times for the antibody had first been determined in pilot experiments. The slides were incubated for 72 h at 4°C in a humidified chamber. The slides were then rinsed with PBS and incubated for 1 h in biotin conjugated horse-antimouse secondary antibody (Vector Lab. Inc. Burlingame, CA) in a dilution of 1:200. The slides were then rinsed again in PBS and endogenous peroxidases in the tissue were quenched by incubating in 0.3% hydrogen peroxide in PBS for 15 min. The slides were then incubated in avidin–horseradish–peroxidase (Vectastain® Elite ABC Kit; Vector Lab. Inc., Burlingame, CA) for 1 hr. Slides were again rinsed in PBS and the HRP reaction was developed in a substrate solution of diaminobenzidine (DAB; Peroxidase Substrate Kit; Vector Lab. Inc., Burlingame, CA). Adjacent sections were stained with hematoxylin and eosin. In each experiment controls were obtained by omitting the primary antibody.

For transplants, we will use the term "luminal layers" or "inner layers" to denote the layers of cells towards the lumen of the rosettes and "outer layers" to denote layers away from the lumen. For developing retinas, the term "basal" or "proximal" and "apical" or "distal" is used to denote localization in terms of how the tissue develops. In the neuroretina, "basal" thus means closer to the vitreous and "apical" closer to the photoreceptors.

RESULTS

The earliest transplant studied corresponded to embryonic age 16 days. The transplanted cells had started to organize in rosettes (Fig. 1A). Brown immunoreactive cells were observed scattered throughout the transplant. On the basis of color, these cells could be differentiated from the black clumps of pigments, that often migrated from the host retinal pigment epithelium. Certain large and deeply stained cells were homogeneously distributed throughout the transplant or occasionally formed small

Fig. 1. Embryonic day 16 rabbit retinal transplant (T) 1 day after the transplantation. (A) Hematoxylin and eosin stained section showing the beginning of rosette formation. Pyknotic cells are visible both in the host (H) retina and a few in the transplant close to the host retina. (B) Immunohistochemical staining with the MIB-1 antibody showing homogeneously distributed immunoreactive cells. Certain large deeply staining cells (arrows) are scattered throughout the transplant (T) but at places they are also found (arrowheads) near the lumen of rosette-like clusters (lumen marked with asterisks). There is some dispersion of pigment from the host pigment epithelium into the transplant (pi). Bar = 50 μm.

rosette-like clusters (Fig. 1B). The size, shape and staining characteristics of the deeply stained cells were identical to those in the late phases of mitosis, described in the normal retina (30,31). Immunoreactive and nonimmunoreactive cells were homogeneously distributed throughout the transplant. On rare occasions, a few cells close to the host retina formed small patches of small deeply stained pyknotic cells (judged by hematoxylin & eosin staining characteristics). These cells were devoid of immunoreactivity. The host retina also showed small and pyknotic cells. No immunoreactive cells were seen in the host retina.

In transplants corresponding to embryonic age 19 days, the cells lying close to the host retina were small, pyknotic and non-immunoreactive (Fig. 2). However, there were a few immunoreactive cells at the host-graft interface and the immunoreactivity in these cells persisted even in older transplant ages (see below). Some cells in the host retina were immunoreactive (Fig. 2). Pyknotic or nonimmunoreactive cells were not observed at the sides of the transplants, even though they were still close to the host retina. Similarly, small transplants covered with the host retina lacked nonreactive pyknotic cells.

By embryonic age 21 or 22 days, the rosettes were more distinct. Large, deeply immunoreactive mitotic cells were often found arranged in the innermost parts of the rosettes, close to the lumen (Fig. 3A and B). Certain cells in the innermost layers of the rosettes had become

Fig. 2. Embryonic day 19 rabbit retinal transplant (T) showing a nonimmunoreactive layer of cells (NR) close to the host retina (H). In the nonreactive layer there are a few cells at the host–graft interface (arrowheads) that are immunoreactive. One immunoreactive cell is visible in the host retina (arrow). Immunohistochemical staining with the MIB-1 antibody. Bar = 50 μm.

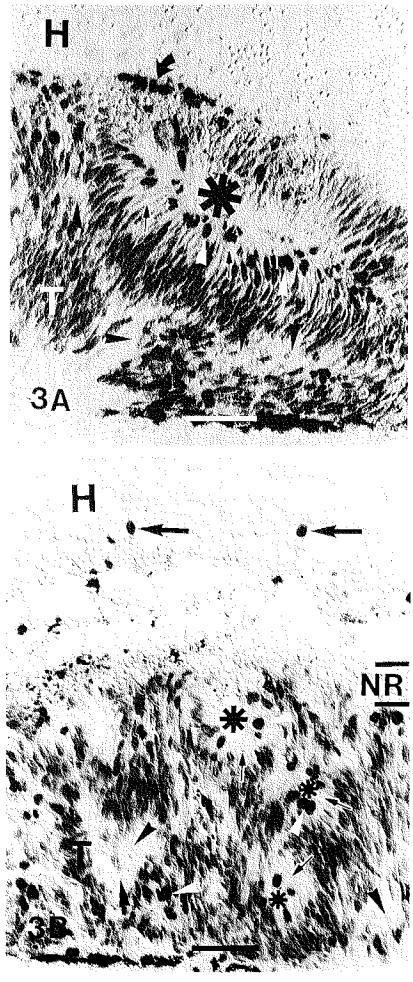

Fig. 3. (A and B) Embryonic day 21 rabbit retinal transplant (T). Some of the cells in the innermost layers of rosettes (lumen of rosettes marked with asterisks) are nonreactive (white arrowheads). There are also clusters of nonreactive cells in between the rosettes (arrowheads). In the layer of nonreactive cells close to the host retina some cells at the host-graft interface are immunoreactive (curved arrows in Fig. 3A), as are some cells (large arrows in Fig. 3B) in the host retina (H). Immunohistochemical staining with the MIB-1 antibody. NR = layer of nonimmunoreactive cells close to the host retina. Bar = 50 μm.

nonimmunoreactive. There were reactive cells in the outer layers of the rosettes. There were clusters of immunoreactive as well as nonimmunoreactive cells in between the rosettes. A layer of nonreactive cells was observed close to the host retina (Fig. 3A and B) and this corresponded to the small pyknotic cells observed with hematoxylin and eosin staining. However, a few immunoreactive cells were present at the host surface of this nonreactive area (Fig. 3A). A small number of immunoreactive cells were present in the host retina close to the transplant (Fig. 3B).

At embryonic age 26 days (Fig. 4), most cells in the inner layers of the rosettes were nonreactive, except a few large, deeply staining cells. Cells were immunoreactive in the outer layers of the rosettes and most of the reactivity was confined to the cells lying in between the rosettes. Some immunoreactive cells were present in the host retina. At embryonic age 29, cells in the inner layers of the rosettes were nonreactive, with certain large deeply reactive mitotic cells and a few other cells being the exceptions (Fig. 5). However, cells in the outer layers of the rosettes and in between them were immunoreactive (Fig. 5). The latter cells correspond to cells in the inner layer of the normal retina. There were also some immunoreactive cells in the host (not shown). On the graft side of the host–graft interface, there was a concentration of immunoreactive cells.

At a transplant age corresponding to postnatal day 2,

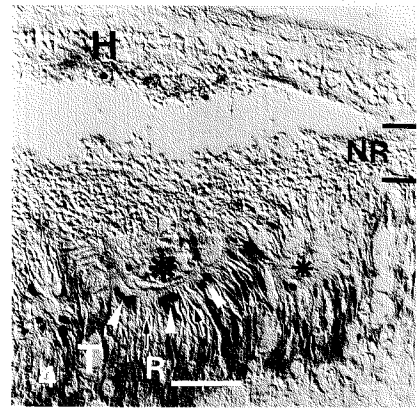

Fig. 4. Embryonic day 26 rabbit retinal transplant (T) showing rosettes (lumen marked with asterisks) with deeply stained cells (white arrowheads) towards the outer limiting membrane. The inner layers of the rosettes are nonreactive (small black arrows), whereas the outer layers are reactive (R). Immunohistochemical staining with the MIB-1 antibody. Bar = 50 μm.

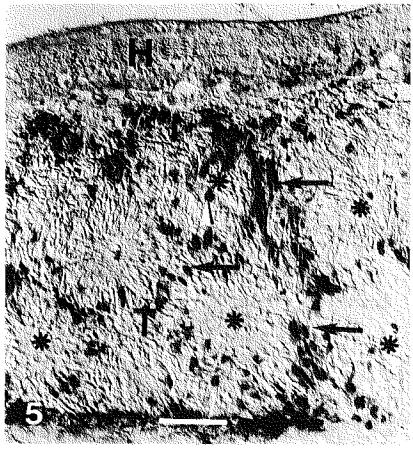

Fig. 5.Embryonic day 29 rabbit retinal transplant (T) showing reactive cells only in between the rosettes (arrows) and some deeply stained cells (arrowhead) towards the lumen of the rosettes (lumina marked with asterisks). Immunohistochemical staining with the MIB-1 antibody. H = host retina. Bar = 50 μm.

Fig. 6. Postnatal day 2 rabbit retinal transplant (T) showing immunoreactive cells in between the rosettes (arrows) and at the host–graft interface (curved arrows). H = host retina. Immunohistochemical staining with MIB-1 antibody. Bar = 50 μm.

the cells in between the rosettes were still immunoreactive and there were immunoreactive cells in the host retina (not shown) and host–graft interface (Fig. 6). At a transplant age corresponding to postnatal day 4, most areas in the transplants were devoid of immunoreactive cells, but occasionally there were areas in between the rosettes in which immunoreactive cells persisted. At the host graft interface some immunoreactive cells were present in the transplant (Fig. 7). Some immunoreactive cells were also observed in the host retina (Fig. 7).

Immunoreactive cells were rare in most parts of transplants obtained at ages corresponding to postnatal days 5, 11, and 12. However, very few immunoreactive cells could be seen distributed in between the rosettes (not shown). Immunoreactive cells were also observed in the transplant at the host graft interface (Fig. 8) and occasionally in the host retina. There were no significant differences in observations in various specimens of the same age.

DISCUSSION

Proliferation and Development of Retinal Transplants

Already 1 day after the transplantation the grafts had started organizing themselves in rosettes. Retinal layers in the form of rosettes became more prominent at later

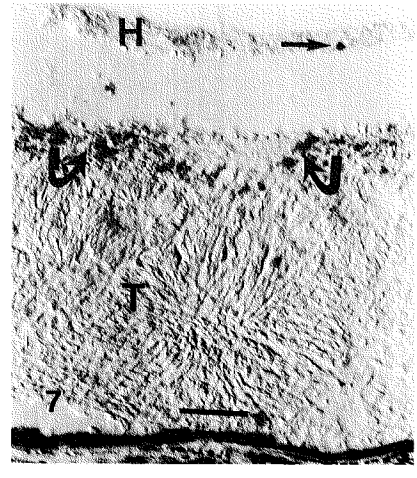

Fig. 7. Postnatal day 4 rabbit retinal transplant (T) showing immunoreactive cells at the host–graft interface (curved arrows). One immunoreactive cell is visible in the thin host retina. Immunohistochemical staining with the MIB-1 antibody. H = host retina. Bar = 50 μm.

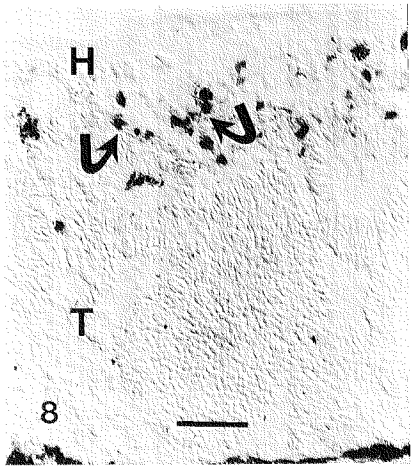

Fig. 8. Postnatal day 11 rabbit retinal transplant (T) showing immunoreactive cells at the host–graft interface (curved arrows). Immunohistochemical staining with the MIB-1 antibody. H = host retina, Bar = 50 μm.

stages. The organization of the graft has previously been studied in detail (6,29,33). It has been shown that the cells of the outer nuclear layer (corresponding to the distal part of the normal retina) are located in the luminal layers of rosettes. The cells corresponding to the inner retinal layers (proximal part of the normal retina) are located in between the rosettes. The phagocytic function of the pigmented epithelium in transplants may be performed by macrophages in the lumen of the rosettes (4).

The random distribution of the postmitotic and the proliferating cells, including the mitotic ones, probably indicates a random organization of the transplants in early stages. Postmitotic cells are expected in this stage because early differentiating ganglion cells are already present in the donor tissue at the time of transplantation (30,31). It has been suggested that the best time to harvest the donor tissue is before the cells undergo terminal mitosis (9,10). This may partly explain why the ganglion cells that have already undergone terminal mitosis at the time of transplantation in E15 donor tissues (30,31) have hitherto not been demonstrated in the grafts. One day after transplantation the mitotic cells appear to take the lead in an effort of organization as these cluster together to form early rosettes.

As the transplanted cells reorganize themselves in rosettes (E19), the proliferating cells also adopt the general proliferation pattern seen in normal retinogenesis. In a previous study (15) proliferation of cells was studied in embryonic diencephalon transplanted to the brain of adult rats. The study shows that the survival of the graft is related to the proliferative activity of the transplant. However, this study did not demonstrate if the pattern of proliferation was maintained after transplantation. It is interesting to note that the mitotic figures in the normal developing retina accumulate at the outer limiting membrane [the distal retina; (30,31)]. This is because proliferating retinal cells normally undergo interkinetic migration, which means that they synthesize their DNA away from the ventricular surface, in the middle layers of the developing retina (30,31), and then migrate to the ventricular surface, where they undergo mitosis. The results in this study show that in retinal cell transplants, the same pattern is maintained. The luminal surface of the rosettes, where in due course the outer limiting membrane will form, corresponds to the ventricular (apical) surface of the normal retina. Cells in metaphase appear in the apical parts of the rosettes, whereas many other cells in the surrounding layers are in other phases of the mitotic cycle, as indicated by their content of the Ki-67 antigen. There must, thus, be a form of interkinetic migration within the rosettes, similar to that which is seen in the normal retina.

Already at E21 and more so at E22, the proliferation of cells is reduced in the innermost layers of rosettes (except for some remaining cells in metaphase). These postmitotic cells are likely to be differentiating photoreceptor cells. In the outer layers of the rosettes there are still many proliferating cells, most likely giving rise to more photoreceptors and cells of the proximal retina. The nonproliferating cells in between the rosettes could be the postmitotic cells differentiating into amacrine and horizontal cells, because these cells are born early in ontogeny (21,22,30,31).

In E26 transplants, the luminal-most layers of the rosettes were largely nonproliferating, with the exception of the mitotic cells. The postmitotic cells in this region are likely to be the differentiating photoreceptors. Otherwise, the pattern of proliferation in all essentials resembles that seen at E21 or E22. The pattern of proliferation thus resembles that in normal development where at E25 and E29 most of the proliferating cells are in a region distal to the postmitotic amacrine cells (30,31). This fits very well with the distribution of immunoreactive cells in the transplants.

In E29 and PN2 transplants, the pattern of the proliferating cells has changed considerably. They were almost completely confined to the regions in between the rosettes, which correspond to the layers of the inner half of the normal retina. This shift of where proliferating cells predominate is also seen in the normal development of the retina (30,31).

In the postnatal day 4 transplants and later, only small patches of proliferation were seen. Again, this is in accordance with the observation in normal retinas that proliferation ceases at birth in the central retina and some days later in the peripheral retina (23,30,31). The regional differences in the mitotic activity in the transplant may be due to the mixing of the central and the peripheral parts of the donor retina at the time of transplantation.

Four days after the transplantation (in E19 transplants), numerous small pyknotic or perhaps apoptotic cells appeared in the parts of the transplant that were closest to the host retina. Such cells have been observed earlier in transplants (6). The MIB-1 immunoreactivity revealed that these were nonproliferating cells. They were also present in transplants with longer survival times. This suggests that a layer of cells at the host–graft interface undergoes degeneration instead of proliferation.

The degeneration of cells is not confined to the transplant alone, because the overlying host retina also is seen to lose its photoreceptor outer segments and many of its cells in the outer nuclear layer (6,29). These observations suggest that the host and the graft have a negative influence on each other. However, the number of pyknotic cells is much smaller at the sides of the transplants than in the middle, notwithstanding that the distance to the host retina is the same in both cases. In addition, the number of nonproliferating pyknotic cells was much smaller in small transplants. Because the choroid is the main source of nourishment for the retina, it is also possible that the transplanted cells farthest away from it may not receive sufficient nourishment for their metabolism and, therefore, cease to proliferate and eventually degenerate.

In rat transplants, migration of host-derived glial cells (probably Müller cells) into the graft begins 2 days after the transplantation, along the host–graft interface (25). In the same transplants, intrinsic graft glial cells (mostly Müller cells) develop approximately according to their normal timetable, becoming partly reactive to glial fibrillary acidic protein (GFAP) after 3 wk, and completely reactive 5 wk after the transplantation. The marked proliferation at the host–graft interface may represent abnormal gliosis, although this has not been much emphasized in previous studies. Nevertheless, in human xenotransplants, cellular retinaldehyde-binding protein (CRALBP) immunoreactivity was found mostly close to the host retina (26) and GFAP immunoreactive fibers were also found mostly at the same location. A glial barrier has previously not been explicitly described in retinal transplants, but neural transplants in brain often become encapsulated by a glial barrier, which interferes with the integration of the graft with the host (3). The observations in the present study suggest that the retinal cell transplants also tend to develop a glial barrier.

Even though the mitotic activity in the transplant is comparable to the normal development in terms of pattern of proliferation, it seems that the transplanted cells do not proliferate as much as the cells in normal development. In E25 and E29 normal retinas, the proliferation of cells was much more (31) than at comparable ages in transplants. For example, at E29 proliferation in the transplant was confined to the areas in between the rosettes corresponding to the proliferation in the inner nuclear layer in the developing retina. In the normal developing retina, there is at this stage much more proliferation in the inner nuclear layer and also proliferation in the outer nuclear layer (30,31). Further, proliferation in postnatal transplants was minimal, whereas significant proliferation persists up to day PN7 to PN11 in normal development. Moreover, cell death is prominent in the transplants and may also be a factor retarding the growth of the transplants.

Cell proliferation and cell death are not the only factors that affect the development of the retina (and hence also transplants). The growth of the eye (and the retina) is a complex process involving both active growth and passive stretching (23). After the retinal cell proliferation ceases in the first postnatal week in rabbits (13,23,24,30–32) the retina passively stretches with the sclera increasing the surface area up to threefold in rabbits at the cost of retinal thickness (23). It is possible that the forces needed for the passive expansion of the retina are lacking in the case of a retinal graft placed in a developed eye, thus, the graft does not undergo the passive stretching and fails to cover the entire surface of the eye.

Proliferation in the Host-Retina

Four days after surgery, proliferating cells were seen in the host retina covering the transplant. To some extent, the proliferation in the host retina could be seen even in older transplants. These cells are likely to be glial cells because Müller cells are known to proliferate after retinal damage (16,19,20). Further, there are indications that host Müller cells respond to the transplantation surgery. For instance, they express GFAP already 4.5 h after the surgery, and this reaction spreads out to the whole retina within 1 day. It lasts for at least 7 wk (25). Hypertrophy and migration of Müller cells within the host retina was also noticed adjacent to the lesion site, commencing 2 days after the transplantation (25). This is in accordance with the proliferating cells being glial cells.

Acknowledgments — This work was supported by the Swedish Medical Research Council (project 14X-2321), the Faculty of Medicine at the University of Lund, Kronprinsessan Margaretas Arbetsnämnd för synskadade, the Riksbankens Jubileumsfond, the Crafoord Foundation, the Segerfalk Foundations, the H and L Nilssons Stiftelse, Stiftelsen Clas Groschinskys Minnesfond, and the T and R Söderberg Foundation.

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Paper III

Histochem Cell Biol (1997) 107:449–458

ORIGINAL PAPER

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Immunocytochemical localisation of neuronal nitric oxide synthase in developing and transplanted rabbit retinas

Accepted: 22 January 1997

Abstract Nitric oxide (NO) acts as a modulator of neuronal transmission in mature neuronal systems, including the retina. Recently, NO has also been suggested to have a trophic function during development. We examined immunocytochemically the distribution of NO-producing cells in developing and transplanted rabbit retinas. An antibody detecting the neuronal isoform of its biosynthetic enzyme, nitric oxide synthase (NOS), was used on normal developing retinas [starting at embryonic day (E) 15] and on rabbit retinal transplants after various survival times (1–139 days after surgery). Weakly stained cell bodies were first observed in the proximal margin of the neuroblastic layer at E 29. Stained processes projecting towards a developing inner plexiform layer were also visible at this time point. Immunoreactive cells were located at later stages in the innermost part of the inner nuclear layer and in the ganglion cell layer, and are likely to correspond mainly to amacrine cells. NOS-labelled cells were also found in retinal transplants. The first NOS-labelled cells appeared, as in normal developing retinas, in ages corresponding to E 29 and were still detected in transplants corresponding to postnatal day 123. NOS-labelled cells were seen in areas between rosettes, where amacrine cells are located. NOS-labelled processes were at times seen to project for long distances, forming very distinct plexuses. NOS-containing amacrine cells thus appear both in the transplants and in developing retinas in the embryonic stages, long before synaptic function involving these cells can be expected, suggesting a role for NO not only in neuromodulation but also in retinal development.

Introduction

A transmitter/modulatory role is currently ascribed to the free-radical gas, nitric oxide (NO) (see, e.g. Bredt and Snyder 1992; Dawson and Snyder 1994; Schuman and Madison 1994). NO is formed from L-arginine, a reaction catalysed by the haem-containing enzyme nitric oxide synthase (NOS), and which requires among other cofactors, calmodulin, Ca^{2+} and the electron donor, NADPH (nicotinamide adenine dinucleotide phosphate reduced) (Bredt and Snyder 1990; Moncada 1992). A constitutive form of NOS has been localised in neuronal cells, and both constitutive and inducible isoforms have been detected in non-neuronal cells (Förstermann et al. 1991; Lamas et al. 1992; Lowenstein et al. 1992).

Cytosolic levels of NO are kept very low, as this molecule can easily diffuse across plasma membranes. The morphological localisation of cell systems capable of producing NO thus relies on the distribution of mRNA coding for its biosynthetic enzyme (NOS) or on the immunocytochemical localisation of NOS itself (Bredt et al. 1990; 1991). The histochemical localisation of NADPH-diaphorase in paraformaldehyde-fixed tissues is also taken as a means of localising NO-producing cells (Dawson et al. 1991; Hope et al. 1991).

Discrete populations of nerve cells have been shown throughout the nervous system to contain basal levels of NOS (Bredt et al. 1991). An increase in the intracellular levels of Ca^{2+} appears to activate the Ca^{2+}/calmodulin-dependent neuronal NOS isoform, stimulating the production of NO (Bredt and Snyder 1990; Schuman and Madison 1994). The NO produced diffuses across the nerve cell membrane and acts on neighbouring cells, stimulating the activity of soluble guanylate cyclases, and thereby elevating cGMP levels (Knowles et al. 1989; Dawson and Snyder 1994). NO can thus be generated in cells in response to increased neuronal activity, and participate in the transmission of signals.

As expected, NO appears to act also in the retina as a neuromodulator. NO-generating compounds are found to affect transmitter release, NMDA-induced currents, coupling between horizontal cells and photoreceptor physiology (Horio and Murad 1991; Miyachi et al. 1991; Margulis et al. 1992; Schmidt et al. 1992; Pozdnyakov et al. 1993; Ujihara et al. 1993; Becquet et al.

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1994; Bugnon et al. 1994; Greenstreet and Djamgoz 1994; Koch et al. 1994; Kurenny et al. 1994). Synthesis of NO by retinal cells has been demonstrated (Venturini et al. 1991; Osborne et al. 1993; Koch et al. 1994) and cells exhibiting NADPH-diaphorase reactivity have been identified in the retina (see Koistinaho and Sagar 1995 for review). Also, the presence of NOS and of NOS mRNA have been demonstrated in retinal cells (Yamamoto et al. 1993; Koch et al. 1994; Liepe et al. 1994; Park et al. 1994; Östholm et al. 1994; Perez et al. 1995).

Besides acting as a neuromodulator, NO may play a role also during synaptogenesis. It has been shown that the loss of ipsilateral retinotectal connections, which is normally seen during development of the chick visual system, is reduced upon inhibition of NO synthesis (Wu et al. 1994). These observations suggest that NO may function as a cellular messenger, also during development of the visual system.

One way of addressing this question is to examine whether the pattern of NOS expression during development differs from that seen in the adult. A transient expression of γ-aminobutyric acid (GABA) has, for instance, been observed in horizontal cells in early postnatal rabbit retinas, suggesting a trophic function for this neuroactive messenger (Redburn 1992). In the present study, we have therefore studied the distribution of NOS-containing cells in pre- and early post-natal rabbit retinas, and how this distribution compares with that seen in the adult rabbit retina. Further, a number of studies have shown that embryonic retinas transplanted to the subretinal space in adult animals survive relatively well (see Sharma et al. 1995, for review), and that the grafted cells are capable of expressing various cellular markers (see e.g. Aramant et al. 1990; Bergström et al. 1994; Seiler and Aramant 1994; Szél et al. 1994). In view of the suggested trophic function of NO, it was our interest to examine whether transplanted retinas maintain their ability to express NOS immunoreactivity, and whether this expression appeared stimulated or inhibited following transplantation.

Materials and methods

Animals

The experiments were conducted after approval by the local animal experimentation and ethics committee (Djurförsöksetiska nämnd i Lund).

Retinas from pigmented rabbits were obtained at embryonic days (E) 15, 20, 25, 29, postnatal days (PN) 0, 3, 5, 7, 11, 15, 20, 46, 60 (at least two animals at each stage) and from adult (n=4). Embryonic tissue was obtained by Caesarean section after killing the pregnant rabbits. Embryos and PN 0–3 animals were killed by decapitation. Young animals (PN 5–20) were anaesthetised with carbon dioxide prior to decapitation. Older animals were killed by an overdose of pentobarbital (72 mg/kg, i.p.). There were no indications that the different methods used in the present study to kill the animals (decapitation, anaesthesia followed by decapitation, or overdose of pentobarbital) had any effect on the distribution of NOS immunoreactivity.

Retinal transplants

Donor tissue

An ordinary mixed strain of pigmented rabbits from stage E 15 (15th postconception day; normal gestational time 30–31 days) were used as donors. Embryos were obtained by Caesarean section after killing the pregnant female rabbits with pentobarbital (as above). The embryos were kept at 4°C in Ames' solution having the following composition: 120 mM NaCl, 3.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 23 mM $NaHCO_3$, 0.1 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 10 mM glucose. Eyes from these embryos were enucleated, and the neural retina was then dissected from the posterior eyecup under an operating microscope. Care was taken not to damage the neural retina, especially when separating it from the pigment epithelium. These retinas were kept in fresh Ames' solution at 4°C until transplanted.

Recipients

Pigmented rabbits (4–6 months old at the time of transplantation) of the same strain as the donors were used as recipients. Fifteen minutes before surgery, the right pupil of the recipient was dilated with 1% cyclopentolate-HCl (Alcon-Couvreur, Belgium) and, if necessary, also with 10% phenylephrine-HCl (Sanofi Winthrop Pharmaceuticals, New York, USA). The animals were anaesthetised with 1 ml/kg Hypnorm (10 mg/ml fluanison, and 0.2 mg/ml fentanyl; Janssen Pharmaceutica, Beerse, Belgium). One to two drops of tetracaine-HCl (Alcon-Couvreur) were instilled into the eye as needed. Thirty-four transplants were performed in this study. The equivalent E or PN age of the transplants (donor age plus the survival time) was: E: 16, 19, 21, 26, 29; PN: 4, 11, 12, 20, 44, 75, 90, 102, 109, 116 and 123.

Details of the transplantation procedure have been previously published (Bergström et al. 1992). Briefly, the donor tissue, which consists of fragmented pieces of both peripheral and central retina, was drawn up in a thin plastic capillary. The capillary was advanced through a small scleral incision behind the limbus and through the vitreous body to the posterior pole of the eye. The capillary was then pushed under the subretinal space, whereby the donor tissue was slowly delivered with the help of a precision microsyringe. Animals were kept in a light/dark cycle of 12 h each. No immunosuppressive drugs were used. Rabbits were later killed at 1–139 days after surgery.

Tissue preparation

Eyes were quickly enucleated and immersed in a freshly prepared solution of 4% formaldehyde in 0.1 mM phosphate-buffered saline (PBS; pH 7.2). The eyes were hemisected in the fixing solution, and the anterior segment, lens and vitreous body were discarded. The remaining eyecups were transferred to fresh fixation medium and kept at 4°C for 2–24 h. All tissue was thoroughly rinsed and cryoprotected by transferring it stepwise through solutions of 5, 10, 15 and 20% sucrose in PBS.

Rabbit eyecups were divided across the optic nerve head, perpendicularly to the myelinated streak and 10- to 15-µm sections were cut on a cryostat. Rabbit retinal pieces were cut along the vertical axis, and the sections obtained included superior and inferior retina, and the myelinated streak. In case of transplants, the area containing the transplant was cut out. The sections were collected on chrome alum-coated slides, air-dried and stored at –20°C until used.

Immunocytochemistry

The NOS antiserum employed was raised in sheep against purified rat recombinant neuronal NOS protein (a gift from I. Charles and P. C. Emson, MRC, Cambridge, UK). The antibody recognises a

protein with a molecular mass of 155 kDa on western blot analysis of rat hypothalamus (Herbison et al. 1996), and is therefore specific for neuronal NOS (Förstermann et al. 1991). Adsorption of the antiserum with the recombinant neuronal nNOS protein abolished all immunoreactivity (Herbison et al. 1996).

Sections were brought to room temperature and air-dried before preincubation for 90 min with 0.1 mM PBS containing 0.25% or 1% BSA, 0.25% Triton X-100 and 1.5% donkey normal serum. The sections were then incubated with the anti-NOS serum (1:3500), diluted in PBS/BSA/Triton X-100 for 12–18 h at 4°C. After rinsing, the sections were incubated with secondary antibody: Texas Red sulphonyl chloride-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch, West Grove, Pa., USA), diluted 1:80, for 90 min at room temperature. The sections were then rinsed with 0.1 mM PBS and mounted with buffered glycerol containing phenylendiamine (Merck, Darmstadt, Germany) to prevent fluorescence fading. Incubation of sections with the secondary antibody alone resulted in no labelling.

Results

Normal development

Immunolabelling corresponding to NOS was not detected in rabbit retinas at E 15, E 20 or at E 25. However, a few weakly stained cells were found at E 29 (Fig. 1A). The la-

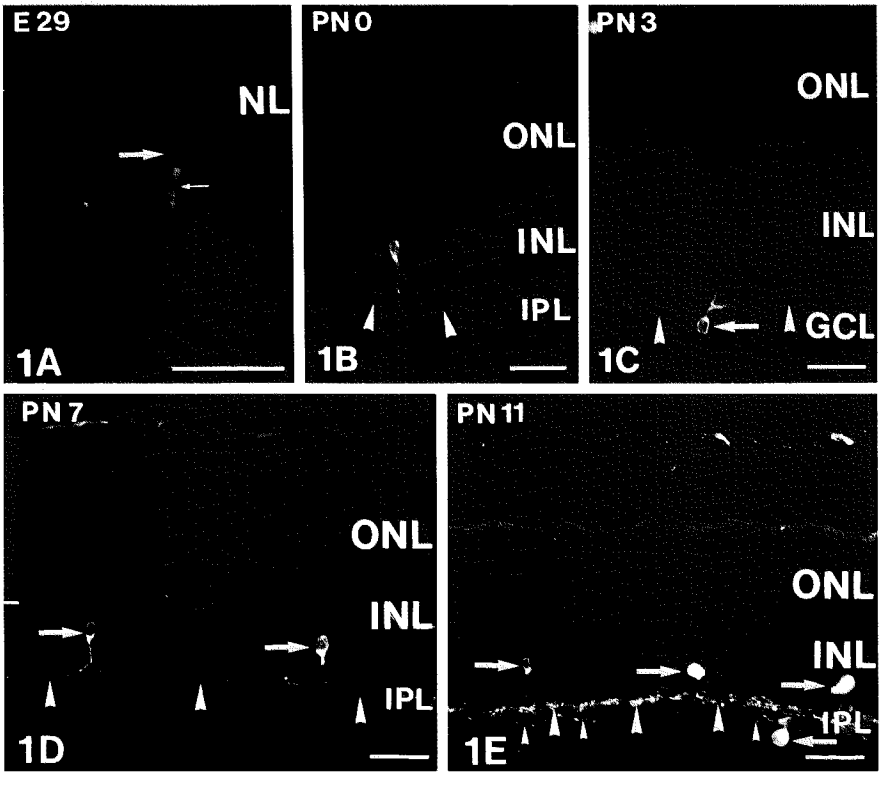
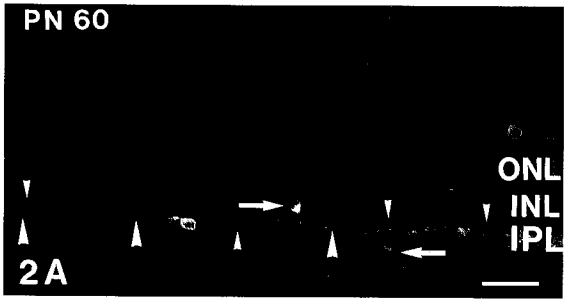
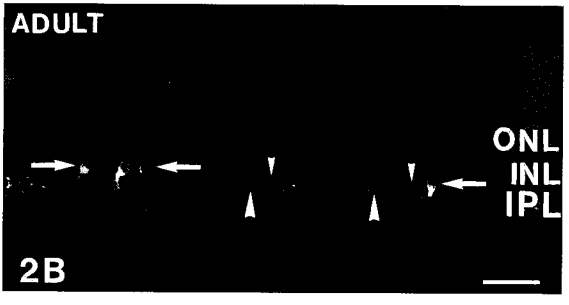
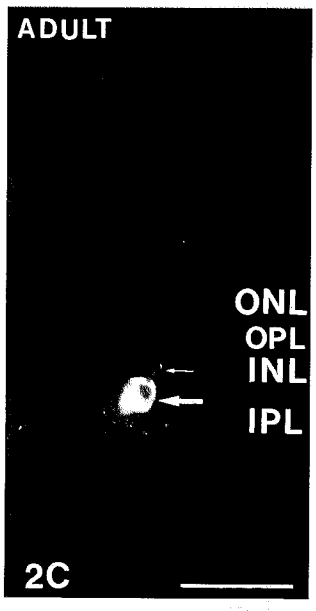


Fig. 1A–E Nitric oxide synthase (NOS) immunoreactivity in normal developing retinas (fluorescence micrographs). **A** Embryonic day 29 (*E 29*) retina showing a faintly labelled cell (*arrow*) in the innermost part of the neuroblastic cell layer (*NL*) projecting a short process (*small arrow*) vitreally. **B** On the day of birth (*PN 0*), NOS immunoreactive processes are seen in the inner plexiform layer (*IPL*; *arrow heads*). (*ONL* outer nuclear layer, *INL* inner nuclear layer). **C** Postnatal day 3 (*PN 3*) retina showing an immunoreactive cell in the ganglion cell layer (*GCL*; *arrow*), projecting immunoreactive fibres onto the IPL (*arrowheads*). **D** postnatal day 7 (*PN 7*) retina showing immunoreactive cells (*arrows*) and a distinct, continuous immunoreactive plexus in the IPL (*arrowheads*). **E** Postnatal day 11 (*PN 11*) retina showing immunoreactive cells on both sides of the IPL (*arrows*). Note also that the immunolabelled cell body to the left (with a continuous process projecting towards the IPL) is smaller than the other immunoreactive cells. Labelling in the IPL appears in distinct sublayers (*small* and *large arrowheads*). *Scale bars* 40 µm







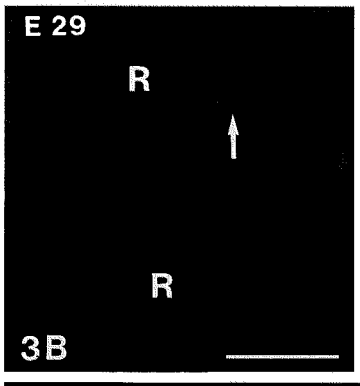
belled cells were seen in the proximal margin of the neuroblastic layer in the inferior retina, near the optic disc. Short, stained processes could be seen to project vitreally.

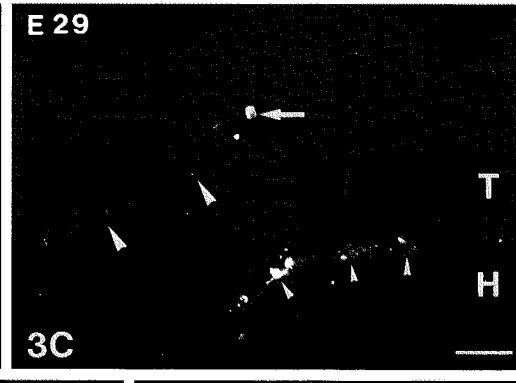
At PN 0, NOS-positive cells were seen in the central retina, both in the inferior and superior retina. Long and well-stained dendrites could at times be seen to project towards the developing inner plexiform layer, where weakly stained dots could be distinguished (Fig. 1B). At PN 3, more strongly positive cells were seen, and could at times also be found in the ganglion cell layer (Fig. 1C). All stained cells were found to project towards the inner plexiform layer, where continuous, labelled processes were seen. At PN 5 and PN 7, immunolabelled cells were seen in the inner nuclear and ganglion cell layers in the central as well as peripheral retina, but were still more abundant in the central areas. Distinct labelling of processes was seen mainly in a band located in the middle of the inner plexiform layer (Fig. 1D). At PN 11, it became possible to distinguish labelled cells of at least two types. One larger cell type exhibiting stronger immunoreactivity and another, smaller and more weakly labelled. Labelling in the inner plexiform layer was seen in a thick band located in the middle of the layer, and in a weaker and more proximal sublamina (Fig. 1E). Some lightly stained dots could at times be seen also in the most distal sublamina of the inner plexiform layer. No further changes were observed at PN 15, PN 20 or PN 46.

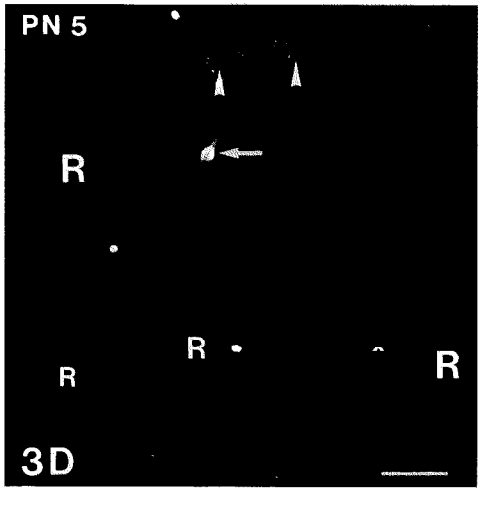
Fig. 2A–C NOS immunoreactivity at postnatal day 60 (*PN 60*) and adult retinas (fluorescence micrographs). **A** PN 60: immunoreactive cells are seen on both sides of the IPL (*arrows*). A continuous and multilayered immunoreactive plexus is seen in the IPL (*small* and *large arrowheads*). **B**, **C** Adult retinas. In **C** a labelled cell (*arrow*) is seen in the INL projecting towards the IPL and the outer plexiform layer (*OPL; small arrow*). *Scale bar* 40 µm

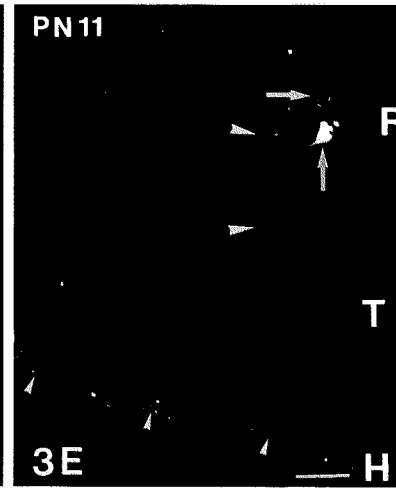
Fig. 3A–E Rabbit to rabbit retinal transplants. **A** Bright field micrograph showing a typical subretinal transplant (*T*) obtained by the technique used in the present study (corresponding to PN 12; haematoxylin and eosin staining). The darkly stained cells of the ONL (*black arrowheads*) are located in the luminal layers of the rosettes (*R*) and the more lightly stained cells of the inner retina (*white arrowheads*) between the rosettes. These two layers of cells are separated by a plexiform layer (*small arrows*).The host retina (*H*) is often thin and degenerated near the transplant. **B–E** Fluorescence micrographs illustrating the distribution of NOS immunoreactivity in retinal transplants. **B** Retinal transplant 14 days after surgery (corresponding to E 29) showing a faintly immunoreactive cell (*arrow*) in between rosettes (*R*). **C** Another transplant of the same age as in **B** showing a labelled cell (*arrow*) and immunoreactive processes (*arrowheads*) in the transplant (*T*). **D** Retinal transplant corresponding to PN 5 showing an immunoreactive cell (*arrow*) between rosettes (*R*) and an immunoreactive plexus (*arrowheads*). **E** Retinal transplant (*T*) corresponding to PN 11 showing immunoreactive cells (*arrows*) and a plexus (*arrowheads*) between rosettes (*R*). In **C** and **E**, an immunoreactive plexus (*small arrowheads*) is also visible in the host retina (*H*). *Scale bars* **A** 100 µm, **B–E** 40 µm


PN 12
R
R
R
R
R
R
T
H
3A

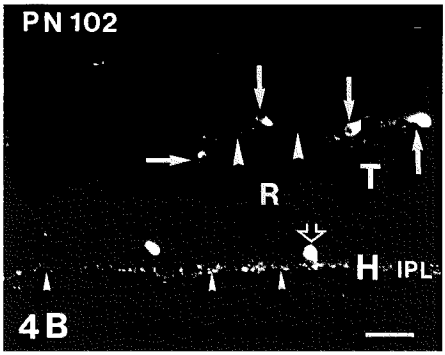

E 29
R
R
3B

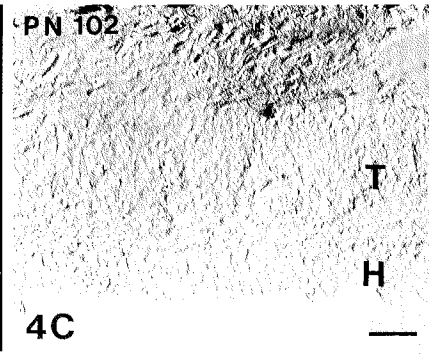

E 29
T
H
3C

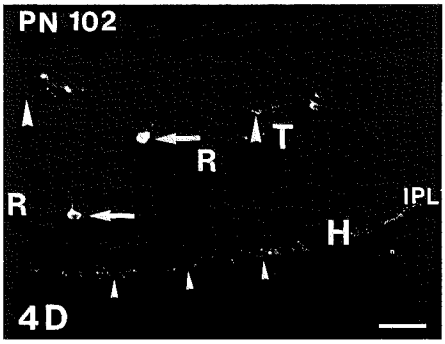

PN 5
R
R
R
R
3D

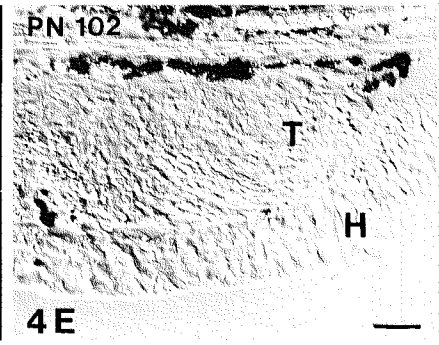

PN11
R
T
H
3E


PN 75
R
R
R
T
IPL
H
4A


PN 102
R
T
H
IPL
4B


PN 102
T
H
4C


PN 102
T
R
R
IPL
H
4D


PN 102
T
H
4E

At PN 60, the pattern of distribution of immunoreactivity was similar to that observed in the adult (Fig. 2A, B). NOS-immunoreactive cells were seen both in the inner nuclear and ganglion cell layers, and at times even within the inner plexiform layer (Fig. 2A). The largest number of NOS-positive cells was seen in the visual streak. Most stained cells were seen to project towards the inner plexiform layer, where a distinct band of stained processes was seen in the middle of the layer. Thinner immunolabelled processes were also seen in the most distal and most proximal sublaminae of the inner plexiform layer. At times, immunolabelled cells located in the inner nuclear layer were seen to emit processes towards the outer plexiform layer (Fig. 2C). However, no specific labelling was detected in the latter.

Transplanted retinas

All subretinal transplants were seen to have developed. The transplanted cells tended to cluster in spherical structures, which, at the light microscopic level, could be seen in cross sections as so-called rosettes (Fig. 3A). These consisted of somewhat organised structures where photoreceptor cells were found located nearest the lumen, with the inner and outer segments projecting into the lumen. Cells of the inner retinal layers could be found between the rosettes.

No NOS immunoreactivity was detected among transplanted cells of ages corresponding to E 16–26. In transplants corresponding to E 29, a few stained cells were occasionally observed (Fig. 3B, C). The labelled cells were found between rosettes, where cells normally located in the inner retina are expected. The same observations were made at later stages (transplants corresponding to PN 0–123; Fig. 3D, E, 4). The number of stained cells was, however, seen to vary between the specimens and at times also between sections. Nontheless, a relatively large number of distinctly labelled cells could be seen, even in older transplants (e.g. PN 75 and PN 102; Fig. 4A, B, D).

NOS-immunoreactive cells were not seen to be located in any particular area of the graft, in terms of proximity to the host retina or to the choroid. At least two subtypes of NOS-containing cells appeared to be present in the grafts, but it was not possible to establish whether there was any difference between the two types regarding survival.

Immunoreactive processes were seen in the grafts and could at times be found to project from the labelled cells to one region, forming what seemed to be an equivalent of an inner plexiform layer within the graft (Fig. 3C–E, 4A, B, D). This layer was at times made of a dense plexus of stained fibres which often ran for considerable distances. In a few cases, labelled fibres were seen to project towards the host retina (Fig. 4A, D). However, we were not able to detect NOS-labelled fibres bridging between the host and the graft.

Immunolabelled cells were seen in the host retinas at all time points. Unless the inner nuclear layer had degenerated following transplantation, NOS-positive cells could also be seen in the host retina in the region contacting the graft (Fig. 4A, B). These had a normal appearance and were seen to project towards the inner plexiform layer, as seen in intact retinas. There was no indication that the number of NOS-labelled cells was altered in the host retina following transplantation.

Discussion

In the adult rabbit retina, cells located in the proximal inner nuclear layer and in the ganglion cell layer were found to exhibit NOS immunoreactivity, indicating that most of them are likely to correspond to amacrine cells. These results agree with our previous observations on the adult rabbit retina (Perez et al. 1995). In this earlier study, an antibody against a portion of the C-terminus of rat neuronal NOS was employed, whereas an antibody raised against the whole rat neuronal NOS protein was used in the present study (Herbison et al. 1996). Yet, the same results were obtained with the two antisera. NOS immunoreactivity has also been demonstrated over photoreceptor inner segments and cone outer segments employing a different antibody against the whole rat brain molecule (Koch et al. 1994). Differences in sensitivity between the antisera or differences in the handling of the retinal tissue before processing for immunohistochemistry could perhaps explain the discrepancy. There is also the possibility that the antigen expressed by the photoreceptor cells is not recognised by the antibodies which we have employed (Perez et al. 1995; present study).

Normal development

The results obtained in the present study on the presence of NOS in developing rabbit retina are in good agreement with those previously reported for the localisation of NADPH-diaphorase (Mitrofanis et al. 1992). It was found in the latter study that NADPH-diaphorase reactivity could be detected first at E 28, which conforms with the presence of NOS immunoreactivity at E 29 seen in the present study. Although no quantitative measurements are available, the number of NOS-labelled cells

◀ **Fig. 4A** Retinal cell transplant 91 days after transplantation (corresponding to PN 75). NOS-immunoreactive cells (*large arrows*) are seen in the transplant (*T*). One of the cells projects (*small arrow*) towards the host (*H*) and towards what appears to be an equivalent of an IPL in the transplant (*large arrowheads*). Immunoreactive processes are seen also in the host retina (*H*) in the inner plexiform layer (*IPL*; *small arrowheads*). **B–E** Retinal transplants corresponding to PN 102. **C** and **E** (Nomarski optics) represent the same fields as in **B** and **D**, respectively. Immunoreactive cells (*arrows*) in the transplant (*T*) are seen to project and form a plexus (*large arrowheads*). Host retina (*H*) also shows a labelled cell (*open arrow* in **B**) and an immunoreactive plexus (*small arrowheads*) in the IPL (*R* Lumen of rosettes) *Scale bars* **A** 100 μm, **B–E** 40 μm

appeared to increase during the first 2 postnatal weeks, which agrees with the distribution of NADPH-diaphorase-reactive cells previously observed in the developing rabbit retina (Mitrofanis et al. 1992).

It is likely that most of the NOS-containing cells found in the proximal margin of the neuroblastic layer in embryonic and early postnatal ages correspond to amacrine cells. Their morphology and location was seen in later postnatal ages to be equivalent to that seen in the adult retina. The two main types of NO-synthesising cells were distinguishable at around PN 11, which again agrees with the observations made with NADPH-diaphorase (Mitrofanis et al. 1992).

The ability of amacrine cells to express NOS, even prenatally, cannot be associated with a role of NO in the processing of visual information, as maturation and synaptogenesis involving these cells are not established until later (Dacheux and Miller 1981). It suggests, rather, that the early onset of NOS expression is genetically determined and that NO may participate in the process of retinal development. Other transmitter/neuromodulator compounds, such as GABA and glycine, as well as tyrosine hydroxylase have also been previously detected in embryonic or neonatal rabbit amacrine cells (Casini and Brecha 1992; Pow et al. 1994). GABA has, in addition, been shown to be transiently expressed in developing horizontal cells (Redburn 1992), further supporting the notion that GABA may play a trophic role in the retina. Although it is not possible to determine if the same cells express NOS both during development and in the adult, there were no indications in the present study that NOS might be overexpressed during early stages, or that a particular cell type might express NOS only during development. The early expression of NOS suggests therefore, as pointed out above, that NO may play a role in retinal development.

It is, however, thus far uncertain how NO may participate in neuronal development. Inhibition of endogenous NOS has been seen in vitro to inhibit cerebellar granule cell proliferation and migration, suggesting that NO may be important for the differentiation of these cells during cerebellar cortical development (Tanaka et al. 1994). Further, the application of nerve growth factor (NGF), a neurotrophin, which, for instance, in the retina, is seen to promote neurite outgrowth of embryonic ganglion cells (Lehwalder et al. 1989), has been shown to upregulate the expression of NOS mRNA in brain cells (Holtzman et al. 1994). It is thus possible that one of the functions of NO during development is to mediate, at least in part, the actions of NGF and/or of other neurotrophic factors.

Transplants

The development and the organisation of the transplants examined in the present study was found to be similar to that observed previously (Bergström et al. 1992).

Transplanted embryonic retinas need not only to develop, but must also overcome the stress caused by the transplantation procedure, which could lead to an up- or to a down-regulation of neuronal NOS expression. NOS immunoreactivity was detected in the transplants 14 days after surgery, which corresponds to E 29, assuming that the graft matures at the same pace as normal retina. Also in the normal retina, NOS immunoreactivity was detected at E 29, indicating that, in at least some of the transplanted cells, the ability to express NOS is maintained and follows the same timetable as in the normal developing retina.

Similarly to what was seen with normal developing retinas, there were no indications that a certain retinal cell type might express NOS only during development. It is thus reasonable to assume that the NOS-immunoreactive cells found in the transplants correspond, as in normal retinas, mainly to amacrine cells. Further, similarly to what was seen during normal development, there were no signs that NOS would be overexpressed among the developing transplanted cells. However, an accurate quantitative analysis was not possible. A relatively large variation in the number of NOS-stained cells was seen not only between different specimens, but also between sections and in the same section. In some sections, large numbers of NOS-positive cells were indeed seen. However, as the transplants consisted of a non-uniform mixture of both central and peripheral embryonic retinas and in normally developing retinas most NOS-labelled cells were found in early stages in the central retina, the number of NOS-immunolabelled cells can be expected to vary in the grafts. It is possible that at times retinal pieces derived mainly from the central retina were represented in a section, which may explain the apparently larger number of labelled cells.

An inducible form of NOS can be expressed in macrophages and astrocytes in response to endotoxins and cytokines (Goureau et al. 1994). It can thus not be excluded that the inducible NOS isoform is also expressed by the transplanted cells. However, the antibody employed in the present study is specific for the neuronal form of NOS, which excludes the possibility that an increased expression of NOS immunoreactivity in the transplants would correspond to the inducible form.

The transplanted retinas were obtained at E 15, a time at which connectivity is not yet established. Many of the grafted cells are also damaged in the process of transplantation. Yet, in spite of the disruption which can be expected to occur during the transplantation procedure, a certain degree of organisation could be observed in the transplants in the following days. Although grafted photoreceptors tended to arrange themselves in rosettes, NOS-labelled cells were often seen to emit processes so that the equivalent of an inner plexiform layer was identifiable. The labelled cells were found in the periphery of the rosettes, projecting mainly towards a particular area of the graft. NOS-labelled processes were found in the transplants to project at times for long distances, and even towards the host retina. However, it was not possible to establish whether NOS processes actually entered the host retina.

In summary, we found no evidence that NOS may be produced in any retinal cell type only during development. Nevertheless, we have shown that NOS immunoreactivity can be detected prenatally in the rabbit retina, making it possible that, in some way, NO plays a role in retinal development. NOS-immunoreactive cells were also found in transplanted retinas. A number of neurochemical markers normally localised in normal retinas (e.g. choline acetyltransferase, tyrosine hydroxylase, glutamic acid decarboxylase and GABA itself) have been previously detected in transplanted retinas (Aramant et al. 1990; Bergström et al. 1994), suggesting that many of the factors required for normal development are preserved after transplantation. NO produced by transplanted cells is likely to act in the same way as it does in a normal developing retina, perhaps as a mediator of a neurotrophic factor. Further, the ability to synthesise NO appears to be maintained in transplanted retinas for at least almost 5 months after transplantation. The study of NOS immunoreactivity in transplanted retinas also allowed the detection of a distinct inner plexiform layer within the grafts.

Acknowledgements The authors wish to thank Karin Arnér, Ingela Liljekvist-Larsson and Katarzyna Said for technical assistance. This study was supported by grants from the Swedish Natural and Medical Sciences Research Councils (B-BU 9981-303-309; B96-14X-2321), the Faculty of Medicine, University of Lund, Hjärnfonden, the Swedish Society for Medical Research, the Riksbankens Jubileumsfond, the Clas Groschinskys Minnesfond, the Crown Princess Margareta's Committee for the Blind, and the Crafoord, Lundbeck, Anna Lisa and Sven-Eric Lundgren, Segerfalk, Åke Wiberg, Greta and Johan Kock and Thelma Zoéga Foundations.

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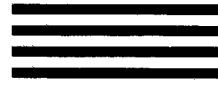
Paper IV



Nitric oxide synthase immunoreactive processes from retinal transplants to the host

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Purpose. To demonstrate the presence of graft-host connections in rabbit-to-rabbit subretinal transplants by examining a chemically and functionally defined cell type, the nitric oxide synthase (NOS) containing neurons.

Methods. Embryonic day 15 rabbit retinas were transplanted to the subretinal space of adult rabbits. After various survival times (grafts reaching the equivalent ages of postnatal days 5, 12, 20, 45, 90, 102), the transplanted retinas were processed for immunocytoche-mistry. An antibody against the neuronal form of nitric oxide synthase, which is expressed in retinal neurons presumably capable of producing nitric oxide, was employed.

Results. In the normal rabbit retina, NOS was expressed in wide-field amacrine cells and in long processes that ramify within the inner plexiform layer. NOS immunoreactive cells were seen in transplants at all survival times examined. Immunolabeled processes were also seen to converge to one or several regions within the grafts. In addition, single labeled fibers originating in the graft were seen to cross the graft-host interface. In a few cases, the bridging fibers could be seen to reach the NOS-immunolabeled host inner plexiform layer.

Conclusions. NOS-containing cells developed in subretinal rabbit-to-rabbit transplants and extended processes that at times could be followed for long distances, making these cells good candidates for studies of graft-host connections. Some of the connecting processes reached the host inner plexiform layer, the target within the host for NOS-containing fibers. The presence of synaptic contacts could not be determined with the technique employed. However, the fact that connections are established by nitric oxide-producing cells, a compound known to play a modulatory role in the retina, suggests that graft-host functional integration could potentially occur.

Key words: Retina, Transplant, Connections, Nitric Oxide Synthase, Rabbit

Introduction

Studies in the past have given ample evidence that transplanted embryonic retinas survive and develop *in oculo,* especially when placed in the subretinal space. [1-5] Most cell types and synapses develop in the transplants, and structural

proteins and neurotransmitters found in the normal retina are also expressed by the transplanted cells. [1,5-11] This indicates that retinal transplants may be capable of carrying out some basic functions. Electrophysiological experiments have shown that 'ON' and 'ON-OFF' spike-like responses can be recorded from the surface of the grafts. [12] However, this does not mean that the information is passed on from the graft to the host.

Tracing studies have demonstrated that in retinas transplanted close to target brain areas, surviving ganglion cells project into the host target tissue. [13-17] However, for *in oculo* retinal transplants to be functional, it is required that they integrate and form connections with the host retina. A few processes belonging to cell aggregates placed epiretinally have been shown to project into the host retina. [1,6,18] However, ideally, the donor tissue should be placed subretinally, whereupon the grafted cells should connect with their partner cells in the host retina. Transgenic photoreceptors cells expressing the *lacZ* reporter gene product, ß-galactosidase, transplanted subretinally, have been shown to contact bipolar cells which did not express this gene. [19,20] However, as pointed by the authors, the demonstration of connections using this approach is equivocal if the donor tissue in these experiments contained also non-photoreceptor cells. In another study, a few synaptic contacts were found between grafted photoreceptors expressing the *lacZ* transgenic marker and labeled host bipolar cells. [16,21]

Thus, connections between transplanted retinal cells and the host retina seem to occur. However, they appear to be few. One of the difficulties in demonstrating connectivity lies in the fact that processes in the grafts normally cannot be followed for long distances. We have previously demonstrated that in rabbit-to-rabbit transplants, nitric oxide synthase (NOS) containing cells are found in subretinal grafts and that these can occasionally be seen to extend long labeled processes. [22] With the antibody used in this and in the previous study [22] we have found that in the normal rabbit retina, NOS is expressed by subpopulations of wide-field amacrine cells, and it is likely that the NOS-containing cells found in grafts are of this type. Taking advantage of the morphological features of NOS-containing amacrine cells, we were in this study able to detect the presence of labeled fibers originating in the graft and extending into the host. Further, the projecting fibers were in some of these cases seen to reach the host inner plexiform layer. Since a functional role has been ascribed to nitric oxide in the retina [23] we thus demonstrate graft-host integration established by a chemically and functionally defined cell type.

Material and Methods

The experiments were conducted with the approval of the local animal experimentation and ethics committee (*Djurförsöksetiska nämnden i Lund*). Animals were handled according to the guidelines on care and use of experimental animals set by ARVO and the Declaration of Helsinki.

Retinal transplants

Outbred pigmented rabbits of mixed strain (embryonic stage (E) 15; normal gestation 31 days) were used as donors for the transplantation. Embryos were obtained by Cesarean section after sacrificing pregnant dams with an overdose of sodium pentobarbital. The embryos were placed in Ames' solution at 4° C containing (mM) NaCl (120), KCl (3.6), $MgSO_4$ (1.2), $CaCl_2$ (1.2), $NaHCO_3$ (23), NaH_2PO_4 (0.1), Na_2HPO_4 (0.4) and glucose (10). The eyes were removed and the neural retinas were dissected free from the retinal pigment epithelium. Both peripheral and central areas were used for transplantation. The isolated retinas were stored in Ames' solution at 4° C (composed as above) until used for transplantation (4 hours at most).

Eleven adult rabbits of the same breed as the donors (4-6 months old at the time of transplantation) were used as recipients. The transplantation technique has been described earlier. [4] In brief, thirty minutes before the surgery, the right pupil of the recipients was dilated with 1% cyclopentolate-HCl (Cyclogyl®, Alcon-Couvreur, Belgium) and one drop of 10% phenylephrine-HCl (Sanofi Winthrop Pharmaceuticals, New York, USA). The recipient rabbits were anaesthetized with 1ml/kg Hypnorm® (10 mg/ml fluanison and 0.2 mg/ml fentanyl; Janssen Pharmaceutica, Beerse, Belgium). One to two drops of tetracaine-HCl (Alcon-Couvreur) were instilled into the eye. One to four embryonic retinas (in up to 10 µl total volume) were drawn up into a thin polyethylene capillary mounted on a special instrument which was connected to a precision microsyringe. A small scleral incision was made in the recipient eye 2-4 mm behind the limbus. The capillary was advanced through the vitreous to the posterior pole of the eye where the retina was penetrated. The embryonic retinas were slowly deposited in the subretinal space below the myelinated streak in the central retina. The animals were kept in light/dark cycles (12 hours each) and were allowed to survive for 21-118 days after the surgery. The transplants thus reached ages equivalent to postnatal 5, 12, 20, 45, 90, and 102. No immunosuppressive drugs were used.

Tissue preparation

Eyes carrying transplants were quickly enucleated and immersed in a freshly prepared solution of 4% formaldehyde in Sörensen's buffer (0.1 mM; pH 7.2). The eyes were bisected in the fixation medium, and the anterior segment, lens, and vitreous body were removed. The posterior segments were transferred to fresh fixa-

tion medium (as above) and kept at 4° C for 2 hours. The tissue was subsequently rinsed and cryoprotected in Sörensen's buffer with increasing concentrations of sucrose (5, 10, 15, 20, and 25%). The area containing the transplant was cut out, embedded in an albumin-glycerin medium (30 gr. egg albumin, 3 gr. gelatin, 100 ml distilled water) and frozen. Sections were obtained on a cryostat (12 μm), collected on gelatin/chrome alum-coated slides, air-dried, and stored at -20° C until used. Certain sections were stained with hematoxylin and eosin.

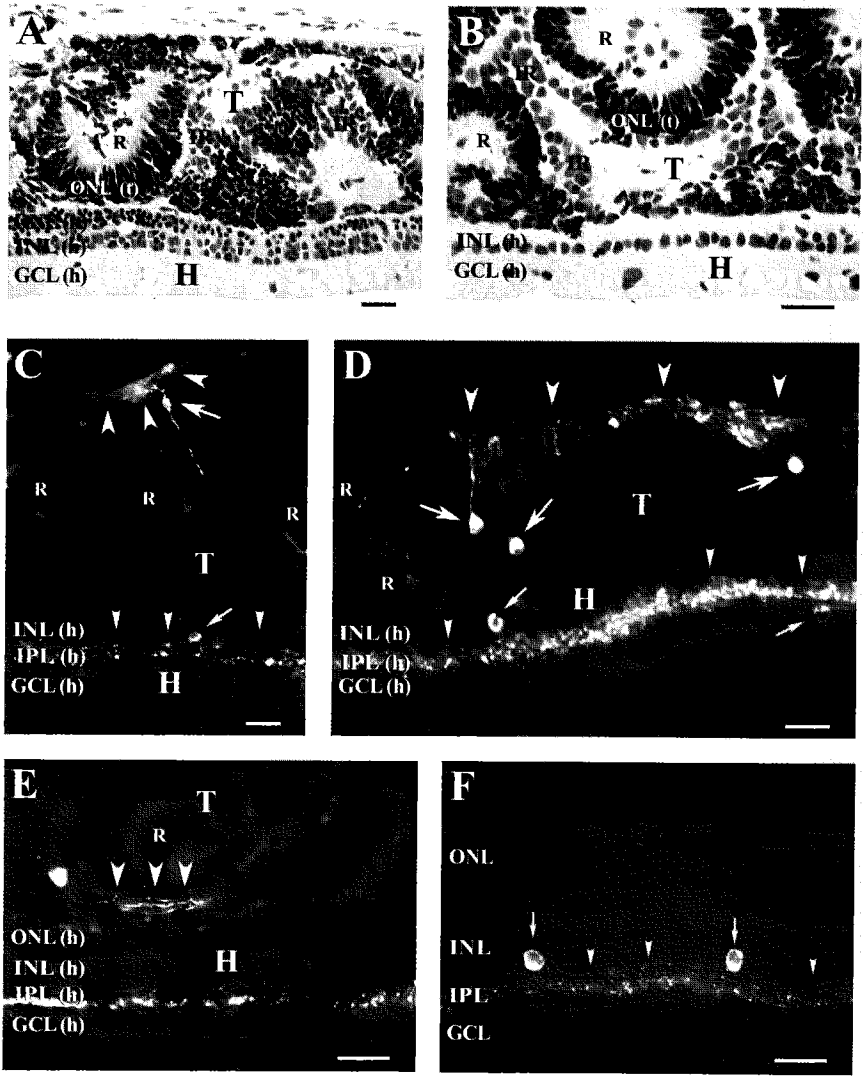


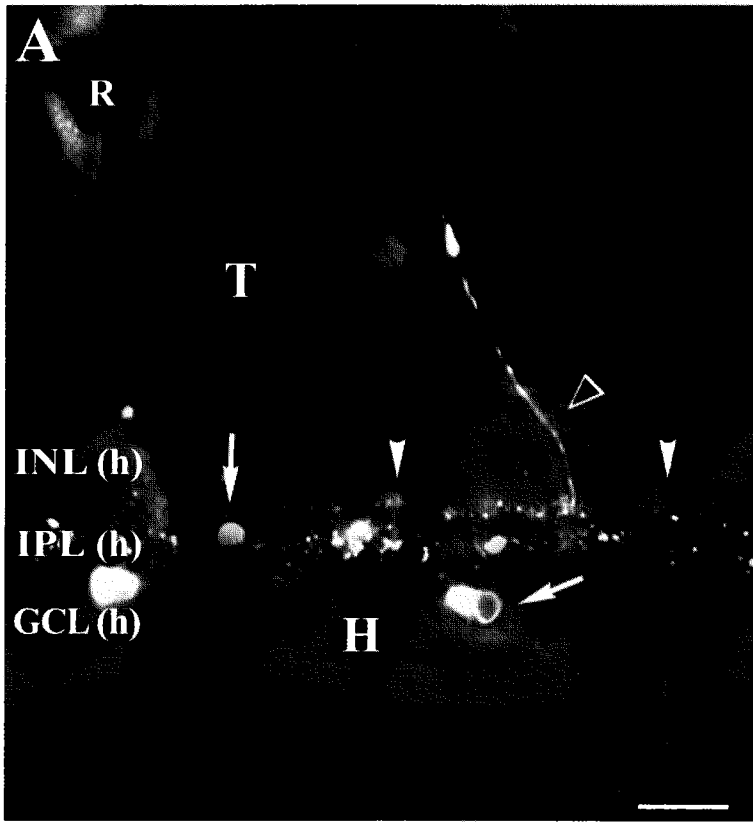
Figure 1: (A-B) bright field micrographs (hematoxylin-eosin) showing typical rabbit-to-rabbit subretinal transplants: (A) transplant [T] corresponding to PN 5 and (B) transplant corresponding to PN 20. In the transplants, cells belonging to inner retinal layers [IR] are located between photoreceptor cells [ONL (t)], which are organized in rosettes [R]. In (A), the outer nuclear layer of the host retina [ONL (h)] is partially degenerated. In (B), the host outer nuclear layer and part of the host inner nuclear layer [INL (h)] have degenerated. (C-F) fluorescence micrographs showing the distribution of NOS-immunoreactive cells. (C) retinal transplant corresponding to PN 5 showing a NOS-immunoreactive cell (arrow) that projects towards a region within the transplant where a NOS immunoreactive plexus is seen (arrowheads). (D) a PN 12 retinal transplant showing several NOS-containing cells in the transplant (arrows). One of these labeled cells is seen to send a long process towards a plexiform layer within the graft (arrowheads), which developed parallel to the host inner plexiform layer [IPL (h)]. (E) shows a specimen where a few cell rows are left of the host outer nuclear layer [ONL (h)]. Immunoreactive fibers in the transplant run parallel to the graft-host border (arrowheads). In (C-D), immunoreactive cells (small arrows) can be seen in the host retina (H) in the inner nuclear layer [INL (h)] and in the ganglion cell layer [GCL (h)]. The host inner plexiform layer [IPL (h)] is also labeled (small arrowheads). (F) a normal adult rabbit retina showing NOS immunoreactive cells (small arrows) in the proximal inner nuclear layer [INL] and their processes in the inner plexiform layer [IPL] (small arrowheads). [T], [t] = transplant; [H], [h] = host retina; [ONL] = outer nuclear layer; [INL] = inner nuclear layer; [IPL] = inner plexiform layer; [GCL] = ganglion cell layer; [R] = rosette. Scale bars = 30 μm.

Immunocytochemistry

Cryostat sections were incubated for 90 min with 0.1 mM PBS containing 0.25% BSA, 0.25% Triton X-100 (PBTx), and 2% normal serum, followed by overnight incubation at 4°C with sheep anti-neuronal NOS serum (1:4200 in PBTx containing 0.5% normal serum). After rinsing, sections were incubated for 90 min with texas red sulfonyl chloride- (TRSC-) conjugated donkey anti-sheep IgG (1:80; Jackson ImmunoResearch, West Grove, PA, USA). Following immunostaining, sections were rinsed and mounted with buffered glycerol containing the anti-fading phenylendiamine (Merck, Darmstadt, Germany). The NOS antiserum employed (gift from Dr. I. Charles and Dr. P. C. Emsom) was raised against purified rat recombinant neuronal NOS protein and was found to be specific for neuronal NOS in control experiments. [22]

Results

Pieces of embryonic retina transplanted to the subretinal space developed all the retinal layers. The cells of the outer nuclear layer formed rosettes, and in between the rosettes were situated cells of the inner retina (Figs. 1A and B). With time, the outer host retina was degenerated in the areas adjacent to the graft (Figs. 1A and B, hematoxylin-eosin; Figs. 1C, D, 2, and 3, immunofluorescence). As a result, some NOS-containing cells in the grafts were occasionally found relatively near the host inner plexiform layer. In the present study, NOS immunoreactive cells were judged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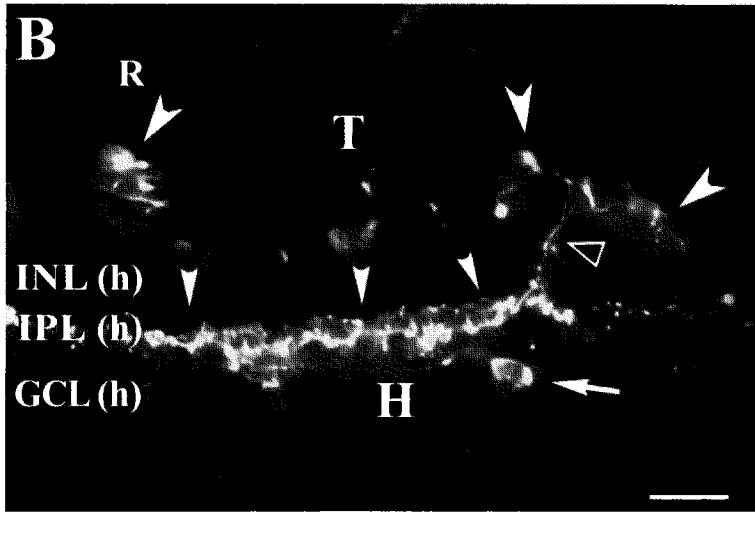
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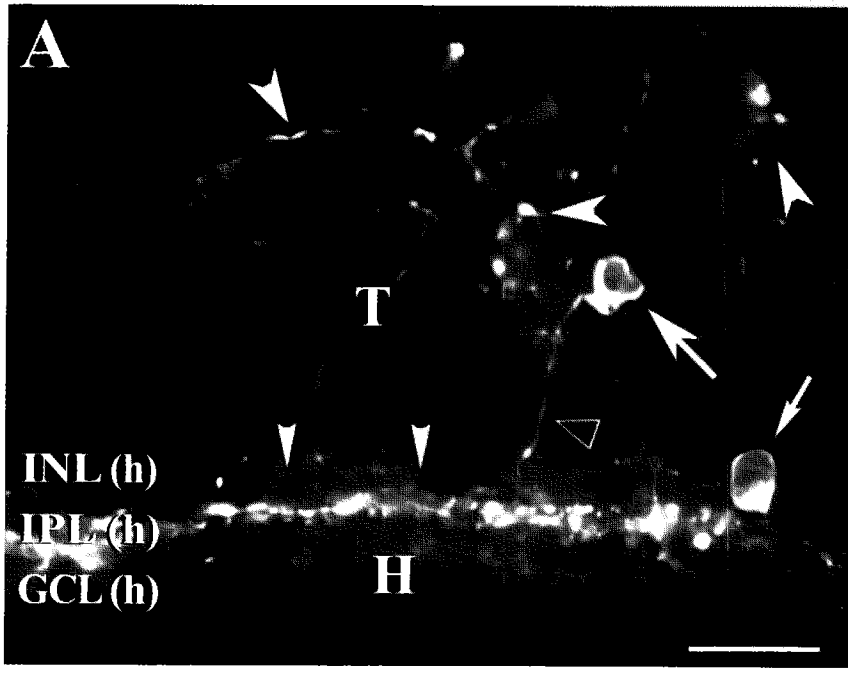
Figure 2: (A-B) fluorescence micrographs showing extension of NOS immunoreactive fibers from the transplant [T] to the host retina [H]. (A) a PN 12 rabbit retinal transplant with a rosette [R]. The host retina exhibits immunoreactive cells located within the inner plexiform layer [IPL (h)] and ganglion cell layer [GCL (h)] (small arrows), and an immunoreactive plexus (small arrowheads) in the inner plexiform layer [IPL (h)]. A long immunoreactive process (open arrowhead) is seen to cross over from the transplant to the host inner plexiform layer [IPL (h)]. (B) a PN 20 retinal transplant showing a short process (open arrowhead) connecting the immunoreactive plexus in the transplant (large arrowheads) to the immunoreactive plexus in the host inner plexiform layer [IPL (h)] (small arrowheads). [T] = transplant; [H], [h] = host retina; [INL] = inner nuclear layer; [IPL] = inner plexiform layer; [GCL] = ganglion cell layer. Scale bars = 30 μm.

to belong to the host or the transplant depending upon their location and presuming that the adult retinal neurons do not migrate.

In normal, adult rabbit retinas (the age of the recipient animals), NOS-containing cells were found at the border of the inner nuclear layer and inner plexiform layer. These constitute a subpopulation of large, sparsely distributed, wide-field amacrine cells (Fig. 1F), and at least one more population of smaller and more weakly labeled cells (not shown), all located in the proximal inner nuclear layer. A few immunoreactive cell bodies are seen also in the ganglion cell layer (not shown). NOS immunoreactivity is also seen in processes distributed mainly in 3 bands in the inner plexiform layer of the normal rabbit retina (Fig. 1F). With the antibody we have used, NOS immunoreactivity is thus restricted to the inner plexiform layer, the innermost cell row of the inner nuclear layer, and the ganglion cell layer.

NOS immunoreactive cells were seen in all grafts examined. Immunoreactive processes originating in these cells were also seen. Most labeled cells found in the grafts projected to the equivalent of an inner plexiform layer within the transplant, which in many cases was located next to the host retinal pigment epithelium (Figs. 1C and D). At times, immunolabeled processes were also found in the grafts close to the graft-host border. In regions where one or more photoreceptor cell rows of the host outer nuclear layer remained, such processes could be seen to run parallel to the border, without entering the host retina (Fig. 1E). Photoreceptor cell rows could be observed in the host retina at the shorter survival times (Fig. 1A) and at the edges of the bleb created in the host retina by the graft (Fig. 1E).

Nevertheless, at times, labeled fibers originating in the graft could be seen crossing the graft-host border. This was seen in regions where the host photoreceptor layer was absent. Such bridging was observed at all survival times, but not in all specimens examined (in 8 out of 11). Further, it was in some cases possible to follow a labeled process originating in the graft all the way into the host inner plexiform layer. Examples of this are presented in Figs. 2 and 3. A long thin process is seen in a graft corresponding to PN 12 (Fig. 2A) and a shorter in a transplant corresponding to PN 20 (Fig. 2B). However, also in the latter case the fiber originates in the graft,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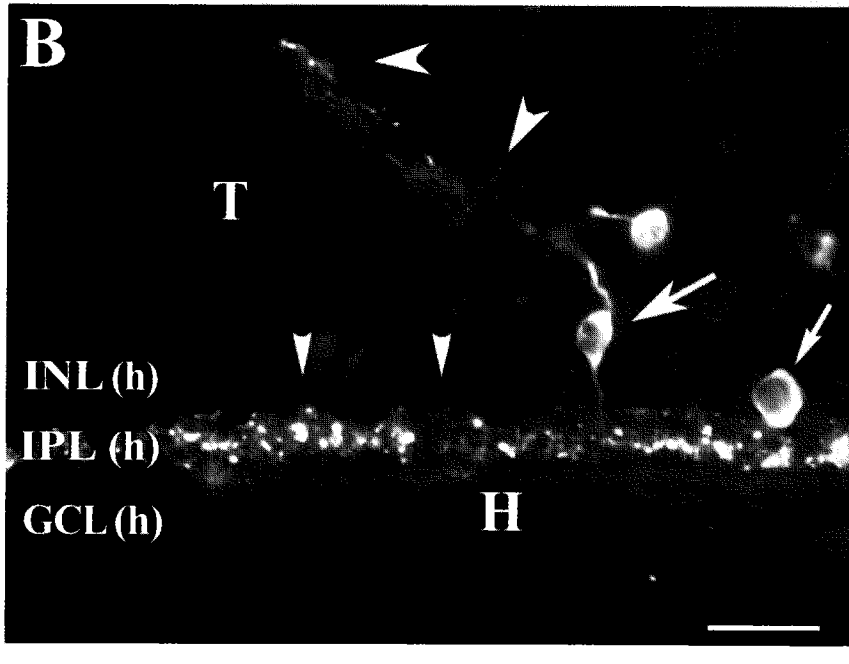
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Figure 3: (A-B) fluorescence micrographs showing NOS immunoreactive cells connecting the transplant [T] to the host retina [H]. (A) a PN 45 specimen showing several stained processes (large arrowheads) in the transplant [T]. One of the processes (open arrowhead) belonging to a NOS-immunolabeled cell located in the transplant (arrow) is seen to project towards the immunoreactive plexus in the host inner plexiform layer [IPL (h)] (small arrowheads). A stained cell body is also seen in the host inner nuclear layer [INL (h)] (small arrow), next to the host inner plexiform layer [IPL (h)]. (B) a PN 90 retinal transplant showing a strongly labeled cell body (large arrow), connecting the inner plexiform layer of the host [IPL (h)] (small arrowheads) to an immunoreactive plexus in the transplant (large arrowheads). An immunoreactive cell is seen in the inner nuclear layer of the host [INL (h)] (small arrow). [T] = transplant; [H], [h] = host retina; [INL] = inner nuclear layer; [IPL] = inner plexiform layer; [GCL] = ganglion cell layer. Scale bars = 30 μm.

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at the level of the graft-host interface, where several labeled processes are seen. No NOS immunoreactive fibers are seen in the normal rabbit retina external to the inner plexiform layer with the antibody employed in this study (Fig. 1F), indicating that the staining seen at the interface (Fig. 2B) is associated with structures within the graft. Further, it should be noted that the inner host retina appears relatively well preserved in the areas adjacent to the transplant (see Figs. 1C, D, E, 2, and 3). In the host inner plexiform layer, the immunoreactive plexus is continuous and parallel to the inner border of the retina and the graft-host interface. Thus the processes that crossed this border perpendicularly could be judged as originating in the graft and not in folds of the host retina.

In Fig. 3A, several stained processes are seen in the graft. In addition, a process belonging to a NOS-immunolabeled cell located in the graft (corresponding to PN 45) is seen to project towards the host inner plexiform layer. Serial sections confirmed the location of the cell. A stained cell body is seen in the amacrine cell layer of the host and strong and continuous labeling is noted in the host inner plexiform layer, reflecting the relative integrity of the inner host retina. The observations indicate that the connecting fiber seen in this specimen indeed originates in the graft.

In Fig. 3B, stained cell bodies are seen in the host retina in a graft corresponding to PN 90. One strongly labeled cell body is seen in the transplant to project to a region within the graft where faintly stained fibers are seen, similar to what was shown in Figs. 1C and D. In addition, one large NOS-immunoreactive cell body is seen to project to the same area within the graft, and to emit a weakly labeled process towards the host inner plexiform layer. This specimen was taken 106 days after transplantation, and, as mentioned above, a progressive loss of the outer host retina is normally observed, which with time brings the graft closer to the host inner layers. Further, a strongly labeled cell is seen in the host retina in the expected position, next to the inner plexiform layer. The connecting cell could therefore, judging from its position, belong to the transplant. However, it is not possible to determine conclusively whether this stained cell indeed belongs to the graft.

In the host retina, the number of labeled cells did not appear to be altered in areas adjacent to the graft or compared to normal retinas, unless also the host inner nuclear layer was disrupted. There were also no indications that following transplantation, cell types other than those seen in a normal rabbit retina expressed NOS immunoreactivity. However, the labeling over the host inner plexiform layer tended to appear stronger and more dense than in non-operated animals.

DISCUSSION

NOS-containing cells (likely to be amacrine cells) were seen in rabbit transplants corresponding to PN 5, which agrees with our previous report. [22] Labeled processes were also identified and were often seen to converge to one or more regions within the graft, forming small and at times dense plexuses of fibers, likely to be the equivalent of an inner plexiform layer. In addition, a few processes could be seen to project also towards the host retina, and occasionally, a process originating in the graft could be seen entering the host. Such bridging was more often seen at long survival times, which is as expected, considering that also in normal developing rabbit retinas, outgrowth of NOS fibers is a relatively slow process. [24] Further, a better graft-host fusion was observed in old transplants, which conceivably should also favor the formation of connections. Using NOS immunocytochemistry, we have thus demonstrated that NOS-containing cells in rabbit transplants are capable of projecting into the host retina, and that their processes reach the host IPL, the target for NOS-containing fibers within the host retina.

We have also shown an example of a NOS immunoreactive neuron projecting to both the host inner plexiform layer and to an equivalent region within the transplant. In the case illustrated in Fig. 3B, it was not possible to determine the origin of the cell, and the possibility that it belonged to the host can not be ruled out. Whatever the origin of the neuron might be, it appears to connect the host inner plexiform layer to that of the graft. If so, information from the graft could be conveyed to the host retina not only by those neurons which directly project to the host, but also indirectly.

The number of NOS immunoreactive cells found in the transplants and of fibers crossing over was not very large and varied between specimens and also between sections from the same specimen. It may be noted that even in normal retinas, NOS is expressed in only a small population of cells, and high numbers of bridging fibers are not necessarily expected. The small number of NOS-containing cells and fibers within the grafts may be explained in part also by the random organization of the transplants which results from the transplantation of pieces of embryonic retina.

However, it has consistently been difficult to demonstrate graft-host connections. [16,19-21] It may thus be speculated that the formation of connections is limited in some way. As mentioned earlier, a rapid loss of the outer layers of the rabbit host retina was normally observed. Numerous dying cells are found also in the transplants,

mainly in areas adjacent to the host retina. [25,26] A gliotic response is seen to follow neuronal cell death, and has, as expected, been observed in the host retina and with time also in grafts. [25,27,28] Factors associated with reactive glia have been identified which inhibit neurite outgrowth. [29-31] Gliosis is therefore likely to influence negatively not only the development and survival of the grafted cells (including NOS-expressing cells), but also the formation of connections between graft and host retinas.

We have in the present study observed also that no connections were ever found in areas where one or more cell rows were left of the host outer nuclear layer. Processes located next to the graft-host border were in these cases seen to run parallel to the border, without crossing over, in spite of the fact that the host outer nuclear layer was thinned and that the host inner plexiform layer was only a few microns away from the graft. Whether this is a limitation of NOS containing fibers only or it reflects the presence of some barrier cannot be established at this point.

Nevertheless, despite conditions that might limit the formation of connections, we have demonstrated here that they do occur. The connections are established by cells in the graft which maintain their ability to synthesize NO, one of the recognized retinal neuromodulatory compounds. [23,32-38] We wish to emphasize that the term connection used here does not imply that synaptic contacts are established by the projecting NOS-containing cells. However, there is evidence that neurites projecting from retinas transplanted to the brain are capable of forming synaptic contacts upon reaching the host target tissue. [39-41] As shown here, NOS-containing processes not only project into the host retina, they were also seen to reach the host IPL. This indicates that subretinally grafted cells are potentially capable of contacting their partner cells in the host retina. If true for other grafted cell types as well, a good basis is then provided for the functional integration of the transplants.

Acknowledgments

The authors thank Karin Arnér and Katarzyna Said for excellent technical assistance. Supported by grants from the Swedish Medical Research Council (K97-14X-12209-01A); Hjärnfonden; Swedish Society for Medical Research; Medical Faculty, University of Lund; Crown Princess Margareta's Committee for the Blind; Crafoord Foundation; Greta and Johan Kock Foundations; EU BioMed 2 Program; Foundation Fighting Blindness.

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Paper V

Influence of technique and transplantation site on rosette formation in rabbit retinal transplants

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ABSTRACT. In order to determine mechanical and host-graft related interactions in the histogenesis of retinal transplants, a new technique for transplanting flat and comparatively large pieces of embryonic rabbit retina into adult rabbit eyes was elaborated. With the procedure, free-floating grafts in the epiretinal space survive, develop and differentiate largely without rosette formation, suggesting that the dissection and transplantation procedure is adequate for obtaining a normal development. On the other hand, subretinal transplants mature at an apparently faster pace than epiretinal transplants, but do not become regularly laminated. Outer segments do not develop well in the epiretinal transplants, whereas they do so in the subretinal ones, suggesting host-graft interactions by means of yet unknown diffusible factors.

Key words: retinal transplantation - development - rabbit - rosette formation - photoreceptor development.

Acta Ophthalmol. Scand. 1997: 75: 3-10

The initial transplantation studies of fetal retinas to the anterior chamber showed by Royo & Quay (1959) were followed by similar experiments by del Cerro et al. (1985). At about the same time, Turner & Blair (1986) demonstrated that the transplanted tissue survives in the epiretinal space, as is also the case when it is placed between the photoreceptors and the pigment epithelium (Aramant & Turner 1988; Bergström et al. 1992).

Retinas have been transplanted in different physical states. Cell suspensions have been found to give transplants that show no or only very little organisation and no, or hardly any photoreceptor outer segments (del Cerro et al. 1988, 1989; Gouras et al. 1991a, 1991b, 1992; Juliusson et al. 1993). Transplanted tissue fragments and microaggregates survive well (del Cerro et al. 1985; Blair & Turner 1987; Seiler et al. 1988a; Bergström et al. 1992; Gouras et al. 1994) and also show better layering than transplants produced with the suspension technique (Juliusson et al. 1993). The fragmented tissue transplant often becomes organised into small rounded structures, usually referred to as rosettes. Within the rosettes, there are usually concentric cell layers that strongly resemble the normal layers of the retina, with well developed photoreceptor outer segments pointing inwards into the central lumen.

The reasons for rosette formation in retinal transplants are not well understood, but it is usually thought of as a general injury response (Lahav et al. 1975; Milam & Jacobson 1990). Mechanical factors have also been suggested to lead to rosette formation (Caffé et al. 1989). Another possibility is that growth factors and similar agents may be released by the host, affecting the transplants, but only very little is known about such interactions. The extent of the rosette formation differs in the various transplantation models. When fragments of retina are transplanted, rosettes are a prominent feature (McLoon et al. 1982; del Cerro et al. 1985; Bergström et al. 1992), whereas they are much fewer or even absent in cell suspension transplants (Gouras et al. 1992; Juliusson et al. 1993). Very little rosette formation was seen in carefully mounted in vitro explants of fetal tissue (Caffé et al. 1989) and there are preliminar reports that transplants of photoreceptor layer embedded in gelatin also show no or only little rosette formation (Silverman & Hughes 1989; Silverman et al. 1991, 1994). In an effort to obtain transplants which develop architecturally similar to the normal retina in terms of lamination and development of outer segments, we have modified the tissue fragment procedure so that comparatively large sheets of minimally disturbed fetal retinas can be transplanted. Since it requires less mechanical handling of the transplant tissue to place it epiretinally than between the photoreceptors and the pigment epithelium, we have compared the results of transplantation at these two places. We have also compared the results obtained with the new procedure with results of fragmented tissue transplants.

Material and Methods

Donor tissue

Ordinary mixed strain pigmented rabbits from stage E15 (15th post-conception day) were used as donors. Embryos were obtained by caesarean section after sacrificing the pregnant female rabbits with barbiturates. They were kept at +4°C in Ames' solution having the following composition (mM): NaCl 120, KCl 3.6, $MgSO_4$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 23,

NaH_2PO_4 0.1, Na_2HPO_4 0.4 and glucose 10 (Ames III & Nesbett 1981). Eyes from these embryos were enucleated, and the neural retina was then dissected from the posterior eyecup under an operating microscope. Care was taken not to damage the neural retina, especially when separating it from the pigment epithelium. In this way we were able to obtain close to all of the undamaged fetal neural retina in the form of a cup. These retinas were kept in the same solution until transplanted.

Recipients

Adult rabbits of the same strain as the donors, weighing 2.5-3 kg were used as recipients. Fifteen minutes before surgery, the right pupil of the recipient was dilated with 1% cyclopentolate and, if necessary, also with 10% phenylephrine HCl. The animals were anaesthetized with Hypnorm®, 1 ml/kg (fluanison 10 mg/ml and fentanyl 0.2 mg/ml). 1-2 drops of tetracaine HCl were instilled into the eye as needed. Twenty-four transplants were performed in this study. In 11 eyes large sheet retinal transplants we replaced subretinally, and in 3 both subretinally and epiretinally. In 10 eyes fragmented tissue transplants were placed subretinally.

Transplantation cannula

A special instrument (Fig. 1) was developed for transplanting the slightly cupped pieces of embryonic rabbit retinas into the adult eyes. The instrument comprises a cannula made from an 18 gauge injection needle, which serves as a jacket around a thin-walled flat polyethylene tube (about 1.8 × 0.6 mm outer and 1.6 × 0.4 mm inner dimensions). This polyethylene tube and the cannula is mounted on a specially designed instrument that can push the tube out of the cannula in a controlled fashion when desired. Further, the plastic tube is attached to a precision micro syringe. Donor tissue is sucked into the polyethylene tube with the help of the micro syringe. In this process, the slightly cup-shaped neural retina enters the tube as a single piece. There is inevitably some damage to the transplant tissue at the cut margins, but the central portion remains as an intact sheet.

For transplanting fragments of the donor tissue the instrument described by Bergström et al. (1992) was used. The difference between the instrument used for transplanting the fragmented pieces of the tissue and the large sheets of tissue is that the plastic cappilary used is narrower (with inner and outer tip diameter of 0.15-0.4 and 0.3-0.5 mm) for transplanting the fragmented donor retina.

Transplantation technique

For transplanting large sheets of donor tissue, the cannula with the polyethylene tube containing 3 to 4 pieces of the donor tissue was introduced into the eye through a scleral incision about 5-6 mm behind the limbus and advanced transvitreally until it reached the predetermined transplantation site in the central retina. The procedure was monitored under an operating microscope, using a standard contact lens. On reaching the retina, the polyethylene tube was pushed out of the cannula, and if required introduced into the subretinal space by doing a retinotomy with the tube containing the tissue. The donor tissue was then ejected at the appropriate site by pushing the piston of the microsyringe attached to the cannula.

For transplanting the fragmented tissue, 3-4 embryonic retinas were drawn into the instrument with the fine capillary and transplanted (Bergstöm et al. 1992). The donor tissue got fragmented while passing through the cappilary.

After the transplantation, sutures are usually not needed. The animals were allowed to wake up and transferred to their normal cages with 12/12 hour light/dark cycles. No antibiotics or immunosuppressives were given postoperatively. All animals were sacrificed 6 weeks after transplantation. Appropriate permits for the study were obtained from the Swedish government Animal Experimentation Ethics Committee at the University of Lund. The experiments were conducted according to the rules set forth in the ARVO resolution on animal experimentation.

Fixation and staining

At the decided post transplantation time, the animals were sacrificed. Their eyes were enucleated and fixed in 4% formaldehyde for 24 h. After fixation, the eyes were hemisected and the part carrying the transplant was excised. The tissue was washed with Sörensen's phosphate buffer (0.1 M pH 7.2) and then with the same buffer containing 5%, 10% and 20% sucrose before sectioning on a cryostat (12 µm). For paraffin sections, the fixed tissue was washed in 30, 50 and 80% ethanol in which it was also stored until embedded in paraffin wax and sectioned (6 µm).

Sections were stained with haematoxylin and eosin for light microscopy. Mouse monoclonal antibodies against rhodopsin (R2-15, diluted 1:6000) and vimentin (Dakopatts, diluted 1:100) were used for immunohistochemistry on cryostat sections. Antibodies were diluted in phosphate buffered saline (PBS) containing 0.25% Triton X-100 and 1% bovine serum albumin. Sections were washed for 15 min in 0.01 M PBS (pH 7.2) containing 0.25% Triton X-100, which was used for all the washes in the immunohistochemical staining. Sections were then incubated with normal blocking serum (rabbit 1:10, Dakopatts) followed by incubation with primary antisera for 20-25 h. Sections incubated with anti-vimentin antibody were then washed, incubated with secondary antibody (rhodamine-conjugated rabbit immunoglobulins to mouse immunoglobulins, Dakopatts) for 30 min, washed again and mounted with Vectashield (Vector Lab. Inc.). Sections incubated with anti-rhodopsin were subsequently incubated in secondary antibody (rabbit-anti-mouse immunoglobins, Dakopatts). To develop the peroxidase reaction, sections were first washed for 15 min in 0.05 M Tris buffer (pH 7.4) and then developed for 10 min in a substrate solution of 0.05% diaminobenzidine (DAB) and 0.015% H_2O_2 in 0.05 M Tris buffer (pH 7.2).

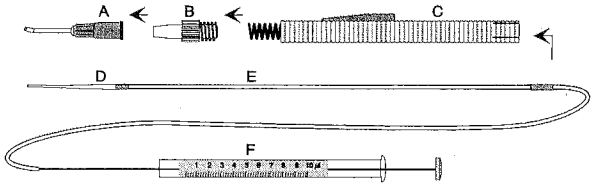


Fig. 1. Drawing of the instrument used for transplantation. The plastic tube (D) fits inside the main part of the instrument (B and C). This part of the instrument pushes the plastic tube (D) out of the cannula (A) which shields it during the passage through the vitreous. The other end of the plastic tube (D) is connected to a glass capillary (E) which in turn is connected to the precision syringe (F) that controls the injection of the donor tissue.

Observations and Results

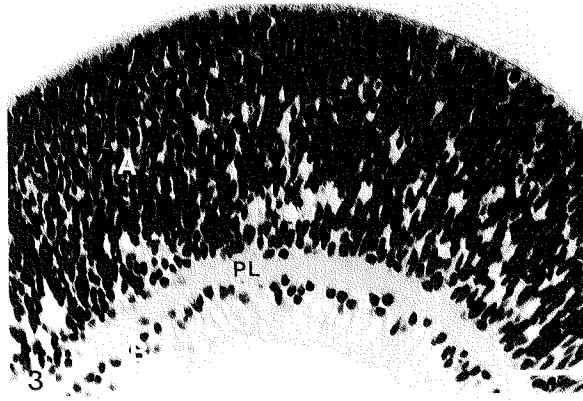
With the described technique it was possible to place flat whole pieces of embryonic retinal tissue in the adult rabbit eyes either epiretinally or between the photoreceptors and the pigment epithelium. The last piece of donor tissue in the transplantation cannula is at times difficult to eject, but otherwise the procedure is straightforward and works well. If the animal is too lightly anaesthetized, insertion of the transplantation cannula may elicit a withdrawal reaction due to the sensory nerves in the choroid, which may ruin the surgical results.

Six weeks after transplantation, which is equivalent to 8 weeks after conception, the epiretinal transplants had grown into a more or less spherical or cup-shaped laminated sheet as in normal retinogenesis. This piece of donor tissue did not show rosette formation except at the margin of the transplant, where a few, small rosettes could be seen (Fig. 2). Transplant cells differentiated in two distinct nuclear layers. The apical layer was comprised of dark and oval nuclei of neuroblastic cells. The convex surface of this layer showed no photoreceptor outer segments (Fig. 3), which was confirmed by negative immunoreactivity for rhodopsin (not shown). On the basal side this nuclear layer was followed by a layer with the appearance of the normal inner plexiform layer. A cell layer equivalent to the normal ganglion cell layer was also present, but the type of cells in this layer remained undetermined (Fig. 3).

Subretinal large sheet transplants in the same eye showed a different organisation than the epiretinal large sheet transplants. Here the cells were predominantly arranged in irregular arcuate arrays or rosette-like clusters (Fig. 4). The photoreceptors in these arcuate arrays always faced the host retina, whereas the photoreceptors in the rosettes faced their centres. Both in the arrays of cells and in the rosettes, the photoreceptors most often had well-developed outer segments, confirmed by light microscopy (Fig. 4) and immunohistochemistry for

Fig. 2. A 6-week-old E 15 large sheet retinal transplant in the epiretinal space developing as a spheroid without any rosettes except at the margins (black asterisks with white dots) where the donor tissue was damaged during the process of transplantation. Differentiation of the transplant in two layers is visible. Hematoxylin and eosin, bar = 200 μm.

Fig. 3. Magnified view of a neighbouring section of the same transplant as in Fig. 2 showing no outer segments, and differentiation of the transplant in two nuclear layers. Neuroblastic cell layer (A) is seperated from the layer that resembles ganglion cell layer (B) by a plexiform layer (PL). Hematoxylin and eosin, bar = 50 μm.



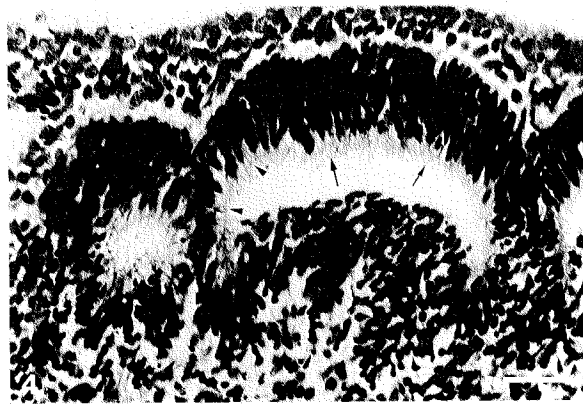
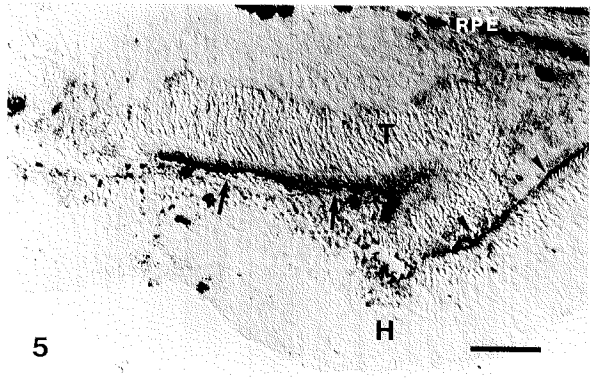
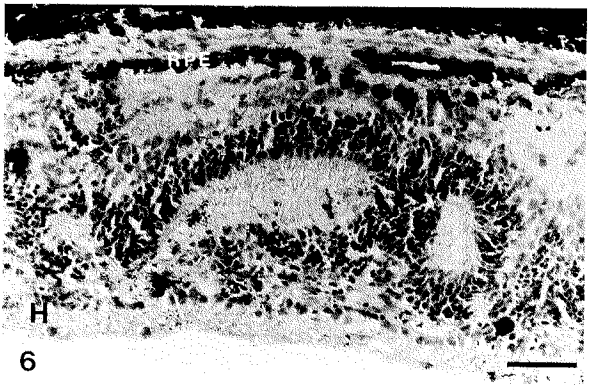
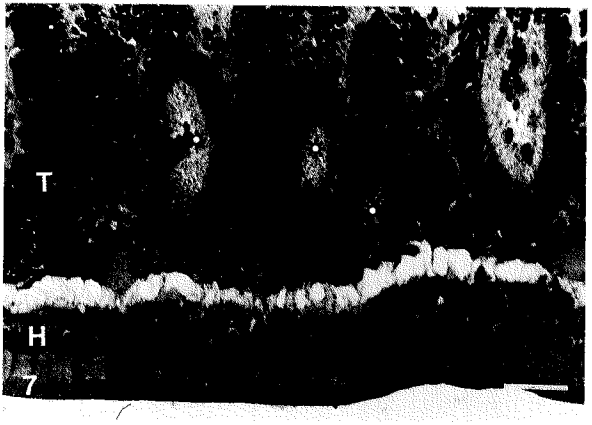


Fig. 4. A 6-week-old E 15 large sheet retinal transplant in the same eye as the transplant in Fig. 2, showing the formation of arcuate structures and relatively well developed outer segments (arrows) with outer limiting membrane (arrow heads). Hematoxylin and eosin, bar = 50 μm.







rhodopsin (Fig. 5). Immunostaining was also present in the host retina adjacent to the transplant but was disrupted in the host retina overlying the transplant, in accordance with previous reports (Bergström et al. 1992). Particularly in cell arrays, photoreceptors were also associated with a well developed outer limiting membrane as judged by light microscopy (Fig. 4). The arcuate arrays appeared not only in places where the retinal pigment epithelium had been removed by the surgery, but also where it was still present (Fig. 6). They were at times very large (up to about 300 μm). The regular rosettes dominated in fragmented tissue transplants (Fig. 7), but rosette free areas were rarely found in places where the host pigment epithelium was disturbed.

Both, the arcuate arrays in the large sheet retinal transplants and the rosettes in the fragmented tissue retinal transplants showed two distinct layers of cell bodies. The first was comprised of comparatively small, oval cell bodies, apparently developing photoreceptor cells. The second consisted of cells with rounder and more lightly stained nuclei, which resembled cells of the inner nuclear layer. A plexiform layer was also present between the two nuclear layers, as was in places another plexiform layer, most likely equivalent to the inner plexiform layer. Thus the rosettes had two cell-rich layers and two layers resembling the

Fig. 5. A 6-week-old E 15 large sheet subretinal transplant (T), showing rhodopsin staining in the photoreceptor outer segments (arrows) in arcuate arrays and in the host retina (arrow heads). Note that the rhodopsin immunoreactivity in the host retina (H) disappears when it apposes the transplant. Host retinal pigment epithelium (RPE) is visible at the right hand corner of the figure. Immunostaining with rhodopsin antibody R2-15, bar = 50 μm.

Fig. 6. A 6-week-old E 15 large sheet retinal transplant (T), covered by the host retina (H), showing arcuate arrangement of the photoreceptors also at places where the host pigmented epithelium (RPE) is still present. RPE shows some irregularities and moulding presumably due to surgical trauma. Dark dots in the transplant are the pigmented cells from the host RPE. Hematoxylin and eosin, bar = 50 μm.

Fig. 7. Fragmented donor tissue transplant (T) in the subretinal space showing the rosette formation (black asterisks with white dots) and not the arrays of cells. H = host retina. hematoxylin and eosin, bar = 50 μm.

outer and inner plexiform layers, respectively. The organisation was more regular and prominent in the arcuate arrays than in the rosettes. A cell layer resembling the ganglion cell layer was also present, but it was not established whether it contained true ganglion cells or some other cell type, such as displaced amacrine cells.

Müller cells contain vimentin, and staining for this substance therefore reveals their overall morphology. As seen in Fig. 8, vimentin staining of transplants which are not covered by the host retina reveal a Müller cell organisation with relatively regular palisade-like arrangement of the Müller cells. A similar organisation is noticeable in the arcuate arrays (Fig. 9), but not in fragmented tissue transplants (Fig. 10) where Müller cells show a radial arrangement.

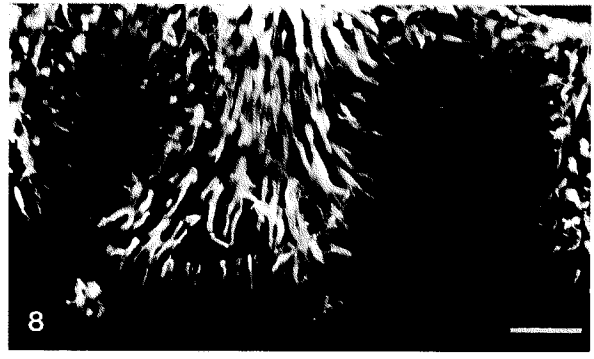


Fig. 8. Vimentin staining in epiretinal large sheet transplant not covered by the host retina showing a palisade-like arrangement of the Müller cells. Bar = 50 µm.

Discussion

The large sheet retinal transplantation technique described in this paper differs from other techniques in transplanting relatively large pieces of whole retinas as compared to fragmented pieces (Bergström et al. 1992), dissociated cells (Juliusson et al. 1993), or the photoreceptor layer (Silverman & Hughes 1989). This new technique enables relatively undisturbed, large pieces of retina to be transplanted.

At the time of transplantation, embryonic day 15, the donor tissue is known to be immature, consisting largely of undifferentiated cells (Bergström et al. 1992). Development of an inner plexiform layer in the epiretinal transplants and formation of layers and photoreceptor outer segments in the subretinal transplants shows that the donor tissue continues to mature at both the transplantation sites, as reported by many authors in various models (Royo & Quay 1959; Turner & Blair 1986; Seiler et al. 1990; Gouras et al. 1990; Bergström et al. 1992, 1994).

In this study, epiretinal and subretinal

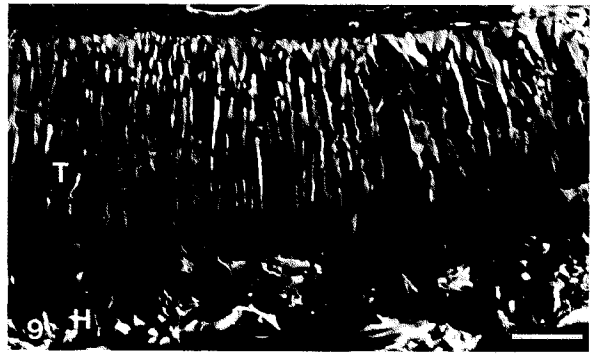


Fig. 9. Vimentin staining in a 6-week-old E 15 large sheet retinal transplant (T) in the subretinal space (H = host retina), showing palisade-like arrangement of Müller cells in arcuate arrays. Bar = 50 µm.

Fig. 10. Vimentin staining in a fragmented tissue transplant (T) in the subretinal space (H = host retina), showing the radial arrangement of Müller cells in the rosettes (lumen indicated by black asterisks with white dots). This arrangement is different that that of arcuate arrays in the large sheet retinal transplant as shown in Fig. 9. Bar = 50 µm.

transplants were placed in the same eye under identical conditions. Nevertheless, only the subretinal transplants developed outer segments demonstrated by light microscopy and immunostaining with rhodopsin. Further, in subretinal transplants, an outer limiting membrane was found (Fig. 4), whereas this structure were not seen in epiretinal transplants (Fig. 3). Most photoreceptor outer-segments appear post-natally in the normal development (Greiner & Weidman 1982). The rosette-free areas of the epiretinal transplants did not show any outer plexiform layer, even at 4 weeks postnatal age. During normal development, the outer plexiform layer develops in the neuroblastic cell mass shortly before birth and is distinct at the time of birth (Greiner & Weidman 1982; Stone et al. 1985; Sharma & Ehinger, unpublished results). Retinal layering and the photoreceptor outer segment development is completed in all parts of the retina by post-natal week 4, which is the equivalent age of the transplants in this study. Thus, the retinal grafts developed after transplantation but could only reach up to a pre-natal stage in the epiretinal space, whereas they reached a post-natal stage in the subretinal space, when the graft tissue age was 4 weeks post-natal. This suggests that the subretinal transplants more rapidly develop mature cells than the epiretinal ones do. It is possible that proximity of the graft to the host retina influences the development. In human embryonic retinal transplants in the epiretinal space of rat eyes, rosettes closer to the host retina stain better for the photoreceptor specific proteins and cellular retinaldehyde binding protein which marks the glial cells (Seiler & Aramant 1994; Aramant et al. 1990).

The lamination of the large sheet retinal transplants was different from that of fragmented tissue transplants. The lamination in these transplants was largely rosette free in the epiretinal space and developed arcuate arrays in the subretinal space, unlike rosettes in the fragmented tissue transplants. The difference in the organisation of the transplant was confirmed by vimentin immunoreactivity of the Müller cells. Müller cells are radially arranged in the outer nuclear layer of the rosettes where as they appear abnormal in the inner retinal layers of the transplant (Seiler & Aramant 1994). In cell suspension transplants also the Müller cells are disorganised (Juliusson et al. 1993). Glial fibrillary acidic protein (GFAP) and S-100 staining also reveals radial organisation of the glia fibers in fragmented tissue retinal transplants in rats (Seiler & Turner 1988b). In the present study also, rosettes in the fragmented tissue transplants showed radial arrangement of the Müller cells, but the arcuate arrays that dominate the large sheet retinal transplants, and most parts of the epiretinal transplants, showed a palisade-like arrangement of the Müller cells.

It is apparent from the results presented here that transplants done with two different techniques can develop different morphologies. Fragmented tissue transplants developed rosettes with the photoreceptors facing their centres. This is similar to the picture seen in the regenerating central retina of chickens in which there is a transdifferentiation of the pigmented epithelium into neurons, both in vivo (Coulombre & Coulombre 1965, 1970; Park & Hollenberg 1989) and in vitro (Layer & Willbold 1989, 1994). In these models of regeneration, the initial aggregation process causes clustering of the most adhesive cells. The initial interaction of the embryonic cells is mediated by variety of cell adhesion molecules (Edelman 1984; Rathjen 1991). Such molecules also play a role in cellular and histological differentiation (Takeichi et al. 1990, 1991). In the fragmentation technique also the donor tissue has to undergo an initial aggregation, like in the regeneration model of central retina, which is important for the eventual cytoarchitecture of the tissue.

Large sheet retinal transplants developed a different morphology. Here the donor tissue architecture was minimally disturbed at the time of transplantation, especially in the epiretinal transplants which were not even covered by the host retina. After transplantation, no or little re-aggregation of the cells took place in the minimally disturbed donor tissue and therefore they developed a largely rosette-free lamination when placed in the epiretinal space. When transplanted to the subretinal location, arcuate arrays appeared, which are structurally different from rosettes, as shown by vimentin staining. However, rosettes developed at places in large sheet retinal transplants where the donor tissue was disturbed and thus needed re-aggregation, like at the margins of the epiretinal transplants. Rosettes are known to form early in fragmented tissue transplants (Sharma et al. 1995). Since the rosette formation is already apparent at the margins of the epiretinal transplants, further maturation of these transplants is not likely to give rise to more rosettes.

A different mechanism of regeneration is present at the cilliary margin in the chicken retina and results in correctly laminated stratospheroids (Mckeehan 1961; Coulombre & Coulombre 1965, 1970; Willbold & Layer 1992). Here, the pigmented cells induce neuroblasts to proliferate, and aggregation of cells plays a minor role. Factors, yet unknown, mediating this process also induce correct laminar orientation in the newly formed cells. Spheroids thus formed do not contain rosettes but laminated spherical structures with correct polarity, that is, the photoreceptors face the outside, similar to the in vivo regeneration from the peripheral retina.

One of the techniques to minimize rosette formation is to transplant cell suspensions or very small pieces, called microaggregates. Cell suspension transplants fail to develop different retinal layers (Juliusson et al. 1993) and the photoreceptor outer segments (Gouras et al. 1991a,b). One to two postnatal day mice neuro-retinal microaggregates, transplanted by using narrower glass pipettes (inner diameter of 0.15 to 0.2 mm) than the ones used to make the fragmented tissue transplants (0.15 to 0.4 mm), resulted in decreased rosette formation (Gouras et al. 1994). Though these transplants had photoreceptor outer segments, there was a relative paucity of the non-photoreceptor cells in the transplants. Another approach to minimize the rosettes is to transplant a gelatine embedded photoreceptor layer, but these grafts also fail to develop healthy photoreceptor outer segments (Silverman & Hughes 1989).

The present results also suggest that the dissection and handling of fetal tissue to be transplanted can be performed carefully enough to prevent rosette formation. However, inserting the transplant between the photoreceptors and the pigment epithelium prevents the appearance of proper lamination. It has been shown previously that mechanical factors may induce rosette formation (Caffé et al. 1989), and the added mechanical handling needed to put the transplant in place may therefore be one of the causes for the disorganization. However, it cannot be ruled out that the pigment epithelium and/or the host retina release substances that prevent the development of proper layering. Pigmented epithelium has been shown to promote the survival and organisation of cell layer in the retinal transplants (Seiler et al. 1995). On the other hand, for unknown reasons, absence of pigment epithelium has been found in association with the rosette free areas in the

fragment tissue transplants (Bergström 1992). However, in the large sheet retinal transplants, arcuate arrays were present even in places where the pigment epithelium was in place, suggesting that in these transplants this effect was due to mechanical handling of the donor tissue and not the pigmented epithelium.

In conclusion, we find that it is important to maintain the tissue architecture at the time of dissection and transplantation in order to get a desirable lamination. In addition, the difference in the degree of maturation of the transplants suggests that other factors may also be of importance.

Acknowledgments

Part of the work was presented at the 1995 annual meeting of the Association for Research and Vision in Ophthalmology.

This work was supported by the Swedish Medical Research Council (project 14X-2321), the Faculty of Medicine at the University of Lund, the Riksbankens Jubileumsfond, the Crafoord Foundation, the Segerfalk Foundations, the H and L Nilssons Stiftelse, Stiftelsen Clas Groschinskys Minnesfond, and the T and R Soderberg Foundation.

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Received on October 4th, 1995

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Paper VI

Survival of Long-term Retinal Cell Transplants

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If retinal cell transplants are to be used in the management of retinal degeneration, they will need to survive in the eye for a significantly long time period. This study was conducted to assess the fate of the neural retinal transplants in the eye after long survival times. Fragmented pieces of neural retinas from embryonic day 15 rabbits were transplanted in the subretinal space of adult animals of the same stain. The transplants were allowed to survive for up to 583 days prior to sacrifice and light microscopical examination. In most cases, both neural and glial cells survived in these transplants. However, some of the grafts seemed to contain predominantly glial cells. Only when the photoreceptors in the graft were apposed to the host pigmented epithelium did the graft photoreceptors survive along with their outer segments and the outer limiting membrane. Otherwise the neuronal components of the grafts were largely cells typically found in the inner retina. In grafts lacking photoreceptors, the rosettiform layering in the grafts was less conspicuous than in short term transplants. Good physical integration of the host and the graft was seen at the host/graft interface, which was free of debris from dying cells seen in less mature transplants. The long-term transplants were much thinner than short term transplants, with the combined thickness of the graft and the degenerated host retina overlying it being 1 or 1.5 times the thickness of the non-degenerated retina adjacent to the transplant. Even after long survival times, the transplants lacked vascularization. No inflammatory cells were seen in or around the grafts. The results suggest that the cells of the inner retina survive for long periods after transplantation, but the photoreceptor cells seem to need the support of the host pigmented epithelium for long-term survival. It may therefore be important to have a transplantation technique where the graft photoreceptors can be placed in apposition with the host pigment epithelium.

Introduction

In the recent past, research in the field of neural degeneration and regeneration has shown that the degenerative retinal diseases may not be so untreatable as previously believed (Sharma and Ehinger, 1999). One of the possibilities being explored is retinal cell transplantation, which aims at replacing the degenerated photoreceptors with healthy ones from donor tissue (for review see Sharma and Ehinger, 1997c; Sharma, Bergström and Ehinger, 1995c). Retinal cell transplants are already under clinical trials for establishing the safety of the procedure (Kaplan et al., 1998; Das et al., 1996). However, certain vital questions still remain to be answered, and one of them is the long-term fate of retinal transplants.

Intraocular retinal grafts proliferate, survive, and differentiate, protected by the immune privilege of the eye (del Cerro et al., 1989; Sharma, 1998; Sharma and Ehinger, 1997; Aramant, Seiler and Turner, 1988). At the appropriate stage of development, embryonic donor tissue consists largely of neuroblastic cells (Sharma and Ehinger, 1997a). In the host environment, this immature pluripotent tissue, is capable of developing into a structurally retinatypic tissue containing most or all retinal cell types. The transplanted retinas also contain many of the structural and the functional proteins found in the normal retina (Yang et al., 1992; Guo et al., 1991; Seiler and Aramant, 1994; Juliusson et al., 1994; Sharma, Perez and Ehinger, 1997; Aramant et al., 1990; Bergström et al., 1994). At the electron microscopic level, the transplants contain most of the cellular components and synapse arrangements found in the normal retina (Ehinger et al., 1991). If the donor tissue is transplanted in the form of fragments or larger sheets, the photoreceptors outer segments develop reasonably well (Ehinger et al., 1992). The transplants are also capable of converting the absorption of light into a neuronal signal (Adolph et al., 1994). Although retinal transplants form few connections with the host retina (Aramant and Seiler, 1995), there is some evidence that limited information can be conveyed to the brain (del Cerro et al., 1991; Coffey, Lund and Rawlins, 1989). However, the functional implications of such weak connectivity remains to be established.

It has been observed that soon after the transplantation some cells of both graft and host origin, degenerate at the host-graft interface (Sharma and Ehinger, 1997b; Bergström et al., 1992b). It is not clear whether this degeneration is a result of a host-graft interaction, a lack of nourishment reaching this part of the transplant since it, and the now detached host retina, is displaced from the RPE and choricocapilaris, or some other cause. It is thus important to understand the long-term consequences of such cell death on the viability of grafts after long survival time.

One of the reasons for the excellent survival of retinal cell transplants *in oculo* is the relative immune privilege of the eye. Nevertheless, it has been shown that neural retinal grafts are immunogenic (Jiang and Streilein, 1991). Also, the immune privilege of the eye is not perfect and it can break down under some circumstances

(Jiang and Streilein, 1991; Ksander et al., 1991). Evidence from retinal pigment epithelium transplantation also suggests that over long time frames, pigment epithelium transplants in the human eye are affected by the host immune systems (Kohen et al., 1997; Gouras and Algvere, 1996; Algvere et al., 1997). The long-term fate of neural transplants is yet to be established in this respect.

Patients with retinitis pigmentosa are potential beneficiaries from retinal transplants. Retinitis pigmentosa is a group of heterogeneous hereditary disorders that have variable onsets and progression depending upon factors including the location of the mutation. In certain patients visual loss can occur early. Even in cases with a later onset of visual loss, useful retinal transplants will have to survive *in oculo* for extended periods of time. It is therefore important to assess the fate of retinal transplants after long survival times.

Most pervious studies of retinal transplants were aimed at developmental aspects of the transplant, and were therefore typically confined the survival time of a few months, till the transplant reaches optimum growth and differentiation. In this study we have concentrated on the fate of retinal transplants after long survival times.

Materials and Methods

Transplantation

Ordinary mixed strain pigmented rabbits (Dutch Belted) were used as donors. Embryos (from embryonic day 15; E15) were obtained by caesarian section after killing the pregnant female rabbits with barbiturates. They were kept at +4° C in Ames' solution containing (in mM): NaCl 120, KCl 3.6, $MgSO_4$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 23, NaH_2PO_4 0.1, Na_2HPO_4 0.4 and glucose 10 (Ames III and Nesbett, 1981). Eyes from these embryos were enucleated, and the neural retina was dissected out. They were kept in the same solution until transplanted.

Adult rabbits of the same strain as the donors and weighing 2.5 - 3 kg were used as recipients. The animals were anaesthetized with Hypnorm®, 1 ml/kg (fluanison 10 mg/ml and fentanyl 0.2 mg/ml). One to two drops of tetracaine HCl were instilled into the eye as needed. The transplantation technique has been described earlier (Bergström et al., 1992). In brief, 3 to 4 embryonic retinas were drawn in to plastic capillary (with inner and outer diameters ranging from 0.15-0.4 and 0.3-0.5 millimeters) of a specially designed instrument. The donor tissue got fragmented while passing through the capillary. The instrument was introduced into the eye through a small pars plana incision and advanced transvitreally until it reached the posterior pole. At an appropriate place on the posterior pole the plastic capillary containing the donor tissue was pushed out of its metal jacket and introduced into the subretinal space by making a small retinotomy with the capillary tip itself. The donor tissue was then deposited into the subretinal space and the instrument drawn out of the eye.

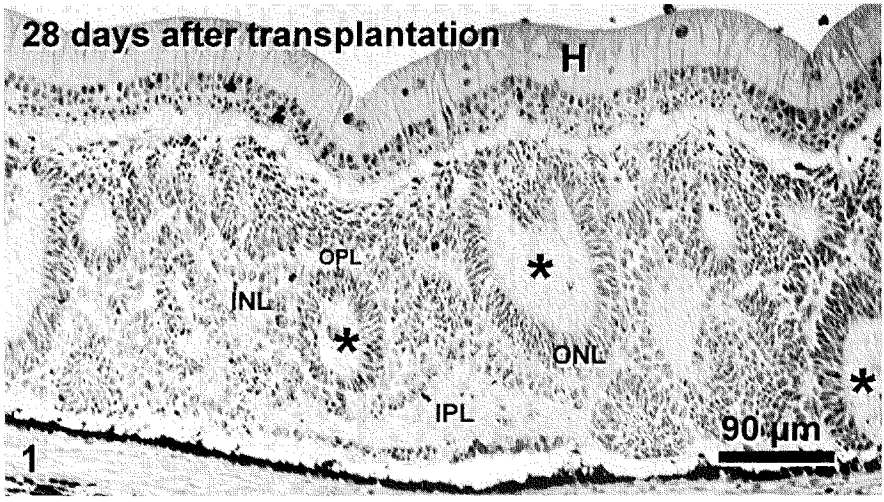

Fig. 1. Rabbit retinal cell transplant 28 days after transplantation (equivalent age 12 days postnatally). The specimen shows differentiated retinal cells. The transplant contains mainly rosettes (asterisk) with dark labeled cells belonging to the outer nuclear layer, forming the inner or the luminal layers of the rosettes. The cells of the outer nuclear layer (ONL) are largely responsible for the rosetted appearance of the transplants. The lightly stained cells belonging to the inner retinal layers (INL) are situated in between the rosettes. A plexiform layer corresponding to the outer plexiform layer (OPL) is found separating these two types of cells. In certain parts of the transplants, especially close to the host retinal pigment epithelium, another plexiform layer is sometimes observed. It probably corresponds to the inner plexiform layer (IPL). H = Host retina. Bar = 90 µu.

After the transplantation, the animals were allowed to wake up and were transferred to their normal cages with a 12/12-hour light/dark cycle. No antibiotics or immunosuppressives were given postoperatively. Eleven animals were allowed to survive for long duration of time after transplantation (n = 11; range = 583 to 470 days; average = 516 days). An additional 10 animals were allowed to survive only for short duration of time (n = 10; range = 12 to 28; average = 22 days). Appropriate permits for the study were obtained. The experiments were conducted according the rules set by the ARVO resolution on animal experimentation.

Tissue processing

The eyes were briefly fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M phosphate, 0.85% NaCl; pH 7.4). The anterior segments of the eyes were removed and the posterior segments were fixed in the same fixative for 24 hours. The tissue was rinsed in the same phosphate-buffered saline. The area containing the transplant was cut out and embedded in plastic or historesin and sectioned.

Results

Transplants

In short-term transplants aged 27 to 31 days, differentiated retinal cells were observed. The transplants showed significant rosettiform structure, with darkly stained cells belonging to the outer nuclear layer forming the luminal layer of the rosettes. More lightly staining cells, representing the inner retinal layers, lay in between the rosettes. A plexiform layer corresponding to the outer plexiform layer was found separating these two cellular layers. In certain parts of the transplants, especially close to the host retinal pigment epithelium, a second plexiform layer was sometimes observed, probably corresponding to an inner plexiform layer (Fig. 1).

In the long-term transplants, graft was identified in 9 specimens. In 3 out of 9, glial tissue (as identified by its overall morphology as well as the pallor of the cytoplasm and the nuclei in the tissue) was found to dominate with very few nerve cells (fig. 2). In other transplants, (6 out of 9), nerve cells were found in abundance (Fig. 3). However, even in these transplants, glial cells were more abundant than in transplants of shorter survival times (compare figures 1, 2 and 3).

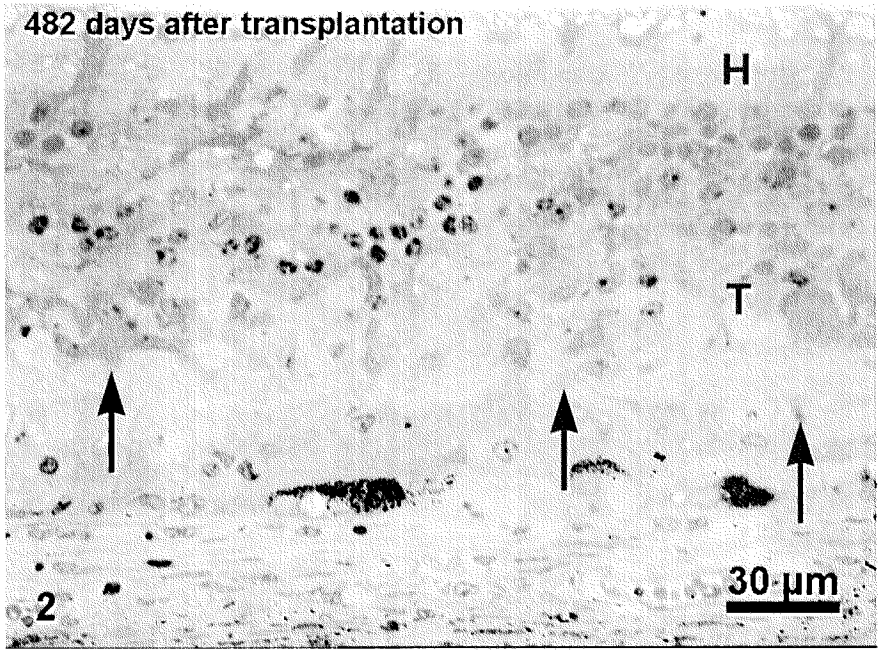

Fig. 2. Long-term rabbit retinal transplant (T), 482 days after transplantation. The specimen shows that the glial tissue dominates the transplant (arrows). Only few nerve cells are present. H = host retina. Bar = 30 µu.

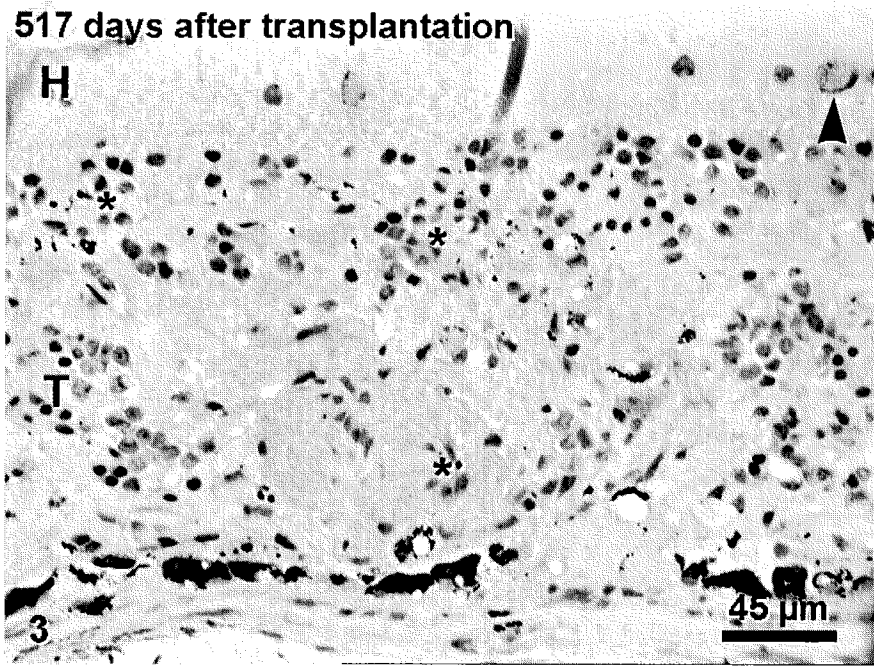

Fig. 3. Long-term rabbit retinal transplant 517 days after transplantation, showing abundance of nerve cells. Even in these transplants the glial cells are more abundant than in transplants of younger ages (compare with fig. 1). In the specimen retinal neurons belonging to the inner retina are found to be arranged around 'ghost lumens' (asterisks). The photoreceptor cells are lacking, and there are thus no easily observable rosettes. The cells of the ganglion cell layer are preserved in the host retina (H) including some large presumed ganglion cells (arrowhead). There is no debris at the host-graft interface, which contrasts with the picture seen in transplants of younger ages (Fig. 5). This suggests a better integration with the host in the long-term transplants than in the short-term ones. Bar = 45 µu.

In long-term transplants photoreceptors were found to survive only when they were well apposed to the host retinal pigment epithelium. The surviving photoreceptors showed outer segments, and, in addition, an outer limiting membrane was also sporadically present (Fig 4). In portions of the graft where the transplanted tissue was not in contact with the host retinal pigment epithelium, surviving photoreceptors were not found. In these parts of the graft, the rosetted layering of the transplant was largely lost. Nevertheless, the outlines of the rosettes and at times a 'ghost' lumen could still be seen (Fig. 3).

At the host-graft interface in transplants of shorter survival time, many degenerating host and/or graft cells were observed (Fig. 5). After longer survival times, the physical fusion between the transplant and the host was excellent as judged by the lack of a discernable border between the host and the transplant. There was no debris of the degenerating cells visible at the host-graft interface. At times it was

difficult to demarcate the border between the host and the graft (Fig. 3).

Retinal grafts were examined for vascularization, especially at places where the host retinal pigment epithelium had been damaged. No blood vessels were observed in the grafts either in the younger or the older age groups. However, in one transplant where the host retinal pigment epithelium was extensively damaged, a vessel could be observed at the junction of the host choroid and the transplant. In this case it was difficult to judge if the vessel was situated in the choroid or the graft. There was no evidence for the presence of inflammatory cells in this graft. The microscopic criteria for macrophages were a small, oval, darkly stained nuclei with scanty cytoplasm.

The thickness of the long-term grafts was much reduced in comparison with the ones that had survived for short duration. In short-term grafts the total thickness of the transplant and the remaining host retina overlying the graft was approximately 3 times the thickness of the intact host retina adjacent to the graft (Fig. 6). In long-term transplants the thickness of the graft and the overlying degenerated host retina was almost equal to, or slightly more (<1.5 times) than the thickness of the intact host retina (Fig. 7).

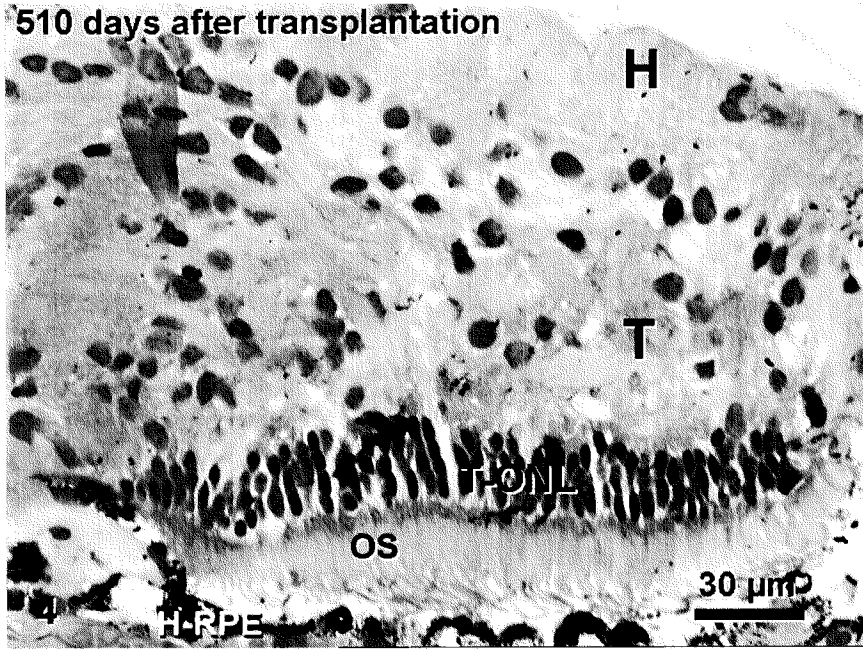

Fig 4. A long-term transplants (T), 510 days after transplantation, showing that the photoreceptors (ONL) survive only when they were well apposed to the host retinal pigment epithelium (H-RPE). The surviving photoreceptors show good outer segments (OS). This suggests that well differentiated transplant photoreceptors can survive for long times if in contact with the pigment epithelium. Bar = 30 µu.

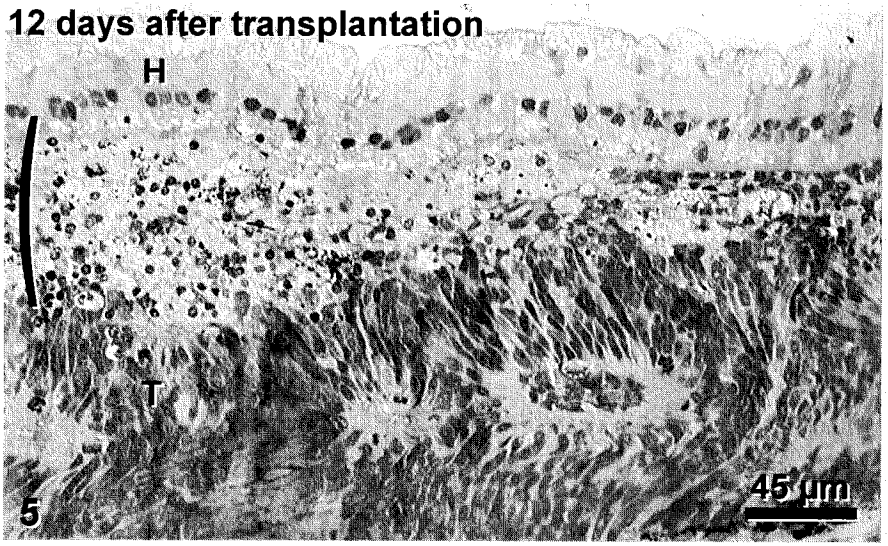

Fig. 5. A transplants of shorter survival time, 12 days after transplantation (equivalent age: embryonic day 27). The specimen shows many degenerating host and/or graft cells at the host- graft interface (parenthesis). In long-term transplants (Fig. 3), the host graft interface was free of such cells. H = host, T = transplant. Bar = 45 µu

Host

The host retina covering the transplants showed degeneration of the photoreceptor and outer nuclear layers. Due to the good integration of the host with the graft, the outer plexiform layer of the host overlying the transplant was not easily distinguishable. The cells of the inner nuclear layer, the inner plexiform layer, and the ganglion cell layer were clearly distinguishable, although the number of cells in the inner nuclear layer appeared to be less than what was seen in the non-degenerated parts of the host retina adjacent to the transplants. Certain large cells (presumed to be ganglion cells) were visible in the host retina covering the transplants (Fig. 3). The deeply stained cells of the outer nuclear layer along with photoreceptor outer segments were seen in the host adjacent to the transplant. In the host retina adjacent to the transplant, the number of cells in the outer nuclear layer and the size of the outer segments tended to taper off (Fig. 6). These observations were also noted in the transplants of younger ages. No pyknotic cells or any identifiable inflammatory cells were found in the host retina.

Discussion

Most clinical situations where retinal transplantation could conceivably be of therapeutic use call for replacement of the photoreceptor layer. As seen in the short-term transplants in this study, as well as in other studies, the photoreceptor cells are capable of developing and differentiating in the rosettes, where they are not in di-

rect contact with the host retinal pigment epithelium (Seiler and Aramant, 1994a; Sharma and Ehinger, 1997a; Bergström et al., 1992a). The photoreceptors in the rosettes have reasonably well developed outer segments and there is an outer limiting membrane, suggesting that the photoreceptors in the grafts undergo advanced degrees of differentiation (Seiler and Aramant, 1994a; Sharma, Bergstrom and Ehinger, 1997a; Ehinger et al., 1991a). The observations in this study suggest that the photoreceptors require the support of pigment epithelium for long-term survival. Only when they were in proper apposition with the host pigment epithelium did they survive. This support is apparently not needed for the initial development and differentiation of the photoreceptor cells, as they develop and differentiate well even in the absence of the pigmented epithelium. Studies on Royal College of Surgeons (RCS) rats also support this finding. In these, there is a genetic defect that renders the pigmented epithelium incapable of phagocytizing photoreceptor outer segments (Mullen and LaVail, 1976; Young and Bok, 1969). The photoreceptors develop normally until the outer segments begin to differentiate. At this stage, debris accumulates at the interface between the photoreceptors and the pigment epithelium (LaVail, Pinto and Yasumura, 1981; Dowling and Sidman, 1962). It is well known that the defect causes the photoreceptors to degenerate, and a healthy pigment epithelium is thus needed for long-term survival of the photoreceptor cells.

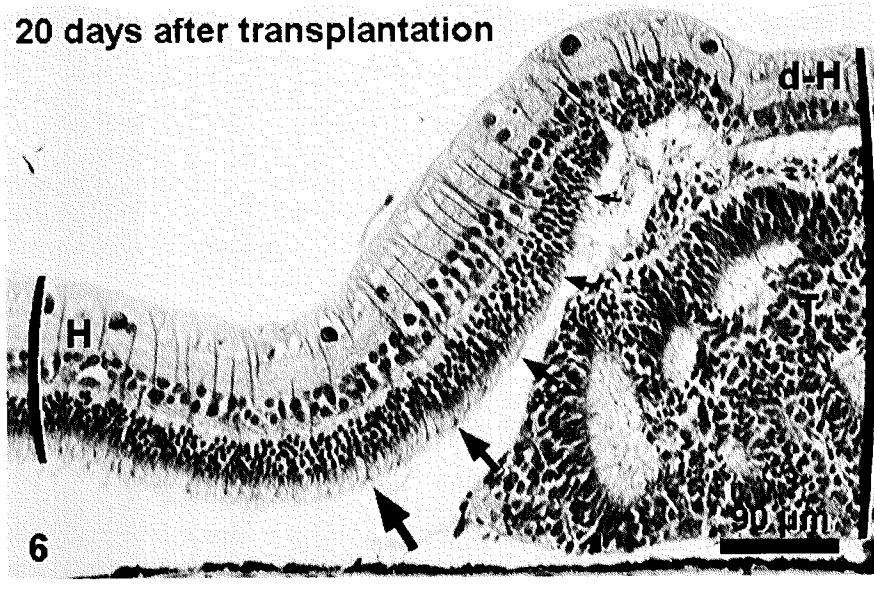

Fig. 6 Short-term rabbit retinal transplant (T), 20 days after transplantation. The combined thickness (large parenthesis) of the graft and the degenerated host retina (d-H) overlying it is approximately 3 times that of the normal retina (n-H) adjacent to it (small parenthesis). Also, the host outer nuclear layer and the photoreceptor outer segments on the side of the graft (arrows progressively decreasing in size) progressively taper off away from the host choroid. Bar = 90 µu.

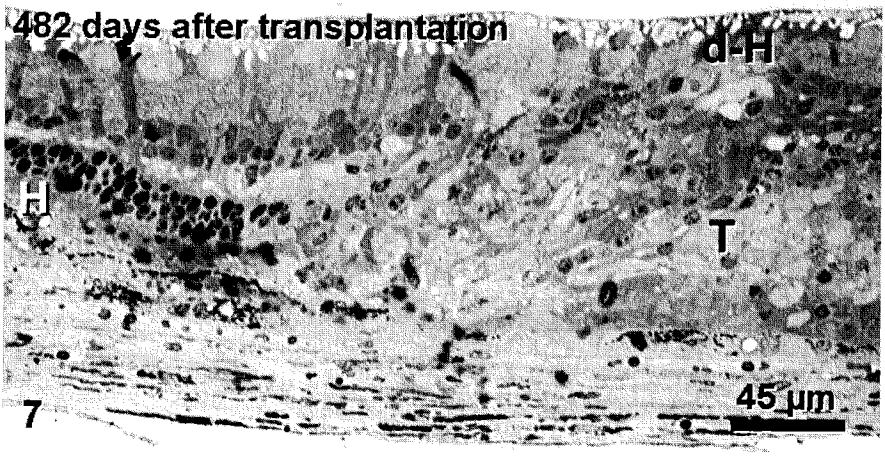

Fig. 7 Long term rabbit retinal transplant 482 days after transplantation showing that the thickness of the long-term grafts is much reduced as compared to the ones that had survived for shorter duration. In this case, the thickness of the graft (T) and the overlying degenerated host retina (d-H) was almost equal to or only slightly more than the thickness of the intact host retina (H). Bar = 45 µu.

However, as is the case in retinal transplants, a lack of contact with or defect in the pigment epithelium does not prohibit the initial development and differentiation of the photoreceptors.

Our results are in agreement with a different study in which microaggregates of mouse neural retina were transplanted into the subretinal space and followed for up to 9 months after the transplantation. Only the photoreceptor cells in contact with the host retina survived (Gouras et al., 1994). In another study, where retinal pigment epithelium was co-transplanted with the neural retina, the inner limiting membrane developed only in the presence of retinal pigment epithelium, and the photoreceptors also survived longer (>12 weeks) when in contact with the pigmented epithelium (Seiler, Aramant and Bergström, 1995).

The cells of the outer nuclear layer located in the inner layers of the rosettes are largely responsible for the histologically identifiable rosettes. In the long-term transplants the reduction of rosettiform lamination is due to the absence of photoreceptor cells. The survival of inner retinal cells, and not those of the outer nuclear layer, demonstrates that the photoreceptor cells are particularly vulnerable to degeneration. It should be noted that the large and varied complement of genetic mutations found in retinitis pigmentosa seem to damage predominantly the photoreceptor cells. In RCS rat retina where the host pigment epithelium is defective, it is also the photoreceptor cells of the neural retina which are effected most, whereas the cells of the inner retinal layers are, to a large extent, preserved (Santos et al., 1997; Sharma, Warfvinge and Ehinger, 1997; Sharma et al., 1998) .

Although the precise mechanism remains unknown, our results indicate that the lack of proximity to the pigmented epithelium results in the degeneration of photoreceptor cells. The subretinal transplants are shielded by the immune privilege of the eye, both in the healthy and the diseased eye (Jorquera et al., 1994). However, the immune privilege of the eye is not perfect, and by no means permanent. For example, some tumor cells transplanted to the anterior chamber of mice induce only a transient ACAID reaction (Ksander et al., 1991). Allogenic retinal grafts placed in the anterior chamber are eventually destroyed, indicating that conventional immunity can overcome the ACAID (Jiang and Streilein, 1991). Histocompatibility antigens are present in only low levels in the normal neural retina, but are usually upregulated after transplantation (Larsson, Juliusson and Ehinger, 1998; Rao et al., 1989). This means that immunity may possibly result in the death of grafted cells. The lack of inflammatory cells either in the graft or the surrounding host retina suggests that immunity probably does not play a role in cell death of the transplants. It has been shown that there is massive cell death by apoptosis in the graft and the surrounding host retina soon after transplantation, but the cell death stabilizes after some time. Nevertheless some apoptotic cell death can still be observed at the longest time (61 days post transplantation) examined in the study (Zhang and Perez, 1998). It is possible that slow apoptosis continuing for a long time may be responsible for the death of the photoreceptors in the transplants. Excessive glial tissue noted in certain transplants could result from prolonged cell death in the transplants. Excessive glial cell proliferation and gliosis has been observed in retinal transplants (Sharma and Ehinger, 1997b; Seiler and Aramant, 1994b) as well as in retinal degenerations (Fan et al., 1996).

In the pathogenesis of subretinal neovascularization, loss of integrity of the Bruch's membrane plays an important role (Dastgheib and Green, 1994; Miller et al., 1990). It is conceivable that some damage to the host retinal pigment epithelium and to the Bruch's membrane must take place during the transplantation surgery. Damage to, and migration of the host retinal pigment epithelium during the transplantation surgery has previously been reported (Sheedlo, Li and Turner, 1993; Bergström et al., 1992). Therefore, the lack of neovascularization in and around the retinal graft is a desirable sign.

It is not clear whether the cell death observed in the graft and the host is due to some host-graft interaction or due to lack of nourishment reaching the cells in the transplant and the host retina which are situated away from the source of nourishment, the host choroid. The long-term transplants tend to stabilize in thickness, suggesting that the thickness reached might be the optimum one, perhaps determined by the availability of nourishment reaching it from the host choroid. It has been noted that subretinal transplants that are small in size undergo minimum degeneration (Sharma and Ehinger, 1997b). Preservation of the cells of the inner retina in the host overlying the grafts indicates that the connections that the graft forms with the host in this area will maintain their functionality for long periods of time.

Patients with retinitis pigmentosa and other hereditary retinal degenerations which can potentially benefit from retinal transplantation essentially need photoreceptor cell replacement. For the long-term survival of photoreceptors in the transplants, a proper apposition of the graft photoreceptors with the host retinal pigment epithelium seems to be important. It will therefore be essential to evolve surgical techniques in which large sheets of donor retinas can consistently and predictably be placed in to the subretinal space well apposed to the host retinal pigment epithelium, or to cotransplant the pigmented epithelium with the neural retina. Progress is already underway in this direction (Sharma, 1999; Sharma, Bergstrom and Ehinger, 1997).

Acknowledgments

This work was supported by the Swedish Medical Research Council (project 14X-2321), the RP Foundation Fighting Blindness, the Swedish Society for Medical Research, EU BioMed 2 program, Kronprinsessan Margaretas Arbetsnämnd, and the Medical Faculty of the University of Lund .

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