Development of Retinal Transplants

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ISRN LUMEDW/MEVL--5001--SE

ISBN 91-628-2082-6

Printed by Reprocentralen, Lund University

Indira Vani

List of papers

This study is based on the following papers which will be referred in the text by their roman numerals:

(I) Sharma, R.K., Bergström, A., and Ehinger, B. (1995). Retinal cell transplants. *Prog. Retinal Eye Res*. **15(1),** 197-230.

(II) Sharma, R.K., Bergström, A., and Ehinger, B. (1996). Influence of technique and transplantation site on rosette formation in rabbit retinal transplants. *Acta Ophthalmol. Scand*. (*In press).*

(III) Sharma, R.K. and Ehinger, B. Mitosis in developing rabbit retina.: An immunohistochemical study. *(Submitted).*

(IV) Sharma, R.K. and Ehinger, B. Cell proliferation in retinal transplants. *(Manuscript).*

Contents

Introduction

Retinitis pigmentosa is a set of genetic diseases leading to progressive photoreceptor degeneration and thus a severe visual impairment. One out of three to four thousand people are affected by the disease (Ammann, Klein and Franceschetti, 1965; Jay, 1982; Hu, 1982; Bunker et al., 1984; Haim, Holm and Rosenberg, 1992). Around the world, about 1.5 million people can be expected to be affected by the disease, which makes it the most common progressive hereditary retinopathy in man.

In the past, various treatments have been empirically attempted on patients with *retinitis pigmentosa* (Duke-Elder and Dobree, 1967; Chatzinoff et al., 1968). Attempted treatments include the use of minerals and vitamins, therapy with placental extracts, steroids, vasodilators, injections of hydrolysates of yeast RNA or transfer factor or methyl sulfoxide, ozone, ultrasound, cervical sympathectomy, and muscle transplants. Success, as judged by the subjective improvement, has at times been claimed, but scientific validation of these claims is difficult since the course of the disease may have spontaneous fluctuations. None of these modalities of treatment have any scientifically proven beneficial effect on the disease.

In the last decades, there have been great advances in research concerning degenerative retinal disorders. The research areas include cell biology (apoptosis), molecular genetics, transplantation research, pharmacological research, and genetic engineering. Clinical transplantation procedures for both retinal pigment epithelium and neural retina are being developed (for review see del Cerro, 1990 and Paper I).

Retina to retina transplantations were first done in 1986 (Turner and Blair, 1986), and since then the transplantation research has come a long way. However, the desirable clinical goals have not yet been reached. At present, most of the scientific efforts concentrate on certain aspects, namely the formation of rosettes in the transplants, the fate of ganglion cells in the transplants, the types and numbers of connections made between the transplants and the host, and the possibility of using transplants as the source of cytokines and other growth regulators (Paper I).

Survival

Donor tissue often consists of neuroblastic cell mass at the time of transplantation. After transplantation the donor tissue survives *in oculo* (Turner and Blair, 1986; Aramant, Seiler and Turner, 1988; Ehinger et al., 1992; Bergström et al., 1992; Sharma, Bergström and Ehinger, 1995), where they are protected by the immune privilege of the eye. The survival of the transplant is affected by various factors. There is a rather wide time window during which donor tissue can be transplanted (Aramant, Seiler and Turner, 1988). Generally speaking, young donor tissue survives better than old. Xenografts survive provided the hosts are immunosupressed. Survival is also affected by the site of transplantation, and the subretinal space is a good one (Sharma, Bergström and Ehinger,

1995). Cryopreserved retina can be used, but it does not form an ideal donor tissue (Aramant and Seiler, 1991).

Neural retina has also been shown to survive and develop in the abnormal environment where the host retina has been damaged by light (del Cerro et al., 1988a; Silverman and Hughes, 1989a), or exposure to high concentration of oxygen (Bergström et al., 1994a). These transplants performed as they would in the normal host environment. Transplants of normal retina to *rd* mouse survive and differentiate, but the survival rate falls with long survival time, suggesting an adverse effect of the mutant environment on the transplant (Jiang and del Cerro, 1989).

Transplantation techniques

Many different techniques are now available for transplanting neural retina in different physical states such as tissue fragments (Turner and Blair, 1986; Bergström et al., 1992), cell suspensions (del Cerro et al., 1988; Juliusson et al., 1993), enzymatically isolated photoreceptors (Gouras et al., 1991a and b), large sheets of whole retinas (Sharma, Bergström and Ehinger, 1995), and sheets of photoreceptors (Silverman and Hughes, 1989). In general immature retina from either an embryo or a newborn animal is dissected and taken up in a fine capillary mounted on a specially designed instrument. The tip of the capillary is introduced into the eye through a pars plana incision and advanced through the vitreous. On reaching the posterior pole, the contents of the capillary are deposited in the subretinal space. Depending upon the caliber of the capillary, the donor tissue may vary from fine fragmented pieces (Bergström et al., 1992; Turner and Blair, 1986) to large pieces of whole retinas (Sharma, Bergström and Ehinger, 1995).

Retinal layers and rosette formation

In most of the transplant modalities being used at present, the transplanted cells organize themselves in various retinal layers, but the layering itself is in the form of so called rosettes (Ehinger et al., 1996; Silverman and Hughes, 1989; Gouras et al., 1992; Gouras et al., 1991; Turner and Blair, 1986; del Cerro et al., 1985). In the rosettes, photoreceptors are located towards the luminal side and the cells of the inner nuclear layer and ganglion cell layer (probably with the exception of ganglion cells), away from the lumen of the rosettes. Rosettes form soon after transplantation.

Rosette formation does not mean that the transplants are rendered incapable of processing light signals, but the quality of the resolution of image thus produced will be poor. It seems likely that the rosette formation in the transplant is the result of mechanical disruption of the donor tissue during the process of transplantation (Sharma, Bergström and Ehinger, 1995).

Cell types and synapses (electron microscopy)

Nerve cell transplants integrate, grow, and differentiate in the host environment according to their intrinsic time table. Electron microscopic studies have shown that the transplanted retinas develop most of the cells found in normal retinas, with a probable exception of ganglion cells (Ehinger et al., 1991; Ehinger et al., 1992). Transplants also form normally occurring synapse types (Ehinger et al., 1991; Ehinger et al., 1992; Zucker et al., 1994; Bergström et al., 1994c). However, transplant photoreceptor outer segments are not entirely normal, especially when dissociated photoreceptor cells are transplanted (Juliusson et al., 1993). Nevertheless, they have been shown to be capable of phototransduction (Klassen and Lund, 1987; Klassen and Lund, 1990; Adolph et al., 1995).

Neurotransmitters and essential proteins

Opsins specific for rods (rhodopsin; Juliusson et al., 1993) and for cones (short wavelength sensitive and medium wavelength sensitive; Szél et al., 1994) are present in the transplants. Other essential proteins such as S-antigen, rod alpha transducin, neuron specific enolase (NSE), and synaptophysin (SYN) are also present (Seiler and Aramant, 1994; Aramant et al., 1990). Immunohistochemical staining for some components of interphotoreceptor matrix has shown that its composition is not entirely normal (Juliusson et al., 1994). IRBP (**i**nterphotoreceptor **r**etinoid-**b**inding **p**rotein) is a protein that is synthesized by the photoreceptor cells (van Veen et al., 1986) and it helps in transporting retinoids between the neural retina and the retinal pigment epithelium. 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.).

A 160 kD neurofilament protein present in the horizontal cells could be seen in retinal transplants (Aramant et al., 1990a) suggesting that these cells survive transplantation.

The HPC-1 antibody marks a special protein, syntaxin, predominantly present in amacrine cells (Barnstable, Hofstein and Akagawa, 1985; Inoue, Obata and Akagawa, 1992; Inoue and Akagawa, 1993), and appropriate numbers of such cells have been seen in transplants. In rats, the first differentiating amacrine cell appears in the graft corresponding to the first postnatal day as seen by immunoreactivity of choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH) (Aramant et al., 1990a). 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 (Aramant et al., 1990a). HPC-1 immunoreactive fibers have also been demonstrated in human transplants (Seiler et al., 1991). Somatostatin-28-immunoreactive fibers, normally present at the junction of the inner plexiform layer and

the inner nuclear layer, appear irregularly arranged due to the rosetted lamination of the graft.

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. It is possible that due to a lack of target derived substances these cells do not survive in *in oculo* retinal transplants.

In order to study their development, glial cells in transplants have been marked with antibodies against the S-100 antigen (a calcium binding protein), glial fibrillary acidic protein (GFAP), and vimentin (Sharma, Bergström and Ehinger, 1995; Seiler and Aramant, 1994; Seiler and Turner, 1988, Seiler et al., 1990). Morphologically, the transplant Müller cells appeared to be normal in the outer nuclear layer and at the outer limiting membrane, but they did not develop end-feet at the inner limiting membrane at the vitreal surface. Müller cells reflecting the architecture of the transplant are found radially arranged in transplants where the rosettes dominate, whereas they are regularly arranged in rosette-free grafts when un-traumatized donor tissue is transplanted (Sharma, Bergström and Ehinger, 1995).

Connections between the host and the graft

Transplants must form meaningful connections if they are to convey visual information, which means that nerve cells from the transplants must send their processes to the host and form synaptic connections there. DiI (a carbocynanine dye) labeling of the transplanted cells showed fiber outgrowth from the transplant to the host retina. Transplant fibers that had grown into the host inner plexiform layer formed synapses there. The labeling also demonstrated that some fibers from the host had grown into the transplant (Aramant and Seiler, 1995). Even though connections between the host and the transplant have been seen, they seem to be few and do not appear regularly. Nothing is known on how to influence the formation of such connections and more work is needed on this problem.

Survival potential of ganglion cells and alternative strategies

Till now there has been no conclusive evidence that ganglion cells survive and develop in the transplants *in oculo* (del Cerro, 1990; Aramant et al., 1990; Bergström et al., 1994). Retinas transplanted to CNS sites close to their target nuclei contain surviving ganglion cells that have been shown to form functioning connections with the target tissue. However, it has been shown that ganglion cell axons will regenerate into peripheral nerve grafts (So and Aguayo, 1985) which can guide the axons all the way to the CNS where they will form connections (Vidal-Sanz et al., 1987; Carter, Bray and Aguayo, 1989; Aguayo et al., 1990).

In *retinitis pigmentosa* and similar diseases, only the photoreceptor layer needs replacement. The possibilities to transplant only an isolated sheet of photoreceptors (del Cerro et al., 1990; Silverman and Hughes, 1989) or isolated photoreceptor cells (Gouras et al., 1992; Gouras et al., 1991) is therefore being explored. Bipolar cells and horizontal cells are the targets of photoreceptors, and there is no need for the transplant to have surviving ganglion cells that should send projections to their targets in the central nervous system. Transplanted photoreceptor sheets have been reported to survive well (Silverman et al., 1992; Juliusson, 1996), and there is a preliminary report that they form functioning contacts with the host retina (Mosinger-Ogilvie et al., 1994).

Function

Many of the prerequisites for retinal functioning are already present in the transplants. Except for certain abnormalities, like the absence of IRBP, most of the essential proteins needed for the phototransduction process (like S-antigen, opsin and α-transducin) are present in the transplants (Seiler and Aramant, 1994; Bergström et al., 1994; Silverman and Hughes, 1989; Aramant et al., 1990; Aramant et al., 1990).

Besides this, most of the neurotransmitters/neuromodulators found in normal retina are also present in the transplants (Aramant et al., 1990a). The presence of horizontal cells, bipolar cells as well as various types of amacrine cells and their synaptic connections suggests that the transplant might process the light signal at the level of both the inner and outer plexiform layers. However, the presence of the various substances is only a necessary prerequisite and not an infallible sign of transplant function.

Direct evidence of retinal cell transplant function has come from electrophysiological experiments. With a single electrode on the surface of isolated grafts, transient ‘ON’ and ‘ON-OFF’ spike-like responses and local electroretinograms could be recorded, which suggest that the nerve cell activity in the transplants is similar to what can be found in the inner and outer plexiform layers of the normal retina (Adolph et al., 1995).

Goal and aims of the present study

The general goal of the present study was to get a further understanding of the biological and clinical basis for retinal cell transplantation. More specifically, information was sought on matters concerning factors affecting the development, and structure of normal and transplanted cells.

Specific aims of the study were:

(1) To comprehend the literature available in the field of retinal cell transplantation and to identify the present problems.

(2) To further develop the surgical procedure for producing retinal cell transplants in rabbits.

(3) To reach a better understanding regarding the rosette formation in the retinal transplants.

(4) To study the influence of transplantation site on the development of donor tissue.

(5) To examine and compare the proliferation of the cells in normal developing and transplanted retinas.

Materials and Methods

Literature (Paper I)

Relevant scientific papers in the field of retinal pigment epithelium transplantation, neuro-retinal transplantation, retinal development, ganglion cell axon regeneration, cell death and ocular-immunology were reviewed.

Tissue

Developing rabbit retinas (Papers III and IV)

Embryonic day (E) 15, 25, and 29 eyes were enucleated from rabbit embryos obtained by cesarean section after sacrificing the pregnant pigmented rabbits with an overdose of sodium pentobarbital. Eyes were also obtained from postnatal day (PN) 0 (= birth day), 3, 5, 7, 11, 15, and 20 pigmented rabbits.

The age of the animals was determined by mating the rabbits on a known date.

Donor tissue (Papers II, III, and IV)

Ordinary mixed-strain pigmented rabbits from stage E 15 (15th post-conception day) were used as donors. Embryos were obtained by cesarean section after sacrificing the pregnant female rabbits with barbiturates. 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 (Papers II, III and IV)

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-HCl and, if necessary, also with 10% phenylephrine-HCl. For surgery, animals were anaesthetized with Hypnorm®, 1 ml/kg (fluanison 10 mg/ml and fentanyl 0.2 mg/ml).

Cannula

For fragmentation technique (Papers II, III, and IV)

For transplanting fragments of the donor tissue the instrument described by Bergström et al. (1992) was used. The plastic capillary used in this instrument is quite narrow (with inner and outer tip diameter of 0.15-0.4 and 0.3-0.5 millimeters respectively), so that the embryonic retinas get fragmented when drawn up into it.

For large sheet retinal transplants (Paper II)

A special instrument was developed for transplanting large pieces of full-thickness 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 1.6 X 0.4 mm inner and 1.8 X 0.6 mm outer dimensions). This polyethylene tube and the cannula are mounted on a especially 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.

Transplantation procedure (Papers II, III, and IV)

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 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.

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. Appropriate permits for the study were obtained from the Swedish Government Animal Experimentation Ethics Committee at the Lunds University. The experiments were conducted according the rules set forth in the ARVO resolution on animal experimentation.

Tissue processing (Papers II, III, and IV)

At the decided post transplantation time the animals were sacrificed. Their eyes were enucleated and fixed in 4% formaldehyde in Sörensen's phosphate buffer (0.1M pH 7.2) for 24 hours. After the fixation, the eyes were hemisected and the part carrying the transplant was excised. The tissue was washed with Sörensen's phosphate buffer and then with the same buffer containing 5, 10, and 20% sucrose before sectioning on a cryostat (12μm). 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). Developing retinas were fixed and processed in the same way.

Staining for light microscopy

Immunohistochemistry (Papers II, III and IV)

Mouse monoclonal antibodies against rhodopsin (R2-15, diluted 1:6000; gift of Dr. P. A. Hargrave), vimentin (Dakopatts, diluted 1:100), and the Ki-67 antigen (MIB-1 monoclonal antibody; Immunotech, Inc., Westbrook, ME; diluted 1:200) 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 minutes in 0.1 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) followed by incubation with primary antisera for 20-25 hours for the rhodopsin and vimentin antibodies and 72 hours for the Ki-67 antibody. Sections incubated with anti-vimentin antibody were washed, incubated with secondary antibody (rhodamine-conjugated rabbit immunoglobulins to mouse immunoglobulins, Dakopatts) 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 of Vector Lab. Inc.

When describing and discussing retinal development we will use the term “basal” or “proximal” and “apical” or “distal” 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 pigment epithelium. For transplants we will use the term “luminal layers” or “inner layers” 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 (containing the cells found in the inner retinal layers of normal retina).

Observations and Results

Architectural organization of transplants (Paper II)

In fragmented tissue retinal transplants

To study the overall architectural organization of the transplants, Müller cells were immunostained for 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, Müller cells were radially arranged in the outer nuclear layer of the rosettes. In the areas between them, which corresponds to the inner retinal layers, Müller 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.

In large sheet retinal transplants

Six weeks after the transplantation (equivalent to 8 weeks postconception) 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 palissade-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 Müller cells were regularly arranged in an arcuate manner, in these transplants.

Development of transplants

In the epiretinal space (Paper II)

In the large sheet retinal transplants 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 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.

In the subretinal space (Paper II)

Both the arcuate arrays and the rosettes showed two distinct layers of cell bodies. The first comprised comparatively small, oval cell bodies, apparently consisting of 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 layer, 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 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, confirmed by light microscopy and immunohistochemistry for rhodopsin. Particularly in cell arrays, photoreceptors were also associated with a well developed outer limiting membrane as judged by light microscopy.

Proliferation of cells as seen with MIB-1 immunoreactivity

In developing rabbit retina

At embryonic day 15, the sensory retina consisted of a thick, multi-layered neuroblastic cell mass and a thin inner anuclear layer. Ki-67 immunohistochemistry showed positive cells in the outer two thirds of the neuroblastic cell mass, whereas its innermost third contained mainly non-reactive cells. Some rounded cells close to the pigment epithelium were more intensely stained. Ki-67 immunoreactivity appeared in most of the pigment epithelial cells.

At embryonic day 25, the ganglion cell layer and the inner plexiform layer had formed. Some cells in the ganglion cell layer or nerve fiber layer were Ki-67 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 region of the neuroblastic cell mass which was adjacent to the non-reactive region. Further outwards, there was a region with elongated cells showing less immunoreactivity. Finally, there was a population of large, rounded and deeply reactive cells close to the retinal pigment epithelium, most likely mitotic. The pigmented epithelium was more heavily pigmented than in E 15. Ki-67 immunoreactivity was present in most of the cells.

At embryonic day 29, some cells in the ganglion cell layer or nerve fiber layer were immunoreactive for Ki-67. The distribution of the Ki-67 immunoreactivity was identical to that seen in the E 25 retina except that the non-reactive layer in the basal part of the neuroblastic cell mass was thicker and corresponded to the rounded cells seen in hematoxylin and eosin stained sections. In the central retina, an outer plexiform layer

could be seen, and the number of immunoreactive cells decreased in the vicinity of the outer plexiform layer. In the embryonic day 29 retina, pigment granules in the pigment epithelium were prominent, making it difficult to judge the Ki-67 immunoreactivity.

At postnatal day 0, an area essentially devoid of Ki-67 immunoreactive cells developed in the central retina. The number of immunoreactive cells increased from this area towards the periphery. In the peripheral part of the retina, the reactivity was generally less than at E 29. Especially in the outer nuclear layer, the reactivity decreased in the regions where the outer plexiform layer had developed. 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 throughout the thickness of the outer nuclear layer. Large, rounded, deeply stained cells were still present close to the retinal pigment epithelium in the outer nuclear layer, and more so in the peripheral areas than in central. The inner nuclear layer was more immunoreactive than the outer nuclear layer and some large, rounded, and deeply stained cells could also be seen in this region. The reactivity in the inner nuclear layer was less in comparison with E 29, and was confined to the elongated spindle-shaped 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. Such immunoreactive cells in the nerve fiber or the ganglion cell layer persisted for a long period of time.

In the postnatal day 3 retina, the outer plexiform layer was clearly demarcated and had almost reached the periphery of the retina. The photoreceptor outer segments were progressively better developed from this stage onwards to PN 15. The central area with no or few immunoreactive cells progressively enlarged in PN 3, PN 5, and PN 7 stages. 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. Cells in the inner and the outer parts of the inner nuclear layer were largely non-reactive. In particular, there were no immunoreactive cells in the cell rows closest to the inner plexiform layer. In the outer nuclear layer, the immunoreactivity was much less than that in the inner nuclear layer, with most of the reactive cells being present close to the outer plexiform layer. In other parts of the outer nuclear layer, scattered immunoreactive cells were present. Most cells in the outermost part of the outer nuclear layer were non-reactive, with some exceptions, including the deeply stained cells. In general, the number of large, deeply stained cells decreased with increasing age, and these cells were more common in the peripheral than in the central retina. Only few immunoreactive cells were present in the ganglion cell layer or the nerve fiber layer, especially close to the optic nerve.

At postnatal day 11 and 15, photoreceptor outer segments were well developed. Only some few immunoreactive cells were still visible at 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 also still immunoreactive. The optic nerve also showed a large number of immunoreactive cells.

In rabbit retinal transplants

The earliest transplant studied corresponded to embryonic day 16 (one day after the transplantation) and showed MIB-1 immunoreactive cells scattered throughout the tissue. Certain large and deeply stained cells were homogeneously distributed throughout the transplant or occasionally formed small rosette-like clusters. Other immunoreactive and non-immunoreactive cells were homogeneously distributed throughout the transplant. In places a few cells, close to the host retina, appeared in small patches devoid of immunoreactivity. No immunoreactive cells were seen in the host retina.

In transplants corresponding to embryonic day 19, MIB-1 immunoreactive cells appeared in places to be organized in rosettes. Cells lying close to the host retina were small and pyknotic. The same layer close to the host retina was devoid of immunoreactive cells. However, there were a few immunoreactive cells at the host-graft interface and the immunoreactivity in these cells persisted even in older transplant ages. Some cells in the host retina were immunoreactive.

By embryonic days 21 or 22, rosettes were more distinct. Small and pyknotic cells were seen close to the host retina as described earlier. Large, deeply immunoreactive mitotic cells were often found arranged in the innermost parts of the rosettes, close to the lumen. A few cells in the innermost layers of the rosettes had become non-immunoreactive, but the deeply stained cells were still present. There were reactive cells in the outer layers of the rosettes and small clusters of non-reactive cells in between the rosettes. There were clusters of MIB-1 immunoreactive cells in between the rosettes as well as small clusters of cells that were not immunoreactive. A layer of non-reactive cells was observed close to the host retina and this corresponded to the the layer where small pyknotic cells could be observed in hematoxylin and eosin stained sections. However, some few immunoreactive cells were present at the host surface of this non-reactive area. A small number of immunoreactive cells were still present in the host retina close to the transplant.

Progressively in embryonic days 26 and 29 , cells in the luminal layers of rosettes became non-reactive and the reactivity was confined to outer layers of the rosettes and in the cells located in between the rosettes. There were also some immunoreactive cells in the host. At the graft side of the host-graft interface, there was a concentration of immunoreactive cells.

At a transplant age corresponding to postnatal day 2, the cells in between the rosettes were still immunoreactive and there were immunoreactive cells in the host retina and at the host-graft interface. At a transplant age corresponding to postnatal day 4, most of 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.

Immunoreactive cells were rare in most parts of transplants obtained at ages corresponding to postnatal days 5, 11, and 12. However, some few immunoreactive cells could be seen distributed in between the rosettes. Immunoreactive cells were also observed in the transplant at the host-graft interface and in some very few cells in the host retina.

General discussion

Identification of present problems in retinal cell transplants

In the last decade, advances in the field of neural transplantation have generated the hope that retinal cell transplantation will become feasible and prove a practical way to treat retinal disorders hitherto untreatable. The history of retina-to-retina transplantation is only about a decade old, but the achievements have been considerable. Pigment epithelial cells and retinal neurons have very recently been transplanted in humans (Algvere et al., 1994; Algvere et al., 1996; Das et al., 1996), but long-term clinical results are not yet available. However, there are certain areas in the field of retinal transplantation where more detailed studies are needed.

In most of the transplant modalities being used at present, the transplanted cells organize themselves in laminae, but this layering is in the form of small spherical balls called rosettes (del Cerro et al., 1985; Turner and Blair, 1986; Silverman and Hughes, 1989; Gouras et al., 1991; Gouras et al., 1992; Paper IV). Rosette-free transplants are highly desirable and 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.

There is also a need to look in more detail into the physiology and development of retinal transplants, as well as to define what is normal and what is abnormal in them. This knowledge will be useful to develop remedies for the limited function 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 enough with the host and form sufficient synaptic connections. More work is needed to find out the factors that can influence the formation of connections between the host and the graft.

There has been no conclusive evidence that ganglion cells survive and develop in the transplants (del Cerro, 1990; Aramant et al., 1990; Bergström et al., 1994), However, it has been shown that ganglion cell axons will regenerate into peripheral nerve grafts (So and Aguayo, 1985), which can guide the axons all the way to the CNS where they will form connections (Vidal-Sanz et al., 1987; Carter, Bray and Aguayo, 1989; Aguayo et al., 1990). It is important to identify the factors that can influence the survival and the projection of the ganglion cells. Intravitreal injection of brain derived neurotrophic factor (BDNF) has been seen to retard the degeneration of adult rat retinal ganglion cells after optic nerve transsection (Mey and Thanos, 1993; Mansour-Robaey et al., 1994). Similar observations have been made in cell cultures (Castillo, Jr. et al., 1994) and retinal transplants in the brain (Panni, Atkinson and Lund, 1994).

Transplanting cells that produce some trophic factor that will prevent neurons from degenerating is an attractive idea which 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 (LaVail et al., 1992). However, cell systems able to deliver the required factors are not available. It is worth looking into the possibility of using the retinal transplants, peripheral nerve transplants, or some other cell line as a source of trophic factors.

Transplantation site and development of donor tissue

The donor tissue at the time of transplantation (E 15) consists largely of undifferentiated cells (Sharma, Juliusson and Ehinger, 1996; Paper III). The development of an inner plexiform layer in the epiretinal transplants and the formation of layers and photoreceptor outer segments in the subretinal transplants show that the donor tissue continues to mature at both these transplantation sites, as reported by many authors in various models (Royo and Quay, 1959; Turner and Blair, 1986; Gouras et al., 1990; Seiler et al., 1990; Bergström et al., 1992; Bergström, 1994).

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 immunostaining for rhodopsin. 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 (Aramant et al., 1990b). The same was found for cellular retinaldehyde-binding protein (CRALBP) which marks the glial cells (Aramant et al., 1990a; Seiler and Aramant, 1994a).

Transplantation technique and rosette formation

The lamination of the large sheet 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 organization of the transplant was confirmed by vimentin immunostaining of the Müller cells, which are radially arranged in fragmented tissue transplants (Seiler and Aramant, 1994; Seiler and Turner, 1988). 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.

It is apparent from the results presented here that transplants done with two different techniques can develop different morphologies. In the fragmentation technique, the

donor tissue is likely to undergo an initial reaggregation, which may be important for the eventual cytoarchitecture of the tissue.

In large sheet retinal transplants, donor tissue architecture was minimally disturbed in the process 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.

Proliferation of cells in developing rabbit retina

The MIB-1 antibody used in this experiment detects the native nuclear protein, Ki-67, or fragments of it (Key et al., 1993). The protein is present only during the cell division cycle (Gerdes et al., 1984; 1983). Immunostaining with the MIB-1 antibody is therefore a reliable means of detecting proliferating cells.

At E 15, Ki-67 reactivity was seen in the outer two thirds of the neuroblastic cell mass. However, mitotic figures visible as large, rounded, and deeply stained cells were found only at the apical border of the neuroblastic cell mass in this (Sharma, Juliusson and Ehinger, 1996; Paper III) and other (Rapaport, Robinson and Stone, 1985; Greiner and Weidman, 1982) studies, indicating that the proliferating cells undergo interkinetic migration.

Ganglion cells are the first cells to leave the mitotic cycle (Sidman, 1961; Fujita and Horii, 1963; Hollyfield, 1971; Kahn, 1973; Jacobson, 1976; Sharma and Ungar, 1980; Walsh et al., 1983), to migrate to the proximal part of the neuroblastic cell mass (Zimmerman, Polley and Fortney, 1988) and to start to project axons towards the optic fissure (Halfter, Deiss and Schwarz, 1985; Silver and Robb, 1979). Therefore, the first group of postmitotic cells accumulating in the most proximal part of the neuroblastic cell mass are likely to be cells that have started to differentiate into ganglion cells (Sharma, Juliusson and Ehinger, 1996; Paper III).

In stages E 25 and E 29, there was a layer of non-proliferating cells in the innermost part of the neuroblastic cell mass (Sharma, Juliusson and Ehinger, 1996; Paper III). These cells correspond to the round, lightly stained cells in hematoxylin and eosin sections (in E 29) and have been described as differentiating amacrine cells in previous studies (Greiner and Weidman, 1982).

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 remaining amacrine cells, Müller cells, bipolar cells and rods (Sidman, 1961; Carter-Dawson and LaVail, 1979; Zimmerman, Polley and Fortney, 1988; LaVail, Rapaport and Rakic, 1991; Reichenbach et al., 1994; Reichenbach and Robinson, 1995).

Differentiated horizontal cells are apparent already at E 27 (Greiner and Weidman, 1982). 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 III).

In 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 III). However, even after the formation of the outer plexiform layer, proliferating cells in the outer nuclear layer could be observed accumulated in the proximal part and the distal-most 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 (Reichenbach and Robinson, 1995).

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 (Sharma, Juliusson and Ehinger, 1996; Paper III). This is consistent with previous reports based on observing mitotic figures (Stone, Egan and Rapaport, 1985).

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 could be seen in the inner nuclear layer (Paper III), as has also been reported by others (Spira and Hollenberg, 1973; Rapaport, Robinson and Stone, 1984; Robinson, Rapaport and Stone, 1985; Rapaport, Robinson and Stone, 1985; Harman and Beazley, 1987; 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 (McArdle, Dowling and Masland, 1977; Blanks and Bok, 1977). It seems that Müller cells are born over a long stretch of time, and that in 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, (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.

The appearance 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 (Sharma, Juliusson and Ehinger, 1996; Paper III). Similar late proliferating cells in the inner nuclear layer of retina have been reported in cat (Rapaport, Robinson and Stone, 1985; Rapaport, Robinson and Stone, 1984). The cells in the nerve fiber layer are probably astrocytes (Schnitzer, 1990; Schnitzer, 1988) 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 (Reichenbach et al., 1991), [^{3}H]-thymidine labeled cells in this region have been shown to contain immunoreactive vimentin, which stains Müller cells (Schnitzer, 1985).

Proliferation of cells in rabbit retinal transplants

In the early transplants, the proliferating cells were randomly distributed, including the mitotic ones, which appeared as large, rounded and deeply stained cells. This most likely reflects the random overall organization seen at this stage. In places, darkly staining mitotic cells appeared to cluster together to form the beginning of what would become a rosette (Paper IV).

Randomly distributed postmitotic cells were also seen, as might be expected from the observation that there are such cells already in E 15 retinas. Since the best time for harvesting CNS donor tissue is before the cells undergo terminal mitosis (Brundin et al., 1986; 1988), E 15 may not be the best time for harvesting the donor tissue.

As the transplanted cells reorganize themselves into rosettes (E 19), the proliferating cells also adopt the general proliferation pattern seen in normal retinogenesis. Mitotic cells 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 IV). This also indicates interkinetic migration within the rosettes, similar to what is seen in the normal retina.

Already at E 21, and more so at E 22, the proliferation of cells is reduced in the innermost layers of rosettes. These postmitotic cells now begin to differentiate into 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 non-proliferating cells in between the rosettes should be the postmitotic cells differentiating into amacrine and horizontal cells. In E 26 transplants, the luminal-most layers of the rosettes were largely non-proliferating (Paper IV). The pattern of proliferation thus resembles that in 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 distribution 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 (Paper IV). This shift of where proliferating cells predominate is also seen in the normal development of the retina (Sharma, Juliusson and Ehinger, 1996; Paper III). Small

patches of proliferating cells seen, in the postnatal day 4 transplants and older, may be due to the mixing of the central and the peripheral retinas. In peripheral retina, cells proliferate for longer period of time.

Soon after the transplantation, numerous small pyknotic or perhaps apoptotic cells appeared in the parts of the transplant that were closest to the host retina as also reported earlier (Bergström et al., 1992; Perez, 1996, personal communications). The Ki-67 immunoreactivity revealed that these were non-proliferating cells. They were also present in transplants with longer survival times (Paper IV). This suggests that a layer of cells at the host-graft interface instead of proliferation undergo degeneration.

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 (Seiler and Turner, 1988). In the same transplants, intrinsic graft glial cells (mostly Müller cells) developed approximately according to their normal timetable. The marked proliferation at the host-graft interface may represent abnormal gliosis, although this has not been much emphasized in previous studies. However, in human xenotransplants, cellular retinaldehyde-binding protein immunoreactivity was found mostly close to the host retina (Seiler and Aramant, 1994), and glial fibrillary acidic protein (GFAP) immunoreactive fibres 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 get encapsulated by a glial barrier which interferes with the integration of the graft with the host (Azmitia and Whitaker, 1983). The observations in the present study suggest that also retinal cell transplants tend to develop a glial barrier (Paper IV).

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.

Conclusions

Although the retinal transplants survive, differentiate, and develop all the retinal layers and most of the retinal cell types, certain aspects of retinal transplants need to be looked into more deeply. It is necessary to find answers to the problems being presently faced, namely the limited growth of the retinal transplants, the rosette formation, the survival of ganglion cells and their projections to the host. There is a need for more thorough investigations on the development of the transplants so that what is normal and what is abnormal in them can be identified. Further, there is a need to study the factors that can influence the retinal transplants towards a normal development. The present study has addressed some of these questions.

(1) Mechanical factors play an important role in the formation of rosettes in retinal cell transplants. Good architectural organization of transplants can be maintained by appropriate handling of the donor tissue at the time of retinal transplantation.

(2) Development of the retinal transplants is influenced by the transplantation site. Donor tissue does not mature as well in the epiretinal space as it does in the subretinal.

(3) 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 normal retina. A layer of cells in the retinal transplants close to the host retina does not proliferate.

(4) Certain cells at the host-graft interface proliferate for a long period of time suggesting that there may be abnormal gliosis at the host-graft interface of the retinal cell transplants.

Svensk sammanfattning (Swedish summary)

Retinitis pigmentosa och andra degenerativa näthinnesjukdomar är en viktig orsak till blindhet över hela världen. Uppskattningsvis 1,5 miljoner människor har retinitis pigmentosa. Näthinnetransplantationer kan bli ett redskap i kampen mot dessa sjukdomar som hittills inte har varit behandlingsbara.

Med dagens teknik för näthinnetransplantation bildar fotoreceptorcellerna små runda rosetter istället för de normala regelbundna näthinnelagren. Skälen till detta är fortfarande bristfälligt kända, men mekaniska faktorer som separation av givarcellerna vid transplantationen och interaktion mellan värd och givare är tänkbara bidragande orsaker.

Resultat från flera laboratorier har visat att transplanterade näthinneceller överlever och fortsätter utvecklas. Dock förblir transplantaten hela tiden små vilket indikerar att de växer sämre än normala näthinneceller. Denna studie genomfördes för att bidra till en bättre kunskap om transplanterade näthinnecellers proliferation och utveckling.

En ny teknik för att transplantera större bitar av intakt embryonal näthinna till vuxna kaniners ögon utvecklades. Sådana transplantat i det epiretinala rummet blir i huvudsak rosettfria förutom i marginalen där givarnäthinnan lätt skadas i samband med transplantationen. Transplantat till det subretinala rummet utbildar bågformade lager som mera liknar den normala näthinnan (Arbete II).

Cellproliferationsmönstret i normal och transplanterad kaninnäthinna studerades immunohistokemiskt med en antikropp (MIB-1) mot ett protein (Ki-67) associerat med proliferation. I normal näthinna ser man de första postmitotiska cellerna (förmodligen ganglieceller) dag E15 basalt bland neuroblastcellerna. Ytterligare postmitotiska celler (troligen amakrina celler) ses distalt om det utvecklande inre plexiforma lagret. Proliferationen avtar i centrala näthinnan vid tidpunkten för födseln, men fortgår i perifera näthinnan upp till en vecka senare. I yttre nukleära lagret fortsätter proliferationen även efter utbildandet av det yttre nukleära lagret vilket talar för att fotoreceptorcellerna bildas under en lång period. Även celler i det inre nukleära lagret prolifererar under en lång tid, och här kan man också se celler i sen proliferationsfas, vilket tyder på en andra proliferationsfas. Ett mindre antal celler i gangliecellslagret/nervfiberlagret, nära synnerven, prolifererar också under en lång tid (Arbete III).

I transplantaten liknar cellproliferationsmönstret mycket det i den normala näthinnan. De delande cellerna genomgår ”interkinetisk migration” i rosettlagren med de senare proliferationsfaserna i de lumennära lagren i rosetterna. En del celler i delar motsvarande inre näthinnan blir postmitotiska först, efterföljda av celler längre in mot lumen. Celler i regioner mellan rosetterna, motsvarande inre näthinnan (sannolikt Müllerceller), prolifererar ända fram till postnataldag 2 (PN2). Närmast värdretina ses ett lager av icke-prolifererande, degenererande celler i transplantatet, men även i detta lager kan vissa celler proliferera. Dessa prolifererande celler indikerar möjligen glios (Arbete IV).

Acknowledgments

I would like to thank my scientific adviser Prof. Berndt Ehinger for his support and teachings.

I would also like to thank every one in the lab and the eye clinic for their help, specially Kennerth Wilke, Anitha Bruun, Anders Bergström, Maria Thereza Rodriguez-Perez, Karin Warfvinge, Sten Andréasson, Elisabet Agardh, Karin Arnér, Sven Jakobsson, Katarzyna Said, Ingela Larsson, Bodil Israelsson, Majvi Persson, Lillian Andersson, Maria Corlin, Gunnel Jahnsén, Bengt Juliusson, Janina Waga, Anna Makár, Sunanda Koul, Thomas Grasbon, Madeleine Selvander, Fredrik Ghosh, Jörgen Larsson, Ken Ninn-Pedersen, Ruth Riise, Hui Zhang, Ming Hu, Yuning Qian, Angelika Skarin, Vesna Ponjavic, and Maria Elena Caminos Benito.

From the department of neurology I would like to thank Prof. Barbro Johansson, Bengt Mattsson, Anna-Lena Ohlsson, Karin Jansner, Li-Ru Zhao, and Muna.

I would also like to Jeppe Stridh and his staff for their help.

I also wish to thank Mats Hugo-Persson for introducing me to Sweden and to Lena, Anna, Kurshid, Jakobssons, Sundströms, Warfvinges and all the friends for absorbing me in this very different culture.

I am also grateful to Bhishm, Adersh, Meenakshi, Yogesh and all the well wishers who have supported me in different times.

For this work I have received financial support from various organisations namely Swedish Medical Research Council, 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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1350-9462(95)00010-0

CHAPTER 10

Retinal Cell Transplants

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CONTENTS

INTRODUCTION

With improving control of the inflammatory ocular disorders and increased life expectancy, degenerative retinal disorders are becoming increasingly important as causes of blindness throughout the world. Based on the belief that the mammalian neurons do not have the capability to regenerate, these diseases have long been considered untreatable, but evidence emerging in the fields of pharmacology, genetics and transplant research suggest that this may not be so.

Recent research has resulted in a number of pharmacological agents that could be useful for treating degenerative retinal disorders. Intravitreal injection of the basic fibroblast growth factor prolongs the photoreceptor life in the Royal College of Surgeons rats (Faktorovich *et al.,* 1990), and more such factors that may serve similar purposes have appeared (LaVail *et al.,* 1992b). A temporally regulated, diffusible activity has been shown to facilitate progenitor cell differentiation into photoreceptors (Altshuler and Cepko, 1992). Such factors have the potential of being useful in the management of retinal degenerative disorders. However, due to their mitogenic activity they are also likely to have side effects like vasoproliferation and other complications. Treatment with moderate doses of vitamin A (Berson *et al.,* 1993) is another example of a recent therapeutical approach, that appears to delay the progress in some forms of retinitis pigmentosa.

In mice with the gene for retinal degeneration (*rd*) or retinal degeneration slow (*rds*), photoreceptor cells have been rescued from degeneration by introducing a healthy gene into the genome of one day old embryos (Lem *et al.,* 1992; Travis *et al.,* 1992). At present the technique needs in vitro fertilization, has a low success rate and incurs the danger of introducing other serious developmental disorders, making it unsuitable for human use.

Corrective genes have also been introduced in cultured retinal pigment epithelium in which the gene for beta-glucuronidase is defective, using a retrovirus as a vector, which requires cell division for gene transfer (Stramm *et al.,* 1990). This system cannot be used in the adult retina, because it is not replicating, but herpes simplex virus or adenovirus can be used to transfect postmitotic cells and may thus be used for subretinal injections bringing the genes to the required site, which is the photoreceptors and the retinal pigment epithelium (Breakefield and DeLuca, 1991; Bennett *et al.,* 1994).

Partly stimulated by the transplantation efforts in brain research directed towards neurodegenerative ailments like Parkinson's, Huntington's and Alzheimer's disease (Ridley and Baker, 1991; Dunnett, 1991; Lindvall, 1991), there has been a rapid progress in developing a similar strategy for retinal degenerative disorders. Factors favoring the retinal transplantation approach include simpler and better understood neuronal connections and microcircuitry (Ehinger and Dowling, 1987; Dowling, 1994) of the retina than in the brain. In the following sections we will review the progress that has been made in this field.

HISTORY

Transplantation and immune privilege of the eye

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 rabbit (van Dooremaal, 1873). Shortly afterwards, whole eye transplants were performed, but the results were not encouraging (see May, 1887 for an overview). However, the anterior chamber technique worked and served as the method of choice for studying tissue growth in an isolated milieu until improved tissue culture methods were developed, resulting in a wide variety of tissues being transplanted to this site (see Olson *et al.,* 1984). The anterior chamber was a good site for such studies not only because of the transparent cornea through which the transplanted tissue can be seen but also because of the unusually weak or absent immune responses experienced in the anterior chamber, making both allotransplants and xenotransplants feasible. The phenomenon is caused by many factors including the active down-regulation of the immune system, known as *the anterior chamber associated immune deviation (ACAID:* Streilein, 1990*)*. Transplants to the anterior chamber have been used to study retinal development by explanting fetal retinal tissues (Royo and Quay, 1959; del Cerro *et al.,* 1985; del Cerro *et al.,* 1987), as well as to examine the development of the human central nervous system (Olson *et al.,* 1988; Henschen *et al.,* 1989; Granholm *et al.,* 1987; Granholm *et al.,* 1989). In such experiments, xenografts of the first trimester human CNS developed histologically according to their genetically determined timetable.

Retinal transplants in the anterior chamber

Retina as the transplanted tissue found its way to the anterior chamber only in 1959 when Royo and Quay implanted the fetal rat retinas into the eyes of the mothers (Royo and Quay, 1959). This light microscopic study showed that the donor tissue could survive, develop and differentiate in the in the host eye. Subsequently, the immunological closeness of donor and the host was found unnecessary for successful transplantations (Fray et al., 1983; del Cerro et al., 1987). These experiments not only strengthened the concept of the so called immune privilege of the anterior chamber (Medawar, 1948), but also provided the initial essential information regarding the development of the retinal transplants. Both neural retina and retinal pigment epithelium from early embryonic and perinatal age (embryonic day 13 to postnatal day 2) were found to grow remarkably well in the host anterior chamber. Under the microscope, the transplants were seen to be vascularized. Photoreceptors and other cells organized themselves into some degree of layering. On electron microscopy, transplanted retinal pigment epithelium showed the polarity of the host with the development of microvilli on its free surface. Investigations with horseradish peroxidase (del Cerro, 1990) demonstrated that the transplant vessels were impermeable, whereas the host vessels in the region were permeable.

Transplants to the posterior eye segment

The first transplantation of retinal neurons to the posterior segment was done by Turner and Blair (1986). They transplanted neonatal rat retina into adult rats, placing it close to a special lesion site, and studied the development with the help of light and electron microscopy. The transplants developed according to their normal timetable but formed rosettes. As will be elaborated, the work was rapidly followed by numerous studies in several different laboratories throughout the world.

Transplantation Technique

Intraocular transplant sites (anterior chamber, epiretinal, subretinal, choroidal).

Royo and Quay (1959) 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 (1983). Under appropriate anesthesia, 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. These transplants could be followed through the transparent cornea with the naked eye, or using a dissecting microscope or a direct ophthalmoscope.

In 1986, Turner and Blair transplanted embryonic rat retina to the epiretinal vitreous space of adult rats (Turner and Blair, 1986), and with some modifications, the same method was used to transplant embryonic mouse and human retinas into rats (Aramant and Turner, 1988; Aramant *et al.,* 1990b; Ehinger *et al.,* 1991a). In this method, an incision was made through the sclera, choroid and retina and the donor tissue was slowly deposited resulting in a transplant similar to the one shown in figure 1. An uncontrolled deposition some time resulted in transplants floating in the vitreous rather than being attached to the retina, especially when the method was used in rabbit.

To overcome this problem in rabbit, a different procedure was developed, using a transvitreal approach with a thin, flexible polyethylene capillary (Bergström *et al.,* 1992) which is brought into the eye with the help of a cannula mounted on a specially designed instrument. With this method, fragmented pieces of embryonic rabbit retina could successfully be placed between the photoreceptors and the pigment epithelium in adult rabbit eyes. According to clinical usage, we call these transplants subretinal, even though the space they occupy is actually intraretinal from an ontogenetical point of view. Using this method, large transplants could be obtained and the misplacement of the transplants into the vitreous became infrequent. Transplants matured, differentiated and formed approximations of the retinal layering, but anomalies like rosettes were always seen (Fig. 2). The cells survived in the host for at least 5

months, although the transplants seemed to shrink with time and to get a simplified organization.

During the surgery, 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, they survive and develop as if they were in the epi- or subretinal location (Bergström, 1994a). 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 (Bill *et al.,* 1980). Therefore, the choroid is usually not considered to be protected by the blood-retinal barrier (Törnquist *et al.,* 1990). 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 (Bergström, 1994a). This seems to cotribute to their unexpectedly good survival.

In another method a whole sheet of outer retina is inserted through an incision at the corneoscleral junction (Silverman and Hughes, 1989a). 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 and discarded 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 which had 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 (1989a) reported 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 (Silverman *et al.,* 1994). With a microsurgical manipulation the sheet can then be unscrolled and adjusted.

Extraocular transplant sites

Mostly for developmental studies, retinas or entire eyes have been implanted at various other sites in the body. Survival of the peripheral retina in the subcutaneous space of guinea pigs was studied by Wyburn and Bacsich (1952). Grafts survived in the absence of direct blood supply and were structurally described to have a ganglion cell layer, a bipolar cell layer and the plexiform layers.

The pathogenesis of anophthalmia has been studied by obtaining the optic cup from E10 fetus of a ZRDCT/an mouse strain that develops anophthalmia and transplanting it into the subcutaneous space of normal mice fetus (Salaun, 1982). 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 has given invaluable knowledge relevant to retinal

transplantation (Freed and Wyatt, 1980; McLoon and Lund, 1980a; McLoon and Lund, 1980b; McLoon and Lund, 1984; Matthews and West, 1982; McLoon *et al.,* 1982; Lund *et al.,* 1987). It has shown not only that retinas can continue to grow in CNS but also that they are also able to form functioning connections (Craner *et al.,* 1989; Klassen and Lund, 1988). This important work is further discussed in section 5.3.1.

Influence of the physical state of the donor tissue

In degenerative retinal diseases, the various disease processes may primarily or secondarily involve specific retinal layers or specific cell types. It is therefore not always necessary to aim for replacing all retinal layers. The concept of specific cell transplantation has gone a long way in other forms of neural transplantation. Replacement of dopaminergic cells for Parkinson's disease is now under clinical trial (Lindvall, 1991). It may be 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 form and type of donor tissue at the time of transplantation may have profound effects on the eventual organization (see Juliusson *et al.,* 1993) and cellular connections in the transplant.

In the past, many modalities of retina-to-retina transplantations have been performed and studied both in rats and rabbits (Turner and Blair, 1986; del Cerro *et al.,* 1985; Seiler *et al.,* 1990; Bergström *et al.,* 1992). It has been shown that tissue fragments (Turner and Blair, 1986), cell suspensions (del Cerro *et al.,* 1988b; Juliusson *et al.,* 1993), and enzymatically isolated photoreceptors (Gouras *et al.,* 1991a; Gouras *et al.,* 1991b) as well as whole sheets of photoreceptors (Silverman and Hughes, 1989a) 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 (Aramant *et al.,* 1990a; Aramant *et al.,* 1990b; Bergström *et al.,* 1992).

Transplantation of dissociated cells might perhaps allow a better integration of the transplanted cells with the host retina than tissue fragments do. Such a strategy has been tried in neural transplantations earlier (Björklund *et al.,* 1983). Based on the same procedure, del Cerro's group (del Cerro *et al.,* 1988b; del Cerro, 1990) dissociated retinal cells from post-natal day 0-2 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 (Notter *et al.,* 1988) is an interesting technique and has been used successfully in brain cell transplantation (Lopez-Lozano, 1987). Work prefatory to retinal cell

transplantation has been published (Notter *et al.,* 1988), 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.,* (1993) 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 heterogenously distributed. In tissue fragment transplants, 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 found to approximately the same extent in the two transplant types, but they 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) and S-100 staining as a marker for the Müller cells (Seiler and Turner, 1988). The amacrine cell staining was very similar in both types of transplants (Juliusson *et al.,* 1993). 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 (Gouras *et al.,* 1991a). 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.

Transplants

Retinal pigment epithelium transplants

Since 1983, when retinal pigment epithelium transplantation was first reported (Gouras *et al.,* 1983), the technique has successfully shown its potential as a possible therapeutic measure. Retinal pigment epithelium grows well in culture medium, forming sheets that can be transplanted, and very recently five patients with macular degenerations and subretinal membranes have received pigment epithelium transplants (Algvere *et al.,* 1994). The observation times are still short, and definite clinical results are not yet available.

Pigment epithelium cultures

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 (Gouras *et al.,* 1985; Edwards, 1982; Flood *et al.,* 1980; Boulton *et al.,* 1982; Hu *et al.,* 1982). 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 (Boulton *et al.,* 1982; Hu *et al.,* 1982) and

non-human (Feeney and Mixon, 1976) retinal pigment epithelium cells have been shown to maintain their ability to phagocytize photoreceptor outer segments. A lack of such activity is known to be responsible for photoreceptor degeneration in at least one strain of rats, the RCS rat (LaVail, 1983).

Cultured cells are able to take up retinol and to synthesize retinyl esters since they have enzymes for esterifying retinol (Flood *et al.,* 1983) and hydrolyzing retinyl esters (Blaner *et al.,* 1984). These enzymes are necessary for the handling of vitamin A in the visual pigment cycle. The cells also synthesize cytoskeletal proteins in culture (Haley *et al.,* 1983b; Haley *et al.,* 1983a) as well as glucosaminoglycans (Edwards, 1982). However, the cultured cells quickly loose their vitamin A stores and subsequent subcultures synthesize more of oleate than palmitate esters (Flood *et al.,* 1983). The retinal pigment epithelial cells divide in the cultures which makes them susceptible to labeling with tritiated thymidine (Gouras *et al.,* 1984; Gouras *et al.,* 1985) The 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.

The transplantation technique

Various techniques have been used to transplant retinal pigment epithelium that include open sky procedure as well as transvitreal and transchoroidal approaches (Gouras *et al.,* 1985; Lopez *et al.,* 1987; Li and Turner, 1988a).

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 since 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 which a suspension of pigment epithelium cells is injected into the subretinal space.

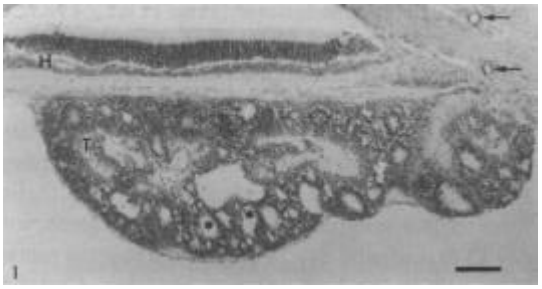

Fig. 1 Example of an epiretinal rat to rat transplant made with the fragmentation technique. The lesion site in the host retina could be identified with the help of a scleral suture. The donor tissue was taken at E15 (embryonic day 15) and the transplant was left in place for 14 days. The figure shows the host retina (H), transplant (T), sutures (arrows) and rosettes (✱). Hematoxylin and eosin staining, bar = 100µm.

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 (Gouras *et al.,* 1985; Li and Turner, 1988a).

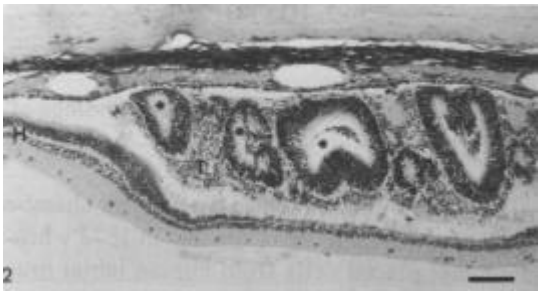

Fig. 2 Example of a subretinal rabbit to rabbit transplant. Embryonic tissue from day E15 was transplanted with the fragmentation technique and left in place for 21 days. The figure shows the host retina (H), transplant (T) and rosettes (✱). Note that the host retina appears healthy on the left side of the figure until it covers the transplant, when it appears degenerated as judged by the reduction in thickness, especially of the outer nuclear layer. Hematoxylin and eosin staining, bar = 100µm.

Retinal pigment epithelium transplants

After being transplanted, cultured retinal pigment epithelium cells undergo a 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 within two hours after the transplantation (Gouras *et al.,* 1985). 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 possess enough plasticity to be influenced by the microenvironment (Schwartz *et al.,* 1985).

Photoreceptor rescue

Retinal pigment cell transplantations have been shown to rescue the degenerating photoreceptors in the RCS (Royal College of Surgeons) strain of rats (Li and Turner, 1988a; Lopez *et al.,* 1989). RCS rats were first described in 1938 (Bourne *et al.,* 1938), and their photoreceptors undergo a rapid photoreceptor degeneration beginning 3 weeks after birth and spreading centripetally to the peripheral retina within 2 months (Dowling and Sidman, 1962). 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 (Hess *et al.,* 1985; Hess *et al.,* 1982; Essner *et al.,* 1979; Caldwell and McLaughlin, 1983; LaVail, 1981; Yamaguchi *et al.,* 1991), but in terms of photoreceptor degeneration, the retinal

pigment epithelium is the most important site affected by the mutation (Young and Bok, 1969; Mullen and LaVail, 1976). Because of the loss of phagocytosis, outer segment debris accumulates at the pigment epithelium cells (Dowling and Sidman, 1962; LaVail, 1981) and eventually the photoreceptors disappear. Transplanted healthy retinal pigment epithelium restores their phagocytic function soon after transplantation, rescuing the photoreceptors. Since the rescuing effect is not limited to the transplantation site, it is possible that the effect is mediated by some diffusible factor, possibly bFGF, because injections of this substance have been shown also to rescue the photoreceptors (Faktorovich *et al.,* 1990).

Sham operations (Silverman and Hughes, 1990) have also been shown to rescue the photoreceptors, possibly because of some factors released during the surgery, but these effects last for a much shorter period as compared to rescuing effects up to one year by the transplants (LaVail *et al.,* 1992a; Gouras *et al.,* 1989; Li *et al.,* 1990; Li and Turner, 1991). 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 and transduction cascade (Gaur *et al.,* 1992). Further, with immunohistochemical methods it has been shown that they contain membrane bound Na^+,K^+-ATPase and opsin at their normal sites (Sheedlo *et al.,* 1989a; Li *et al.,* 1990). These substances are essential for the normal function of the photoreceptors, and the results indicate that rescued cells continue to synthesize them. 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. Alternatively, the effect is mediated by some diffusible factor.

Retinal pigment epithelium transplantation also prevents neovascularization in the RCS rats (Seaton and Turner, 1992). Transplantation of retinal pigment epithelium in rat eyes have delayed age related changes in various retinal layers (Yamaguchi *et al.,* 1993).

There is no human hereditary tapetoretinal degeneration that has been shown to reside primarily in the retinal pigment epithelium, but neither has it been excluded that some forms of retinitis pigmentosa, such as perhaps choroideremia, Leber's amaurosis, macular degenerations, dominant drusen or gyrate atrophy could be diseases of the retinal pigment epithelium. Any such disease may be amenable to transplantation therapy. However, besides the need for further improving the surgical technique, attention should also be paid to potential problems like transplant induced proliferative vitreoretinopathy. It should be noted that proliferating retinal pigment epithelium is at least partly responsible for certain vitreoretinopathies (Machemer and Laqua, 1975; Ryan, 1985), especially when the neural retina is detached (Anderson *et al.,* 1981) or like in tumors (Tso and Albert, 1972; Wallow and Tso, 1973a) or after trauma (Wallow and Tso, 1973b; Marshall and Mellerio, 1971; Tso, 1973; Laqua and Machemer, 1976).

Neural transplants

Survival

When grafted into eyes, immature retinas survive and continue to mature. 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. Parameters used to asses the survival of the cells are inexact, and applying more precise quantitative methods have met with difficulties like 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 (Brundin *et al.,* 1987).

Various factors influencing the survival of the retinal transplants are further discussed below.

Donor age influences

Evidence emerging generally in the field of 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: Brundin *et al.,* 1988a; human: Brundin *et al.,* 1986). Human grafts taken from substantia nigra at 9 weeks of gestation contain dopaminergic cells that survive transplantation in immunosuppressed rats, whereas rats grafted with human nigral tissue from 11 to 19 weeks old embryos contain few if any dopaminergic cells (Brundin *et al.,* 1986). 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. (1988) 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 (Braekevelt and Hollenberg, 1974) which may be responsible for the drop in the survival rate. Grafts with postnatal day 21 tissue degenerated within 1-2 days after transplantation (Aramant *et al.,* 1988). The mechanism behind these 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 (Ffrench-Constant *et al.,* 1988) or some other factor.

Transplants of fetal E60 (embryonic day 60) and E90 monkey retinas have shown good survival, growth and differentiation in rat eyes (del Cerro *et al.,* 1992). In these experiments, the host rats were immunosuppressed.

Xenografts versus homografts

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, and it has for a long time been known that many tissues that will not survive transplantation in other parts of the body will readily grow in the eye, protected by its barriers. As early as 1914, it was found that mucous carcinoma grafts survived in brain but not in subcutaneous tissue (Ebeling, 1914), and many subsequent studies on the development of human embryonic brain tissue have been conducted in different xenograft models (Brundin *et al.,* 1988b; Clarke *et al.,* 1988; Kamo *et al.,* 1987; Nilsson *et al.,* 1988; Redmond, Jr. *et al.,* 1988; Granholm *et al.,* 1989). Similar xenografting studies have also been done with retina to retina grafting.

Embryonic retina of the mouse has been transplanted to the adult rat retina (Aramant and Turner, 1988), sheets of adult human photoreceptor cells have been transplanted into normal and light damaged rats (Silverman and Hughes, 1989b), and fetal monkey retina has been transplanted into adult rats to study the differentiation of cones (del Cerro *et al.,* 1992). 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 (Pollack *et al.,* 1992; Lund *et al.,* 1988; Sefton and Lund, 1988; Klassen and Lund, 1988). 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 postconceptional age of 3-12 weeks and xenotransplanted to immunosuppressed rats (Ehinger *et al.,* 1991a).

Two of the most important observations that have been made in the xenograft studies are that such grafts consistently have been found to develop according to their intrinsic time table, and that the properties of the graft are determined by the donor tissue and not the host environment. Thus, xenografting can serve as a model for studying the transplantation of the human retina for clinical purposes (Aramant *et al.,* 1990b; Ehinger *et al.,* 1991a). It can also be concluded from these studies that the human embryonic and fetal retina can be used as a donor material.

Influence of site

Comparisons of the number of degenerating cells and vacuoles in transplants placed in the epiretinal and the subretinal space show that the subretinal space serves as a better environment for the transplants than the epiretinal site. This is particularly true when the quality of the donor tissue is less than optimal, either in terms of donor age, cryopreservation or dissociation of cells (Aramant and Turner, 1988; Aramant and Seiler, 1991; Juliusson *et al.,* 1993). Currently, the reason for this can only be guessed. Transplants in the subretinal space are likely to have better access to nourishment from the choroid than epiretinal

transplants, at least at the non-vascularized rabbit retina. The limited space at the subretinal site may also have a mechanical effect, holding the cells together. However, it also seems that handling and architectural disturbance of the donor tissue in the process of retinal transplantation plays a crucial role in the eventual histogenesis of the graft. Whole pieces of rabbit embryonic retinas placed in the epiretinal space develop very good lamination but lag behind in maturation as compared to subretinal transplants (Sharma *et al.*, manuscript in preparation).

Influence of cryopreservation

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 timely availability of fresh donor tissue. Aramant *et al.* (1991) 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% dimethylsulfoxide. 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 (1987) and Aramant *et al.* (1988), 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 normal and tends to fragment (Jensen *et al.,* 1984; Sörensen *et al.,* 1986; Collier *et al.,* 1987). There are several reasons for this. Freezing and thawing may change the membrane proteins and thus also the adhesion between the cells. DMSO 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 . However, it survives better in the subretinal space than epiretinally .

Transplantation into damaged retinas

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 (Silverman and Hughes, 1989a) and suspended neuroretinal cells (del Cerro *et al.,* 1988a) 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 (del Cerro *et al.,* 1988a) and show immunoreactivity to opsin (Silverman and Hughes, 1989a). 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 an *rd* mouse differentiated into photoreceptors and expressed S-antigen. The mutant 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 (Jiang and del Cerro, 1992). This could also mean that the site of affection in *rd* mutation may not be the rod cells alone.

Unlike rats and mice (LaVail and Sidman, 1974; LaVail, 1981) 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 (Noell, 1955; Noell, 1958; Bresnick, 1970). 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 (Bergström, 1994b).

Structure

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 is from the donor tissue or the host. To substantiate claims in transplantation research, it is important to mark the cells so that their origin can be defined. There has been a steady increase in the number of tracers that have become available for neurobiological research. They belong to diverse groups and have different modes of action. Certain of them are injected at a particular site along a neuron pathway, and are then transported in the nerve fibers in the anterograde or retrograde direction. Some of these tracers have also been administered by incubating the donor tissue with them. None of the tracers that currently are available serves the ideal needs of transplantation research, but some of them can be used.

Fluoro-Gold, a substituted stilbene, was initially introduced as a retrograde neuron tracer (Bentivoglio *et al.,* 1980; Schmued and Fallon, 1986) and has found widespread use in neurobiological research (del Cerro *et al.,* 1988b; Snyder-Keller, 1988). This cytoplasmic stain has been used in retinal cell suspensions of postnatal day 1-2 rats in a concentration of 0.04% for 30 minutes at 4°C (Schmued and Fallon, 1986). Between 90 to 95 % of the dissociated cells take it up with 80 and 90% viability (del Cerro *et al.,* 1988b). Cryostat or plastic embedded sections of labeled transplanted cells can be studied under the fluorescence microscope. Intravitreal injections of 2 µl 0.04% Fluoro-Gold has also been shown to stain the neural retina of rats in vivo (del Cerro *et al.,* 1990a), producing electron dense lysosomal and lamellar bodies. Similar results have been observed in neurons retrogradely labeled with Fluoro-Gold (Schmued *et al.,* 1989). Another stain, Fast Blue, has been used successfully to mark suspensions of CNS cells for neural transplantation (McConnell, 1985) and retinal cells (del Cerro *et al.,* 1988b). Both these substances mark the cells for as long as up to 100 days, have no toxic effect on the cells, and do not effect

eventual morphogenesis of the transplants (del Cerro *et al.,* 1988b). These substances work well with suspended cells, but when pieces of retinal tissue are incubated in the stain, the margins of the tissue pieces become more intensely stained than the center because of the low diffusibility of the stain. Another drawback with these stains is the risk that if the stain leaks out or is released by the donor cells (as may happen if the donor cells degenerate), the stain may be incorporated by the host cells.

Another 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. This method has some advantages. If the donor cells degenerate and release the isotope, it will have to be incorporated in dividing cells to mimic donor tissue. Cells in an adult retina are not dividing, so this possibility is remote. Dividing macrophages that may take up [^{3}H]-thymidine are usually easily distinguished.

Autoradiography with [^{3}H]-thymidine will obviously mark only dividing cells like the cells in the retina of new-born rats. They divide up to about 1 week after birth (Young, 1985). Being a nuclear marker, [^{3}H]-thymidine does not mark structures like the photoreceptor outer segments or the synaptic structures, which makes it difficult to identify them as belonging to a labeled cell. A more recent approach to this problem has come from the use of certain transgenic strains of mice (Zack *et al.,* 1991; Gouras *et al.,* 1992; Gouras *et al.,* 1991b). 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, β-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. The reactivity decreased over a period of 1 month, possibly due to reduced rhodopsin expression in the rudimentary outer segments of the transplanted cells.

Recently synaptic connections between transplanted photoreceptor cells and the bipolar cells of the host retina have been reported using *lacZ* gene expressed cells and Bluo-gal reaction (Silverman *et al.,* 1994).

Rosettes

Depending upon its age, the donor tissue contains mostly or exclusively neuroblastic cells. When this 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. However, the tissueis often organized around a lumen surrounded by an outer limiting membrane, photoreceptors and other retinal layers, thus forming small spherical structures called rosettes (Fig. 1 and 2). Rosettes have also been reported in other types of transplants like cell suspensions (del Cerro *et al.,* 1989; Gouras *et al.,* 1992; Juliusson *et al.,* 1993) and subretinal transplants of sheets of outer retina (Silverman and Hughes, 1989a).

Rosette formation is nothing specific to retinal transplantation. They have also been described in a variety of conditions such as trauma (Lahav *et al.,* 1975)

and in retinitis pigmentosa (Milam and Jacobson, 1990). Retinal explants cultured in vivo also develop rosettes (Caffé and Sanyal, 1991). 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 (Sahel *et al.,* 1994). Even though the Flexner-Wintersteiner rosettes have a structure which 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 (Sahel *et al.,* 1994). 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 (Ts'o *et al.,* 1970). Nevertheless, the term rosettes is widely used in connection with retinal cell transplantation, and we will continue to use it.

The rosettes develop early in the transplants. We have observed immature cells arranged in rosettes without any central lumen already two days after the transplantation in the rabbits (Sharma *et al.*, manuscript in preparation). The reason for the formation of rosettes in the retinal transplants is not clear. It is possible that mechanical factors (Caffé and Sanyal, 1991) and separation of the retinal cells from their native neighbors in the process of transplantation are among the reasons. Transplantation of flat pieces of rabbit retina has resulted in a diminution of the number of rosettes when the transplants have been deposited in the vitreous, close to the host retina (Sharma *et al.,* 1995). In the subretinal space these transplants form arcuate arrays, which appear different from rosettes on vimentin staining. (Sharma *et al.*, manuscript in preparation). Since the growth of a retinal transplant is a continuation of its regular development, an initial reaggregation of the cells is likely to play an important role in the eventual histogenesis. Therefore, in order to keep the transplants free of rosettes as much as possible, it is important to disturb the donor tissue only minimally at the transplantation.

The extent of rosette formation has been different in various studies. In some models it is a prominent feature (McLoon *et al.,* 1982; del Cerro *et al.,* 1989) and in others it is less and sometimes totally absent, particularly in cell suspension transplants (Gouras *et al.,* 1992; Juliusson *et al.,* 1993). In the latter case, the distorted and small photoreceptor outer segments suggest the transplants remained relatively undeveloped (del Cerro *et al.,* 1989; Gouras *et al.,* 1991a; Gouras *et al.,* 1991b; Du *et al.,* 1992). Further, there was hardly any organization at all, which most likely was the reason why no rosettes were seen.

The proximity of the photoreceptor cells to the retinal pigment epithelium is another factor that may be important for the rosette formation. In tissue cultures of the chick retina, retinal pigment epithelium was found to be important not only for the survival of the photoreceptors but also for the development of the retinal structure (Liu *et al.,* 1988). Co-culturing with retinal pigment epithelium induces normal polarity in the retina whereas the absence leads to rosette formation (Layer and Willbold, 1989). Recently, a similar effect has been shown for

pigment epithelium cells co-transplanted with neuro-retinal cells in rabbits, where transplants containing both types of cells developed a better morphology and survived for a longer time compared to transplants containing only neural cells (Seiler *et al.,* 1995).

Formation of rosettes in the transplants will no doubt interfere with the resolution of the image, but not necessarily with the perception or even localization of light. Retinas with rosettes have been shown to respond to light in vitro (Adolph *et al.,* 1992, 1995) and to send the signals to the higher centers (Klassen and Lund, 1987; Sefton *et al.,* 1989).

Electron microscopy

Ehinger *et al.* (1991a) 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 later age was selected to allow near full term maturation of the transplanted cells, which develop according to their intrinsic timetable. At 40-41 weeks, 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 (Gouras *et al.,* 1990). Zonulae adherentes were seen in the transplants soon after the transplantation (Bergström *et al.,* 1994b).

Horizontal cell perikarya are not easily distinguishable in normal or in transplanted retina because they lack distinguishing features. However, on the basis of their location and lack of postsynaptic membrane specializations 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 (Ehinger *et al.,* 1991a; Ehinger *et al.,* 1991b) and in rabbit and rat homografts (Bergström *et al.,* 1994b; Zucker *et al.,* 1994).

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 (Turner and Blair, 1986; Ehinger *et al.,* 1991a; Ehinger *et al.,* 1991b; Bergström *et al.,* 1994b; Zucker *et al.,* 1994; see also Fig. 3).

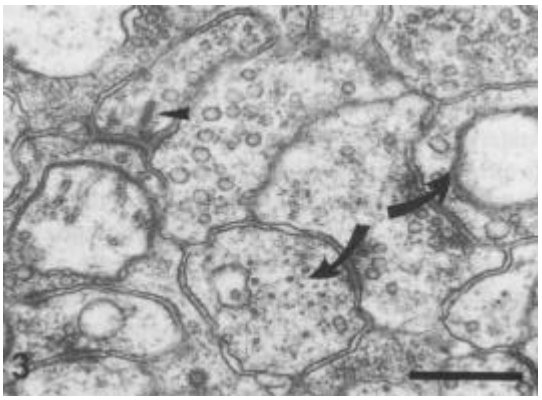

Fig. 3 Electron micrograph of rabbit to rabbit transplant. An embryonic day 15 retina was placed in the subretinal space and allowed to grow for 56 days. The micrograph shows a bipolar cell with a ribbon synapse (arrow head) and conventional synapses between amacrine cells (curved arrows). Bar = 0.5µm.

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 (del Cerro, 1990; Turner and Blair, 1986; Ehinger *et al.,* 1991a; Ehinger *et al.,* 1991b; Bergström *et al.,* 1994b; Zucker *et al.,* 1994; see also Fig. 3). 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 (Ehinger *et al.,* 1991a).

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 (Aramant *et al.,* 1990a; Bergström *et al.,* 1994b; Zucker *et al.,* 1994). In the scanning electron microscope, Turner and Blair (1986) 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, and 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.

Structure of outer segments

The development of photoreceptor outer segment in retinal cell transplants has been described at the electron microscopic level in human (Ehinger *et al.,* 1991a) and monkey (del Cerro *et al.,* 1992) embryonic xenografts, and in mouse to mouse, rabbit to rabbit, and rat to rat homografts (Gouras *et al.,* 1992; Bergström *et al.,* 1994b; Zucker *et al.,* 1994). 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 (Fig. 4) whereas in cell suspension transplantation techniques outer segments are reported to be small and distorted or absent (del Cerro *et al.,* 1989; Gouras *et al.,* 1991a; Gouras *et al.,* 1991b; Du *et al.,* 1992; Gouras *et al.,* 1992; Juliusson

et al., 1993). These results suggest that photoreceptors depend on each other for their proper development. Consequences of this are further discussed in section 4.2.4. Distorted photoreceptors are still capable of phototransduction (Pu and Masland, 1984) as has been shown in the retinas transplanted to the optic tectum (Klassen and Lund, 1987; Klassen and Lund, 1990).

Many authors believe that lack of apposition of photoreceptors to the pigment epithelium results in maldevelopment of outer segments (see e.g. LaVail and Hild, 1971). Photoreceptor layers transplanted along with the retinal pigment epithelium (Seiler *et al.,* 1995) and studies of retinal reattachments (Anderson *et al.,* 1983) 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 (Perry and Lund, 1989; Banerjee and Lund, 1992). The presence of microglial cells has not been confirmed in the retinal transplants in the eye, but other types of phagocytic cells have been observed in the center of the rosettes (Gouras *et al.,* 1992).

Synapses

Short term grafts show only few or poorly developed synapses whereas grafts that have been allowed to grow long enough for the development of the synapses show all the types seen in adult retina (Ehinger *et al.,* 1991a; Bergström *et al.,* 1994b; Zucker *et al.,* 1994). The appearance of the synaptic connections in the developing graft clearly shows that the graft is growing in its new environment. The number of synapses may be less than normal (Ehinger *et al.,* 1991a), but 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 (Ehinger *et al.,* 1991a; Ehinger *et al.,* 1991b; Gouras *et al.,* 1992; Bergström *et al.,* 1994b). 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 (Ehinger *et al.,* 1992). Occasionally, synaptic ribbons were observed with no direct association with any membrane or postsynaptic process (Ehinger *et al.,* 1991a; Bergström *et al.,* 1994b).

Regions corresponding to the inner plexiform layer consistently showed a high density of synapses. Most terminals were filled with conventional small (350-450 nm) synaptic vesicles (Fig. 3), but occasionally large, (>800 nm) dense-cored vesicles were seen (Ehinger *et al.,* 1991a; Bergström *et al.,* 1994b). Conventional synapses, presumed to be made by amacrine cells, were common and were found to make contacts with bipolar cell processes, other amacrine processes and some processes not definitely identified. Infrequently, amacrine cells were been seen to make synapses with small spines or thin intervaricose processes (Ehinger *et al.,* 1991a).

In the regions of the transplant that corresponded to the inner plexiform layer, processes appeared that contained numerous synaptic vesicles and ribbon synapses (Fig. 3). 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 (Ehinger *et al.,* 1992). Some few gap junctions between amacrine cell processes were seen in rat to rat (Ehinger *et al.,* 1992; Zucker *et al.,* 1994) and rabbit to rabbit transplants (Bergström *et al.,* 1994b). 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 (Bergström *et al.,* 1994b; Zucker *et al.,* 1994). 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 chamber (del Cerro *et al.,* 1985; Matthews and West, 1982). When normal donor tissue was transplanted to an eye that has a dystrophic retina, the transplant showed more abundant synapses than the host retina (Gouras *et al.,* 1991a). A similar result has 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 (Mosinger Ogilvie *et al.,* 1994).

Growth and differentiation: Cell types.

At the time of transplantation, the fetal retinal tissue contains undifferentiated neuroblastic cells and it often becomes fragmented, and during development the neuroblastic cells reorient themselves in rosettes. 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 recently been discovered that short wavelength sensitive cones dominate (Szél *et al.,* 1994). It has been proposed that cones in non-primate mammals may during their development start as short wavelength sensitive cells and then at some later point switch to middle wavelength photopigments (Szél *et al.,* 1994). The observations in transplants suggest that this switch has been disturbed, making it a possible tool for analyzing factors that might influence the change.

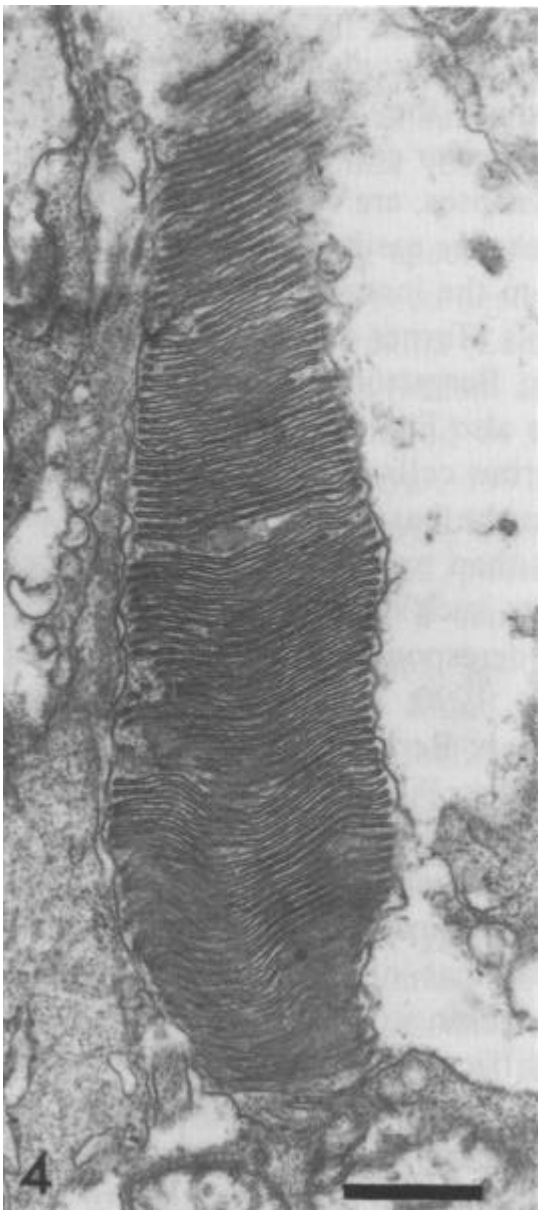

Fig. 4 Electron micrograph showing a relatively well developed photoreceptor outer segment in the same rabbit to rabbit transplant as in Fig. 3. Bar = 0.5µm.

Depending upon their degree of maturation, rods of human, rat or rabbit retinal grafts stain with different intensities with antisera against S antigen, rhodopsin or rod alpha transducin. The staining intensity of the graft photoreceptors varies in different parts of the transplant, and tends to be stronger and more often prevalent in the rosettes closest to the host retina (Aramant *et al.,* 1990a; Seiler and Aramant, 1994). 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 conception. The S antigen and other photoreceptor markers like alpha transducin and rhodopsin express themselves in the photoreceptors at 20 weeks post conception (Aramant *et al.,* 1990a; Seiler and Aramant, 1994).

Some cones stain with neuron specific enolase (NSE) and synaptophysin (SYN), one or two weeks earlier (Seiler and Aramant, 1994). Areas of different degrees of maturation often appear in one and the same transplant, possibly because central and peripheral regions of the donor retina are mixed together during transplantation, and they may develop at different rates. In the normal development, the central retina precedes the peripheral parts by about 6 weeks in humans (Johnson *et al.,* 1985; Provis *et al.,* 1985).

In the normal retina, the interphotoreceptor matrix (IPM) links the photoreceptors to the pigment epithelium and serves purposes like molecule transport (Liou *et al.,* 1982), photoreceptor isolation (Mieziewska *et al.,* 1991), retinal adhesion (Yao *et al.,* 1990), and regulation of phagocytosis of photoreceptor outer segments by the retinal pigment epithelium (Adler, 1989). The interphotoreceptor matrix comprises insoluble and soluble components like proteins, glycoproteins, acid hydrolases, glucosaminoglycans and proteoglycans (Adler and Klucznik, 1982; Adler and Martin, 1983). 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 normal distribution both in the host and in the corresponding structures in the transplant rosettes (Juliusson *et al.,* 1994). However, 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 linkage and binds to interphotoreceptor matrix surrounding the cones and not the rods (Juliusson *et al.,* 1994). Further, 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. IRBP, synthesized by the photoreceptor cells, is a protein found in abundance in the interphotoreceptor matrix (Pfeffer *et al.,* 1983). It is a glycoprotein with a molecular weight of approximately 140 kDa (Chader, 1989), 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 normal (Juliusson *et al.,* 1994).

In rabbits, horizontal cells processes in the outer plexiform layer are immunoreactive to 160 and 200 kD neurofilaments and vimentin, whereas the HPC-1 antibody stains horizontal cells and the MAP 1A antibody stains processes and soma of horizontal cells. The HPC-1 antibody is known to label a special synapse related protein, syntaxin (Inoue *et al.,* 1992; Inoue and Akagawa, 1993). In rats, horizontal cells could be seen with a 160 kD neurofilament antibody at two but not one week after the transplantation (Aramant *et al.,* 1990a). This may mean a delay of one week in the maturation of these cells as compared to their normal development (Shaw and Weber, 1983).

The development of different other retinal neurons in the transplants has been studied by immunocytochemical marking of the specific neurotransmitters or neuron specific substances. The HPC-1 antibody marks a special protein, syntaxin, predominantly present in amacrine cells (Barnstable *et al.,* 1985; Inoue

and Akagawa, 1993; Inoue *et al.,* 1992), and appropriate numbers of such cells have been seen in transplants. Amacrine cells can also be identified according to the different type of neurotransmitters they contain (Tumosa *et al.,* 1984; Eckenstein *et al.,* 1981; Ehinger and Dowling, 1987; Nguyen-Legros *et al.,* 1983; Sagar *et al.,* 1986; Sagar, 1987; Vaughn *et al.,* 1981; Versaux-Botteri *et al.,* 1986; Voigt, 1986), and amacrine cells can be detected with the appropriate antibodies, as will be further elaborated below. Rarely, amacrine cells in the inner nuclear and the ganglion cell layers have been fond to be immunoreactive to somatostatin (Ferriero and Sagar, 1987). Antibodies to the medium weight (160 kDa) neurofilament subunit, a neuron specific substance marks ganglion cells and horizontal cells (Barnstable, 1985; Shaw and Weber, 1983; Sloan and Stevenson, 1987).

In rats, the first differentiating amacrine cell appears in the graft corresponding to the first postnatal day as seen by immunoreactivity to choline acetylase (ChAT) and tyrosine hydroxylase (TH) (Aramant *et al.,* 1990a). In the normal embryonic rat retina, measurable levels of ChAT have been found (Puro *et al.,* 1982), but possibly because of difference in sensitivity of the methods, Mitrofanis *et al.* (1988) 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 (Mitrofanis *et al.,* 1988; Nguyen-Legros *et al.,* 1983). HPC-1 (syntaxin) and glutamic acid decarboxylase (GAD) staining showed that the inner plexiform layer of the graft starts differentiating at 8 post natal day. Further, at this age, the outer plexiform layer of the graft contained neurofilament immunoreactive horizontal cells (Aramant *et al.,* 1990a). In grafts with an age corresponding to 4 and 6 post natal weeks, the staining intensity was the same as in the host cells. Immunoreactive fibers to somatostatin-28 were found mostly at the border of inner nuclear layer and inner plexiform layer in the host retina, but in the retinal fragment transplants, these fibers were randomly distributed in the inner plexiform layer and the inner nuclear layer revealing a abnormal lamination pattern. Marking of human retinal transplants with 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 (Seiler *et al.,* 1991).

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 (Barnstable and Dräger, 1984; Perry *et al.,* 1984), against the micro tubular associated protein (Okabe *et al.,* 1989), against the 200 kD neurofilament (Shaw and Weber, 1983) and against neuron specific enolase (NSE) (Wilhelm *et al.,* 1992) 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 post natal day 1, but no cell bodies were stained (Aramant *et al.,* 1991). Some small cells in the ganglion cell layer of rabbit grafts have been found to be immunoreactive to the antibody against MAP 1A (Seiler *et al.,* 1990). It is possible that small ganglion cells may develop in the graft but not contain immunohistochemically detectable amounts of markers. On the other hand, it has been shown that the

retinal ganglion cells for at least a limited time depend for their development on target derived substances, both *in vivo* (McLoon and Lund, 1984; Perry *et al.,* 1985; Sefton *et al.,* 1987; Sefton and Lund, 1988) and *in vitro* (Armson *et al.,* 1987; Johnson *et al.,* 1986; McCaffery *et al.,* 1982; Nurcombe and Bennett, 1981; Turner, 1985a; Turner, 1985b). When grafted to ectopic sites in the brain not having the target tissue, embryonic retinal tissue does not contain ganglion cells at 5 or more weeks after grafting (Matthews and West, 1982; McLoon and Lund, 1984). It therefore remains unsettled to what extent ganglion cells develop and survive in retinal cell transplants.

In order to study their development, glial cells in transplants have been marked with antibodies against the S-100 antigen (a calcium binding protein), and glial fibrillary acidic protein (GFAP) (Seiler and Turner, 1988; Seiler *et al.,* 1990). In such 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 transplant Müller cells appeared to be normal in the outer nuclear layer and at the outer limiting membrane, but they did not develop end feet at the inner limiting membrane at the vitreal surface. These findings have been confirmed in human xenografts. Vimentin immunoreactivity appeared early in transplants (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 within the graft. CRALBP immunoreactivity begun to appear at 19-20 weeks and was intense at 25 weeks, mostly in the regions near the host (Seiler and Aramant, 1994).

Neurotrophic factors

A number of agents (neurotrophic factors, also known as neurotrophins, cytokines or growth factors) are known to participate in nerve cell differentiation and growth (Korsching, 1993). Some of them have also been found to play a role in the maintenance and survival of certain nerve cell populations even in the mature CNS (Phillips *et al.,* 1990). In the retina, particular cell types have been shown to be responsive to such agents exhibiting neuroprotective functions. 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 transsection (Mansour-Robaey *et al.,* 1994; Mey and Thanos, 1993) and to significantly reduce degeneration of rat photoreceptors following exposure to constant illumination (LaVail *et al.,* 1992b). More recently, genetically engineered astrocytes that synthesize and secrete bioactive BDNF have been shown to promote ganglion cell survival in cell cultures (Castillo, Jr. *et al.,* 1994), but also in transplants to the brain (Panni *et al.,* 1994). These observations suggest that growth factors and cytokines might be used to improve retinal cell survival and differentiation in transplants.

The *rd* mouse is a model for retinitis pigmentosa, with a genetic defect in the rod cGMP phosphodiesterase. This enzyme is expressed in the rod cells (Bowes *et al.,* 1990), leading to a rapid loss of these cells. Even though cones do not express this defect, in due course, the *rd* mouse loses most of its cone cells

also, implying that cones are dependent on rods or an intact retina for their survival. Silverman (1994) has 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. Such a rescuing effect should be mediated by diffusible factors and should not require synaptic contacts between the transplant and the host.

Function in retinal cell transplants

In order to restore vision by retinal transplants, it will be essential that they develop the fundamental retinal cell types and that they contain their functionally important components like neurotransmitters and other specific substances. It is also important 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 (Aramant *et al.,* 1990a; Aramant *et al.,* 1990b; Silverman and Hughes, 1989a; Bergström *et al.,* 1994a; Seiler and Aramant, 1994). In this respect, the photoreceptors thus appear to be reasonably normal. However, IRBP is known to be synthesized by the photoreceptors (Fong *et al.,* 1984; van Veen *et al.,* 1986), and the failure of this substance to appear in retinal cell transplants (Juliusson *et al.,* 1994) 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 absence of IRBP in the transplants is caused by the lack of contacts between the rosette photoreceptor cells and the retinal pigment epithelium.

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 (Aramant *et al.,* 1990a). Recently we have seen nitric oxide synthase immunoreactive cells in the rabbit retinal transplants (Sharma *et al*., unpublished). The fact that horizontal cells, bipolar cells as well as various types of amacrine cells and their synaptic connections are all present, is an important prerequisite for some function(s) in the transplants and suggests 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 (Adolph *et al.,* 1995). 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 what can be found in the inner and outer plexiform layers of the normal retina (Dowling, 1987). In retinal transplants in the CNS of unilaterally enucleated host, cells with 'on' and with 'on-off' responses have also been found (Simons and Lund, 1985). These light evoked responses were similar to the normal ERG. In an abstract, retinal transplants in the anterior chamber were reported to transduce light and to generate electroretinographic responses (Collier *et al.,* 1989).

Cell death in transplants

All the efforts in retinal transplantation have so far been concentrated on studying the development of the cells, and there is therefore almost no knowledge regarding the death of these 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 (Cunningham, 1982; Lund, 1972; Potts *et al.,* 1982). 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 speaking, ganglion cells are the first cells to differentiate (Morest, 1970) and to die (Potts *et al.,* 1982; Cunningham, 1982), 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 ganglion cells have not been found in transplants. Cell death has been reported early after the transplantation and to sharply decline after the first weeks of transplantation (del Cerro, 1990; Bergström *et al.,* 1992). After one month the transplants seem stabilized with some amount of debris. The causes of this early cell death in transplants are not known, but 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 (Clarke, 1990; Ellis *et al.,* 1991; Oppenheim, 1991) 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 the target cells. During the critical period of synapses formation, neurotrophins like NGF and BDNF are essential for neuron survival (Levi-Montalcini, 1987; Barde *et al.,* 1982; Turner *et al.,* 1982; Hofer and Barde, 1988). Later, the same neurons often become independent of it. Recent developments have shown that neuronal damage may lead to the molecular and cellular events which culminate either in death or regeneration of the cell. In mice, the *fos* gene has been found to be associated with neuronal death and the *jun* gene with regeneration (Jenkins *et al.,* 1993b; Jenkins *et al.,* 1993a). 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 (Gall and Isackson, 1989; Dugich-Djordjevic *et al.,* 1992b; Dugich-Djordjevic *et al.,* 1992a; Zafra and Gimenez, 1989; Lindholm *et al.,* 1990; Ernfors *et al.,* 1989; Saika *et*

al., 1993; Saika *et al.,* 1991) suggesting that the genetic programming used during development may be re-utilized in adults.

HOST

Connections

Axon growth. Optic nerve bridges

The capacity of neural retina to regenerate from retinal pigment epithelium persists throughout life in some salamanders and until metamorphosis in some tailless amphibians (Reh and Nagy, 1987). In mammalian vertebrates, this can happen only in early embryonic life, with the exception of regeneration of photoreceptor outer segments under certain pathological conditions (Blight and Hart, 1977).

Will the ganglion cells in the retinal transplants in the eye, far away from their target, be able to send out axons to form meaningful connections?

It is known that the mammalian visual system does not regenerate even though abortive sprouting occurs following injury to the optic nerve (Cajal, 1928; Goldberg and Frank, 1980; Richardson *et al.,* 1982; Allcutt *et al.,* 1984). It is surprising to find that after optic nerve transsection, which leads to the eventual degeneration of all the optic nerve axons, the loss of retinal ganglion cells is incomplete. Studies on optic nerve lesions have reported the survival of about 60% of the neurons of the ganglion cell layer in some species (review: Misantone *et al.,* 1984). This can only partially be explained by the presence of displaced amacrine cells in the ganglion cell layer that may account of up to 50% of the cells in that layer in some species.

Taking the lead from abortive sprouting, many authors studied the regeneration of the optic nerve (Allcutt *et al.,* 1984; Carter *et al.,* 1994; Aguayo *et al.,* 1981; David and Aguayo, 1981; Berry *et al.,* 1988; Politis and Spencer, 1986; Stevenson, 1985). Further, peripheral nerve implants were found to have possible trophic interactions which could enhance ganglion cell survival and axon fasciculation. Following observations in the spinal cord (David and Aguayo, 1981), autologous sciatic nerve grafts were used as bridges for the growth of ganglion cell axons (So and Aguayo, 1985). 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 (Keirstead *et al.,* 1985). This and a subsequent study (Berry *et al.,* 1988) support the finding that ganglion cells have an inherent potential to regenerate if transsected intraretinally.

The extraretinal regenerating potential of ganglion cells has been reviewed earlier by Cajal (1928). More recently, Politis and Spencer (1986) and Berry (1988) have 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 (Stevenson, 1985).

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 have been found to be greater than their normal projections (So and Aguayo, 1985). However, their entry into the CNS has remained limited to a few millimeters (David and Aguayo, 1981; Aguayo *et al.,* 1981). Some studies have indicated that few regenerating axons reach the target tissue through the bridges and form cone-like dilatations suggesting synaptogenesis (Plouet *et al.,* 1986). 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.

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.* (1979) and subsequently by many other investigators.

Retinas transplanted close to the target tissue (Matthews and West, 1982; McLoon and Lund, 1983; Matthews and West, 1982; McLoon and Lund, 1984) in the brain show highly specific axonal outgrowth to a number of subcortical visual centers (Radel *et al.,* 1990; Hankin and Lund, 1990) like the superior colliculus, the pretectal region, the accessory optic nucleus (McLoon and Lund, 1980a; Radel *et al.,* 1990), 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 (Galli *et al.,* 1989).

The extent of the neuritic 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 (Panni *et al.,* 1994). In older grafts, mature ganglion cells are more prone to be damaged by the axotomy caused by the grafting and, consequently, they project poorly (McLoon and Lund, 1980b).

The extent of the neurite outgrowth also depends upon the available synaptic space (McLoon and Lund, 1980a). 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 (McLoon and Lund, 1980b). 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 (McLoon and Lund, 1983). On the other hand, in the neonatal host, the projections were seen to terminate over the whole extent of the superior colliculus. Projections from the graft were not affected by the dissociation and reaggregation of donor tissue prior to grafting (McLoon *et al.,* 1982).

Freed and Wyatt (1980) 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' were thought indirectly to suggest connections between host and the 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 (Simons and Lund, 1985), closely conforming with observations on normal rodent colliculus (Fukuda and Iwama, 1978). Responses could also be recorded from cortical area 18a, and they were most likely mediated by circuitry involving intermediate relays (Craner *et al.,* 1989). Stimulation of the transplant activates regions several intermediate synapses away as seen from results with *c-fos* activation (Craner *et al.,* 1992).

Transplant mediated pupillary reflexes

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 (Klassen and Lund, 1987; Klassen and Lund, 1988). The transplant mediated pupillary responses could be elicited also from long term transplants where the photoreceptor layer was no longer evident, raising the concern that these reflexes may be an indicator of minimal functional capability.

More recently, Silverman *et al.* (1992) have summarily reported on 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 not due to the residual visual function that can be seen in the animal models of retinal degeneration (Nagy and Misanin, 1970; Karli, 1954; LaVail *et al.,* 1974; Dräger and Hubel, 1978).

Psychophysical results

To address the question of whether the animals can see with the retinal transplants, the effect of retinal illumination on the suppression response (Coffey *et al.,* 1989), alerting behavior (Lund *et al.,* 1991) and photophobic behavior (Coffey *et al.,* 1990) was studied in rats with retinal transplants to the brain. Results of conditioned suppression experiments showed that the animals could detect the illumination of the transplant (Coffey *et al.,* 1989). Experiments with photophobic behavior of the rats suggested that the transplant illumination was not experientially similar to the illumination of the normal eyes (Coffey *et al.,* 1990).

In the intra-ocular retinal transplant model if the transplanted retinal cells survive, grow, differentiate and form synapses with the host neuroepithelial cells, then they may provide useful input to the visually handicapped host. However, there is only one report of behavioral tests on the efficacy of retinal cell transplantation. del Cerro *et al.* (1991) analyzed the inhibition of the acoustic startle reflex in rats with light-damaged retinas with or without transplants. Light-blinded rats show 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 the intraretinal transplants may have provided useful visual information to the blinded host.

Changes in the host retina

Many changes in the host retina can be attributed to surgical trauma.Minute scars involving one or several of the layers of retina is the most obvious one. Occasionally, there can be hemorrhages in the subretinal space, in the choroid or in the vitreous. Some pigment laden macrophages in the subretinal space close to the surgical site and a few free red blood cells and macrophages in the vitreous are also the results of the surgical trauma.

The photoreceptors regularly diminish markedly in number in the host retina overlying a subretinal transplant (Bergström *et al.,* 1992; see also Fig. 2). It is possible that the separation of the host photoreceptors from the pigment epithelium is responsible. Outer segments in the detached retina of Rhesus monkey degenerate very quickly whereas the inner segments remain intact for up to 7 days (Guérin *et al.,* 1989). The rate of outer segment renewal is also slower in the detached retina (Kaplan *et al.,* 1990). On the other hand, photoreceptors in the rosettes are separated from the pigment epithelium but do develop outer segments, although they are not entirely normal. In the host retina, photoreceptor inner and outer segments soon undergo extensive changes that are apparent as a thinning of the outer nuclear layer. The reason for the disappearance of the outer layers of the host retina remains undetermined.

Surgical trauma to the host retinas results in the loss of ganglion cells due to axotomy and thinning of retinal layers in the peripheral retina (Turner and Blair, 1986; Turner *et al.,* 1986b; Laedtke and Turner, 1989). In rat retina, up to 50% of the cells larger than 30 µm, presumed to be the ganglion cells, had died 4 weeks after the surgical intervention. The presence of a transplant reduced this loss to 30% (Laedtke and Turner, 1989). Similar results have been documented with embryonic neural grafts which sustain host CNS neurons that would have died (Bregman and Reier, 1986; Sharp *et al.,* 1986). In addition, embryonic retina also exerted a stimulatory effect in the central direction from the lesion site, because the cells in the retina were seen to be hypertrophic. Peripheral nerve implants at the site of retinal lesion site produce strikingly similar results (Turner *et al.,* 1986a; Turner *et al.,* 1987). Since similar hypertrophic influences have been shown to be the result of exogenous growth factor treatment (Hendry and Campbell, 1976), similar factors may be mediating the stimulating action of the grafts.

Photoreceptor rescuing effect of retinal pigment epithelium transplantation in the rats with the hereditary retinal degeneration is well documented (Li and Turner, 1988b; Lopez *et al.,* 1989; Gouras *et al.,* 1989) and is discussed elsewhere in this paper (see section 4.1.4).

Integration with the host

Neural transplants to the brain often become encapsulated by a glial barrier (Azmitia and Whitaker, 1983), but there is no evidence of such a barrier at retinal cell transplants, either at the light or electron microscopic level. In contrast, retinal transplants have been seen to fuse with the cut edges of the host retina and there is relative paucity of glial cells in and around them. Immunohistochemical studies (Seiler and Turner, 1988; Seiler *et al.,* 1990) using glial cell specific antibodies like that against glial fibrillary acidic protein (GFAP), S-100 (a calcium binding protein) or vimentin have demonstrated that within two days after the transplantation host glial cells may invade the transplant to give it architectural support without forming any glial barrier (Seiler and Turner, 1988). Sometimes a vitreal membrane made up of mesenchymal elements and some blood vessels can be observed in the host. The lack of astroglial cells 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. Since astroglial cells are important elements of the retinal histogenesis (Wang *et al.,* 1994; Engele and Bohn, 1991), their normal presence may be important for the development of the transplants.

Immunology

Immune privilege of the eye

It has been known for many years that histoincompatible tissues survive unexpectedly long at certain sites (Medawar, 1948; Kaplan and Stevens, 1975; Jiang and Streilein, 1991a), 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 (Kaplan and Streilein, 1978; Kaplan *et al.,* 1975; Niederkorn *et al.,* 1981) 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 (Niederkorn and Streilein, 1983), preserved humoral immunity and primed cytotoxic T cell response (Streilein, 1990), 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 (Cousins *et al.,* 1991). These 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 (Jiang and Streilein, 1991b). Histocompatibility antigens are scant in the neural retina, but their expression is upregulated after transplantation (Rao *et al.,* 1989). 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 (Streilein *et al.,* 1980), not all of them evoke a permanent state of ACAID. For example, some tumor cells transplanted to the anterior chamber of mice induce only a transient ACAID (Ksander *et al.,* 1991). Allogenic retinal grafts placed in the anterior chamber are eventually destroyed, indicating that conventional immunity can replace the ACAID (Jiang and Streilein, 1991b).

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 (Jorquera *et al.,* 1994). 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 (Tanihara *et al.,* 1993) and that its production by the astrocytes is upregulated in pathological conditions (Wahl *et al.,* 1991). The Müller cells of the retina suppress T cell proliferation by a direct contact mechanism (Caspi *et al.,* 1987). 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, insuring 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 (Streilein *et al.,* 1992).

Influence of host age

There has been little research regarding the effect of host age on retinal transplants. Immunological maturity and hence 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 (White and Gilks, 1993). Neural xeno-transplants into adult rat brains showed a high degree of rejection (Lund *et al.,* 1987), whereas 70% of similar grafts survived in the brain of neonatal rats for at least 90 days (Sefton *et al.,* 1989). 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 (Lund *et al.,* 1987). This indicates the time of development of immunological competence of rats. Other studies on neural transplants including retinal grafts in CNS (McLoon and Lund, 1983) and the growth of fetal brain explants in the anterior chamber of the eye (Eriksdotter-Nilsson *et al.,* 1986) indicate that host age can influence the outcome.

Altering host immunity

Cyclosporin A is a commonly employed drug that suppresses the immunity of the host, and it has been shown to enhance the survival of kidney, bone marrow and liver transplants (Steinmuller, 1985; McGregor *et al.,* 1986; Sheil, 1987). It 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 (Aramant and Turner, 1988). 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 survivals improved if the animal is treated with Cyclosporin A (Aramant and Turner, 1988). FK-506 is a different, potent immunosuppressive drug that acts during the commitment phase of lymphocyte activation to block a subset of calcium-associated events necessary for transcription of certain early lymphokine genes (Sewell et al., 1994). This drug has been found useful in the treatment of non-infective uveitis (Ishioka et al., 1994). In retinal transplants, immunosuppression with FK 506 has also been shown to prolong cross species graft survival (Yamamoto *et al.,* 1994).

Altering the transplant antigenicity

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 (Liversidge *et al.,* 1988), 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 (Streilein *et al.,* 1992).

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 (Koller *et al.,* 1990). 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.

PRESENT PROBLEMS AND FUTURE DIRECTIONS

In the last decade, advances in the field of neural transplantation have generated the hope that retinal cell transplantation will become feasable and prove a practical way to treat retinal disorders hitherto untreatable. The history of retina to retina transplantation is only about a decade old, but the achievements have been considerable. However, the clinical goal has not yet been reached. There is

not yet any method available for transplanting retinal neurons to patients. Pigment epithelial cells have very recently been transplanted in humans, but no long-term clinical results are available yet. Currently, the scientific efforts concentrate on certain problems: the formation of rosettes in the transplants, the fate of ganglion cells in the transplants, the types and numbers of connections made between the transplants and the host and the possibility of using transplants as the source for cytokines and other growth regulators.

In most of the transplant modalities being used at present, the transplanted cells organize themselves in laminae, but this layering itself is in within rosettes (del Cerro *et al.,* 1985; Turner and Blair, 1986; Gouras *et al.,* 1991a; Gouras *et al.,* 1992; Silverman and Hughes, 1989a). This does not mean that these cells are rendered incapable of processing light signals, but the quality of the image thus produced will be poor. It seems likely that the rosette formation in the transplant is the result of mechanical disruption of the donor tissue during the process of transplantation. Studies are under way to find methods of transplanting flat pieces of donor tissue embedded in gelatin or other polymers for protection, so that the process of transplantation is the least damaging.

Transplants must form meaningful connections if they are to convey visual information, which means that nerve cells from the transplants must send their processes to the host and form synaptic connections there. Even though bridges have been seen, connecting the transplant with the host, they seem to be few and do not appear regularly. Nothing is known on how to influence the formation of such bridges, and more work is needed on this problem.

In retinitis pigmentosa and similar diseases, only the photoreceptor layer needs replacement. The possibilities to transplant only an isolated sheet of photoreceptors (Silverman and Hughes, 1989a; del Cerro *et al.,* 1990b) or isolated photoreceptor cells (Gouras *et al.,* 1991a; Gouras *et al.,* 1992) is therefore being explored. Bipolar cells and horizontal cells are the targets of photoreceptors, and there is no need for the transplant to make long distance connections in these experiments. Transplanted photoreceptor sheets have been reported to survive well (Silverman *et al.,* 1992), and there is a preliminary report that they form functioning contacts with the host retina (Mosinger Ogilvie *et al.,* 1994).

Till now there has been no conclusive evidence that ganglion cells survive and develop in the transplants (Aramant *et al.,* 1990a; Bergström *et al.,* 1994a; del Cerro, 1990), although there are indirect suggestions that they may (Turner and Blair, 1986; Ehinger *et al.,* 1991a; Bergström *et al.,* 1994b; Zucker *et al.,* 1994). However, it has been shown that ganglion cell axons will regenerate into peripheral nerve grafts (So and Aguayo, 1985) which can guide the axons all the way to the CNS where they will form connections (Vidal-Sanz *et al.,* 1987; Carter *et al.,* 1989; Zwimpfer *et al.,* 1992).

Transplanting cells that produce some trophic factor that will prevent neurons from degenerating is an attractive idea which 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 candidate factors have been found (LaVail et al. 1992a and b). Further, cones are known eventually to degenerate also when it is the rods that are primarily affected, and the simplest way to

explain this is to assume that cones for their proper maintenance need some trophic factors produced by rods. However in most cases neither the factors themselves nor suitable concentrations or time points for administering them are known. Further, cell system able to deliver the required factors without also damaging the host are not available. As attractive as the idea of a transplant as a source of trophic factors may be, the development of suitable methods and cell line for the purpose is only in its infancy.

It is known that the retinal pigment epithelium is defective in the RCS rat, and that replacing it will retard the degeneration of its retina (Sheedlo *et al.,* 1989b; LaVail *et al.,* 1992a). It appears plausible that human degenerative diseases may also be caused by a malfunctioning retinal pigment epithelium, although very little is known about this. It is therefore important that the first retinal pigment epithelium transplants into human eyes have now been performed. It is too early to assess the final results in these cases, but it is already clear that there are surgical techniques available for pigment epithelium or neuroretinal surgery on human eyes. This makes it important to classify the degenerative disorders in detail in both humans and experimental animals, establishing the cell types that are malfunctioning.

CONCLUSIONS

Retinal pigment epithelium grows well on certain culture media and will under appropriate conditions form monolayers which can subsequently be transplanted. Retinal pigment epithelium transplants possess many of the physical and functional features of the normal retinal pigment epithelium, including phagocytic action on the photoreceptor outer segments. Such transplants have been shown to have photoreceptor rescuing effects in the RCS rat.

Neural retina survives transplantation well. The outcome is affected by donor age, species of the host, site of transplantation and whether the donor tissue has been cryopreserved before transplantation or not.

Nerve cell transplantsdifferentiate, grow and integrate in the host environment according to their intrinsic timetable. They form all the retinal layers and most of the normally occurring cell and synapses types, with the possible exception of ganglion cells. However, the retinal layers and the cells are not entirely normal and usually become arranged in so-called rosettes. The photoreceptor outer segments are also found distorted.

Retina to retina transplants possess the cell types, synapses, neurotransmitters and many essential proteins and other substances that are required for processing light signals. Electrophysiological studies have shown that transplants are able to detect light and to perform at least some simple signal processing. Although surviving ganglion cells have not been convincingly shown in retinal transplants *in oculo*, retinas transplanted to CNS sites close to their target nuclei contain surviving ganglion cells that form functioning connections with the target tissue.

Regeneration experiments have shown the potential of retinal ganglion cells to grow axons through a peripheral nerve graft into the brain.

Thanks to the immune privilege of the interior of the eye, host immune responses do not pose any great problem for observation times up to several months.

ACKNOWLEDGMENTS

This work was supported by the Swedish Medical Research Council (project 14X-2331), the RP Foundation Fighting Blindness, the Segerfalks stiftelse, the H och L Nilssons stiftelse, the Riksbankens Jubileumsfond, Swedish Society for Medical Research, and the Medical Faculty of the University of Lund.

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

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Running headline: Rosettes in 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

Introduction

The initial transplantation studies of fetal retinas to the anterior chamber showed by Royo and Quay (1959) were followed by similar experiments by del Cerro et al. (1985). At about the same time, Turner and 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 and 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; del Cerro et al., 1989; Gouras et al., 1991a; Gouras et al., 1991b; Gouras et al., 1992; Juliusson et al., 1993). Transplanted tissue fragments and microaggregates survive well (del Cerro et al., 1985; Blair and Turner, 1987; Seiler et al., 1988 a; 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, Albert and Craft, 1975; Milam and 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, Lund and McLoon, 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 preliminary reports that transplants of photoreceptor layer embedded in gelatin also show no or only little rosette formation (Silverman and Hughes, 1989; Silverman et al., 1991; Silverman et al., 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 and 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 were placed 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. Instrument comprises of a cannula made from an 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 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 instrument described by Bergström et al. (1992) was used. Difference between the instrument used for transplanting the fragmented pieces of tissue and the large sheets of the tissue is that the plastic capillary used is narrower (with inner and outer tip diameter of 0.15-0.4 and 0.3-0.5 millimetres) for transplanting the fragmented donor retina.

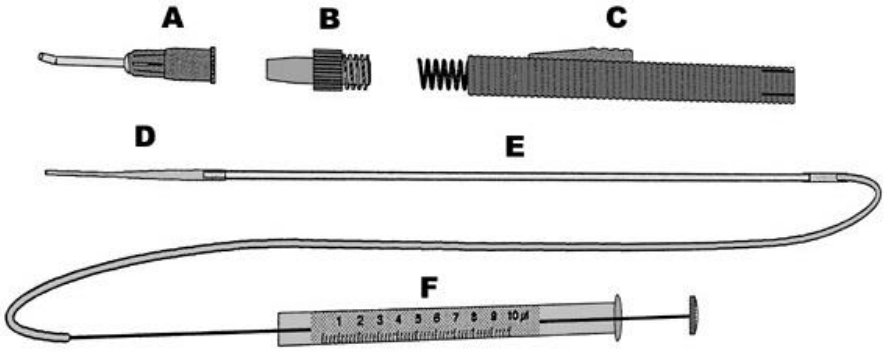

Fig. 1. Drawing of the instrument used for transplantation. The plastic tube (D) fits inside the main parts of the instrument (B and C). This part of the instrument pushes the plastic tube (D) out of the cannula (A) which shields it during the passage through the

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.

Transplantation Technique

For transplanting large sheets of donor tissue, 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 in to the instrument with the fine capillary and transplanted (Bergström et al., 1992). The donor tissue got fragmented while passing through the capillary.

After the transplantation, sutures are usually not needed. The animals were allowed to wake up and transferred to their normal cages with a 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 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 hours. After the fixation, the eyes were hemisected and the part carrying the transplant was excised. The tissue was washed with Sörensen's phosphate buffer (0.1M pH 7.2) and then with the same buffer containing 5%, 10% and 20% sucrose before sectioning on a cryostat (12µm). 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 minutes 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 hours. Sections incubated with anti-vimentin antibody were then washed, incubated with secondary antibody (rhodamine-conjugated rabbit immunoglobulins to mouse immunoglobulins, Dakopatts) for 30 minutes, 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 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).

Observations and Results

With the technique described, 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 the donor tissue in the transplantation cannula is at times difficult to eject, but otherwise the procedure is straightforward and works well. If the animal was too lightly anaesthetized, inserting the transplantation cannula may elicit a withdrawal reaction, due to the sensory nerves in the choroid, which in the worst of cases may ruin the surgical results.

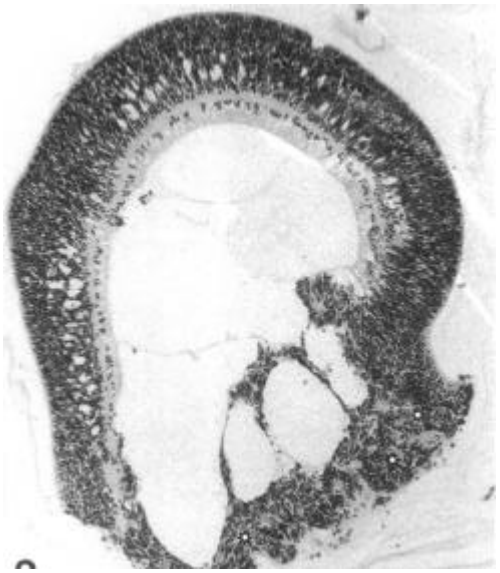

Fig. 2. A 6 week old E 15 large sheet retinal transplant in the epiretinal space developing as a spheroid without any rosettes except at the margins (black asterisks with white dots) where the donor tissue was damaged during the process of transplantation. Differentiation of the transplant in two layers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

Six weeks after the transplantation, which is equivalent to 8 weeks after the conception, the epiretinal transplants had grown into a more or less spherical or cup-shaped laminated sheet as in normal retinogenesis. This piece of the 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 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 the cells in this layer remained undetermined (Fig. 3).

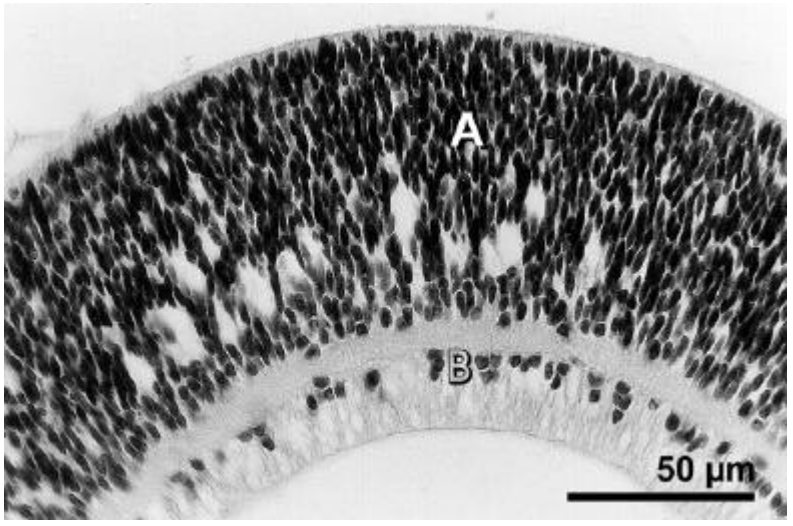

Fig. 3. Magnified view of a neighbouring section of the same transplant as in Fig. 2 showing no outer segments, and differentiation of the transplant in two nuclear layers. Neuroblastic cell layer (A) is seperated from the layer that resembles ganglion cell layer (B) by a plexiform layer (PL). Hematoxylin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

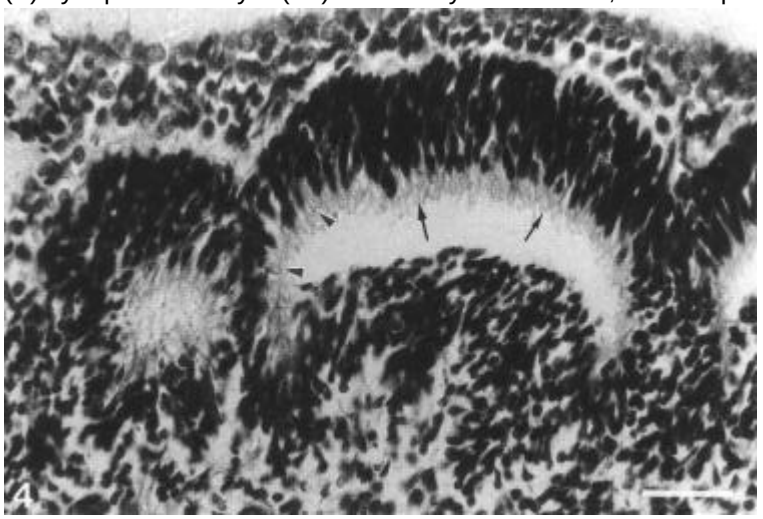

Fig. 4. A 6 week old E 15 large sheet retinal transplant in the same eye as the transplant in Fig. 2, showing the formation of arcuate structures and relatively well developed outer segments (arrows) with outer limiting membrane (arrow heads). Hematoxylin and eosin, bar = 50µm.

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 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 were disturbed.

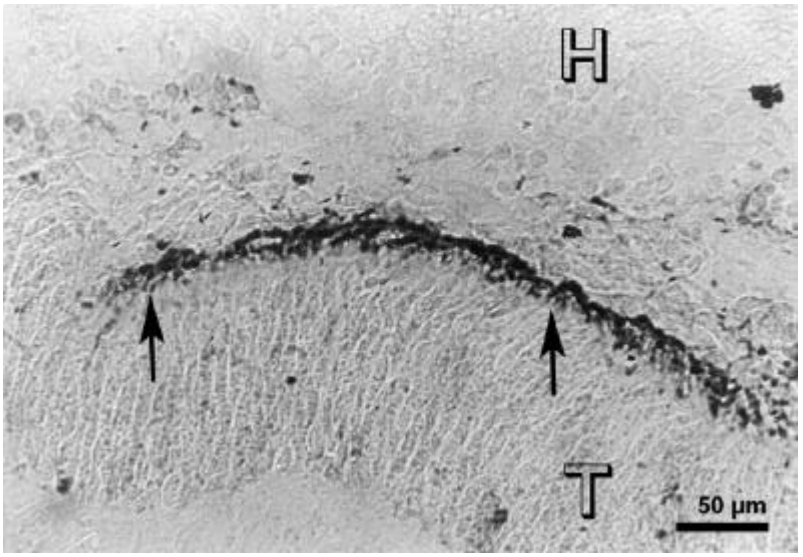

Fig. 5. A 6 weeks old E15 large sheet subretinal transplant (T), showing rhodopsin staining in the photoreceptor outer segments (arrows) in arcuate arrays and in the host retina (arrow heads). Note that the rhodopsin immunoreactivity in the host retina (H) disappears when it apposes the transplant. Host retinal pigment epithelium (RPE) is visible at the right hand corner of the figure. Immunostaining with rhodopsin antibody R2-15, bar = 50µm.

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 comprised comparatively small, oval cell bodies, apparently developing photoreceptor cells. The second consisted of cells with rounder and

more lightly stained nuclei, which resembles cells of the inner nuclear layer. A plexiform layer was also present between the two nuclear layer, 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. 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 relative 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.

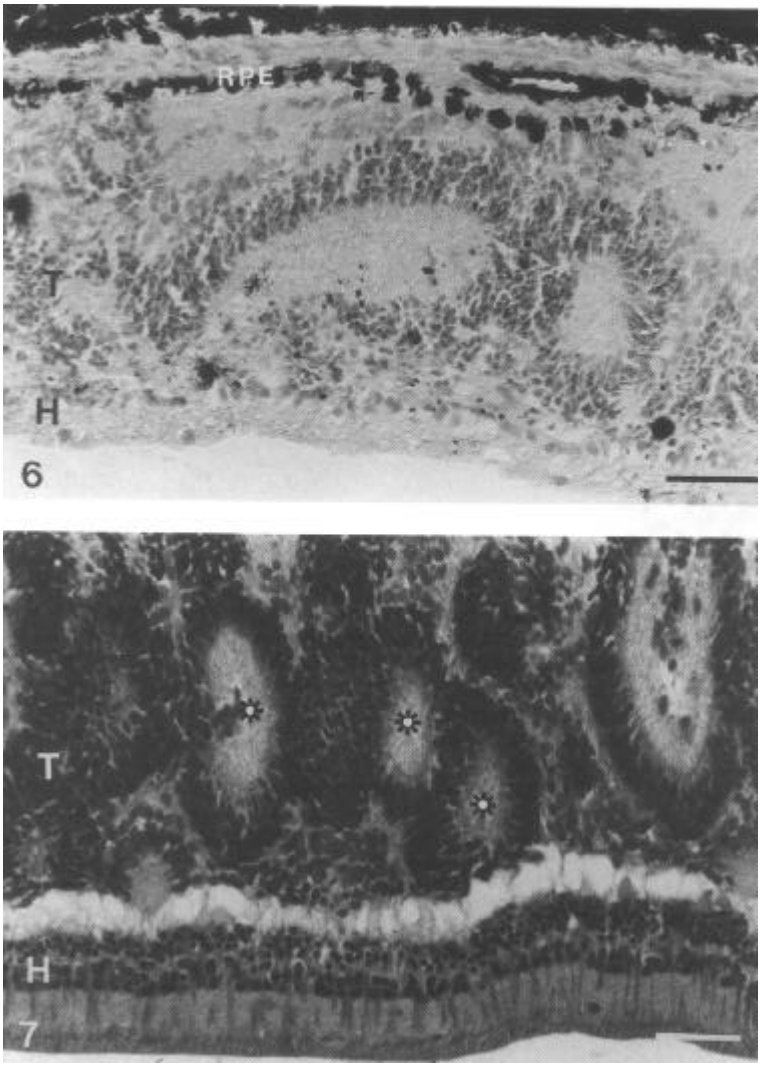

Fig. 6. A 6 week old E15 large sheet retinal transplant (T), covered by the host retina (H), showing arcuate arrangement of the photoreceptors also at places where the host pigmented epithelium (RPE) is still present. RPE shows some irregularities and

moulding presumably due to surgical trauma. Dark dots in the transplant are the pigmented cells from the host RPE. Hematoxylin and eosin, bar = 50µm.

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 = 50µm.

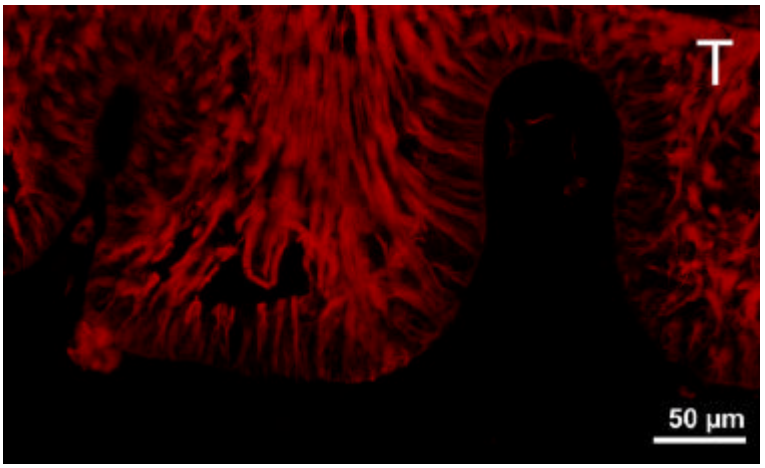

Fig. 8. Vimentin staining in epiretinal large sheet transplant not covered by the host retina showing a palisade-like arrangement of the Müller cells. Bar = 50µm.

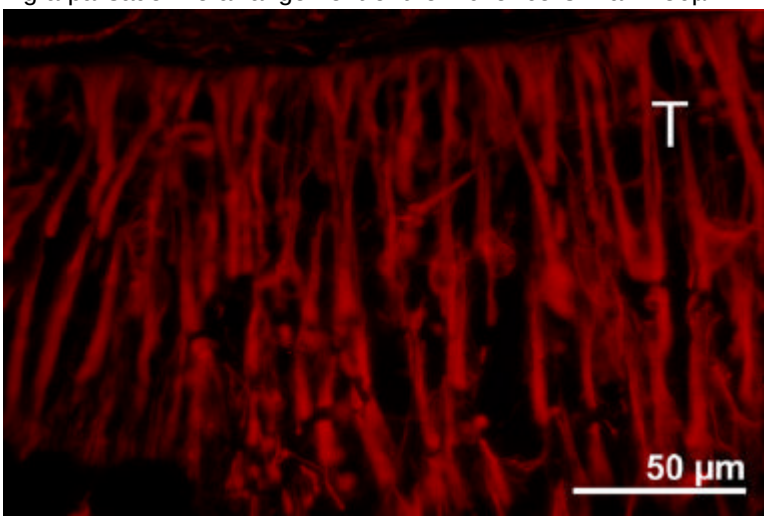

Fig. 9. Vimentin staining in a 6 week old E15 large sheet retinal transplant (T) in the subretinal space (H = host retina), showing palisade-like arrangement of Müller cells in arcuate arrays. Bar = 50µm.

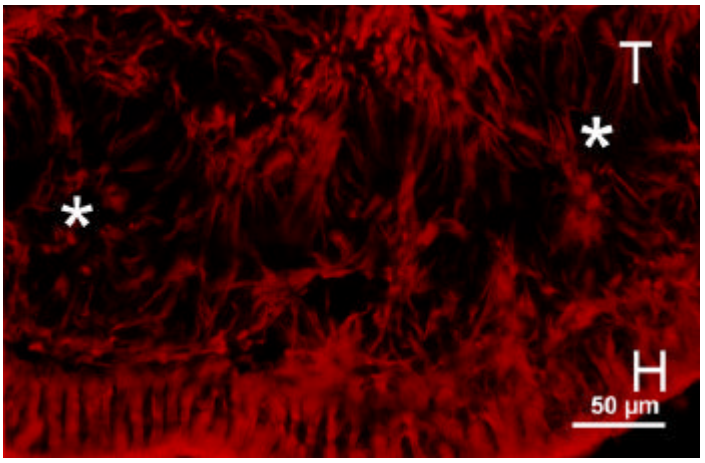

Fig. 10. Vimentin staining in a fragmented tissue transplant (T) in the subretinal space (H = host retina), showing the radial arrangement of Müller cells in the rosettes (lumen indicated by black asterisks with white dots). This arrangement is different than that of arcuate arrays in the large sheet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

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 the fragmented pieces (Bergström et al., 1992), dissociated cells (Juliusson et al., 1993), or the photoreceptor layer (Silverman and 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 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 and Quay, 1959; Turner and Blair, 1986; Seiler et al., 1990; Gouras et al., 1990; Bergström et al., 1992; Bergström et al., 1994).

In this study, epiretinal and subretinal 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 and Weidman 1982). The rosette-free areas of the epiretinal transplants did not show any outer plexiform layer, even at 4 weeks post-natal 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 and Weidman, 1982; Stone et al., 1985; Sharma and 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 week 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 and 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 and 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 and Turner, 1988 b). In the present study also, rosettes in the fragmented tisue 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 chicken in which there is a transdifferentiation of the pigmented epithelium into neurons, both in vivo (Coulombre and Coulombre, 1965; Coulombre and Coulombre, 1970; Park and Hollenberg, 1989) and in vitro (Layer and Willbold, 1989; Layer and Willbold, 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; 1983; Rathjen, 1991). Such molecules also play a role in cellular and histological differentiation (Takeichi et al., 1990; Takeichi, 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 and Coulombre, 1965; Coulombre and Coulombre, 1970; Willbold and 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 minimise 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 and b). One to two postnatal day mice neuro-retinal microaggregates transplanted by using a 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 minimise the rosettes is to transplant a gelatine embedded photoreceptor layer, but these grafts also fail to develop healthy photoreceptor outer segments (Silverman and Hughes 1989).

The present results also suggest that the dissection and handling of the 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 proper lamination to appear. 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 proper layering to develop. 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.

Acknowledgements

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

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 Söderberg Foundation.

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Mitosis in developing rabbit retina.

An immunohistochemical study

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Running Headline: Mitosis in developing retina.

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Summary

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 or nerve fiber 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) do 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.

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 the ventricular surface. 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 mouse (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 layer has been shown (Reichenbach et al., 1991) in rabbits.

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 it is the first time an immunohistochemical approach has been used to study mitosis in the rabbit retina.

MIB-1 is an antibody that recognizes a nuclear-associated antigen, Ki 67, present in all the phases of cell cycle except the resting phase (Gerdes et al., 1984).

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 sacrificing 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) and 20 (n=2) pigmented rabbits.

The age of the animals was determined by mating the rabbits during a 1 hour 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 hours. 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 and hematoxylin & 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) in 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 hours at 4°C in a humidified chamber. After 72 hours, the slides were rinsed with PBS and incubated for 1 hour in biotin conjugated horse-anti-mouse secondary antibody (Vector Lab. Inc. Burlingame, CA,. USA) in 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 minutes. The slides were then incubated in avidin and biotinylated horseradish peroxidase complex (Vectastain® Elite ABC Kit; Vector Lab. Inc. Burlingame, CA,. USA) for 1 hour. 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,. USA). Adjacent sections were stained with hematoxylin and eosin.

At least two slides from each specimen were stained with hematoxylin and eosin and MIB-1, and in each experiment controls were obtained by omitting the primary antibody.

We will in this paper use the term “basal” or “proximal” and “apical” or “distal” to denote localization in terms of how the tissue develops. In the neuro-retina, “basal” thus means closer to the vitreous and “apical” closer to the photoreceptors.

Observations and results

Embryonic stage E15:

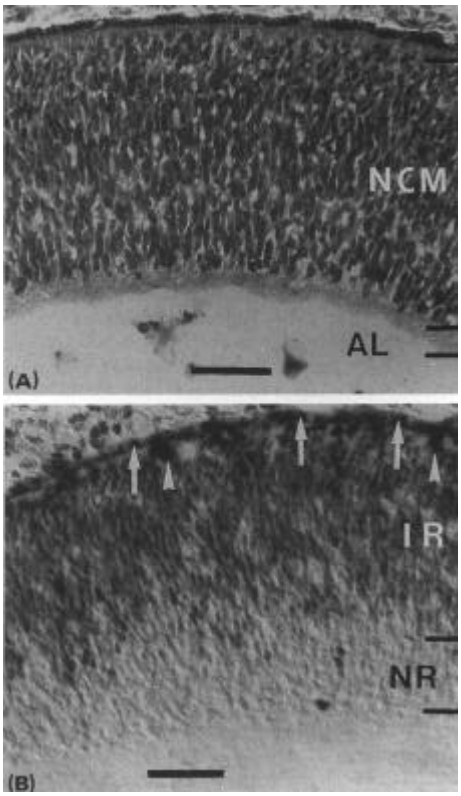

Fig. 1. An embryonic day 15 rabbit retina. (1 A) A haematoxylin and eosin stained section showing the multi-layered neuroblastic cell mass (NCM) and a thin anuclear layer (ACL). (1 B) An immunohistochemically stained section with MIB-1 antibody. MIB-1 immunoreactive cells in the apical two third of the 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.

At this stage, 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. 1A). Both the neuroblastic cell mass and the anuclear layer are thicker in the center than in the periphery, with the anuclear layer almost becoming non-existent in the extreme periphery.

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. 1B). 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. The retinal pigment epithelium is a single cell layer thick, with some pigment granules (Fig. 1B). MIB-1 immunoreactivity appeared in most of the pigment epithelial cells.

Embryonic stage E25

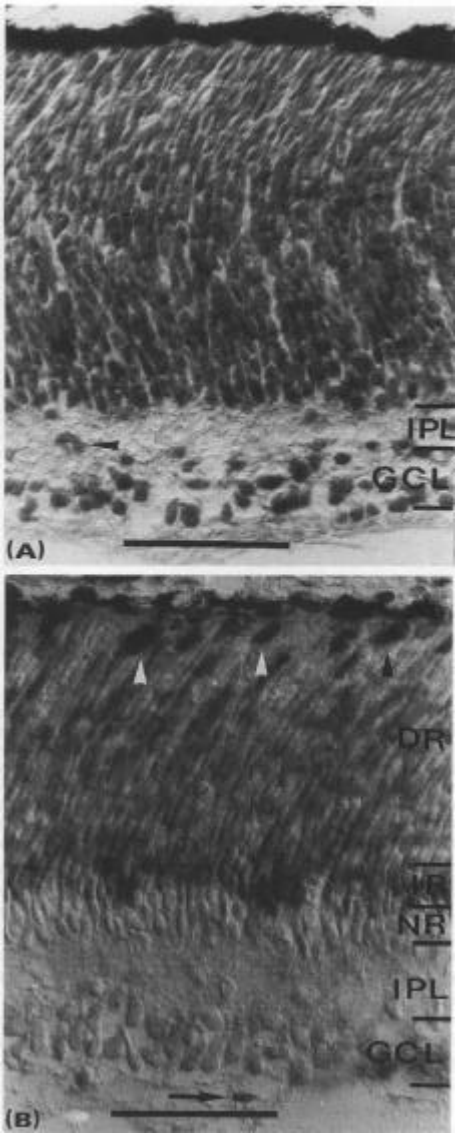

Fig. 2. An embryonic day 25 rabbit retina. (2 A) An haematoxylin and eosin stained section showing the formation of inner plexiform layer (IPL) and the ganglion cell layer (GCL). Some cells are observed in the inner plexiform layer (arrowheads). (2 B) An immunohistochemically stained section with MIB-1 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 (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 .

At this stage, some cells had migrated from the neuroblastic cell mass to form the ganglion cell layer delineated from the neuroblastic cell mass by the inner plexiform layer (Fig. 2A). Some cells were also observed in the inner plexiform layer. Cells in the ganglion cell layer were rounded whereas those in the neuroblastic cell mass were spindle-shaped.

As seen in figure 2B, 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. The retinal pigment epithelium

is more heavily pigmented than in E15 (fig. 2B). MIB-1 immunoreactivity was present in most of the cells.

Embryonic stage E29

The ganglion cell layer at this stage was thinner and better organized than at previous stages, with large, rounded, light stained nuclei. Only few cells were seen in the inner plexiform layer. Some nuclei in the basal part of the neuroblastic cell mass had rounded, lightly stained nuclei (fig. 3A). Also, there were some lightly stained cells at the border of the basal two thirds and the apical one third. The developing outer plexiform layer was apparent in central parts of the retina (Fig. 3A). At this stage they looked like small protrusions in the space between the neuroblastic cell mass and the retinal pigment epithelium. Some cells in the ganglion cell layer or nerve fiber layer were immunoreactive to the MIB-1 antibody.

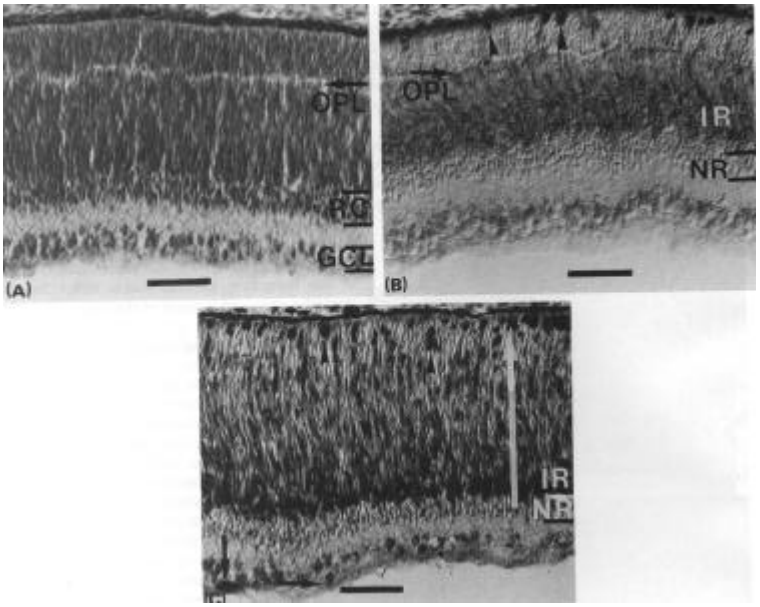

Fig. 3. An embryonic day 29 rabbit retina. (3 A) An haematoxylin and eosin stained section showing the formation of the outer plexiform layer (OPL; arrow). The ganglion cell layer (GCL) is thinner than in E25. Cells in the proximal (basal part) of the inner nuclear layer are rounded (RC). (3 C) An immunohistochemically stained section with MIB-1 antibody showing non-immunoreactive layer in the basal part of the neuroblastic cell mass (NR), corresponding to the rounded cells (RC) in figure 3A, and an accumulation of reactive cells distal to it (IR). The reactivity gradually decreases (in the direction of the large arrow) 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). (3 B) An immunohistochemically stained section with the MIB-1 antibody showing immunoreactivity in the central retina where the outer plexiform layer (OPL; arrow) has formed. Reactivity in the outer nuclear layer is much less than that in the middle of inner nuclear layer (IR; I mark). Bar = 50 µm .

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. 3B). It corresponds 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. 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 region close to the pigment epithelium (fig. 3B). 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. However, especially in the outer nuclear layer, reactive cells could be seen on both of its sides (fig. 3C). In the embryonic day 29 retina, pigment granules in the pigment epithelium were prominent, making it difficult to judge the MIB-1 immunoreactivity.

Post natal stage PN0

In the newborn retina (stage PN0), the outer plexiform layer became distinct and covers a larger part of the retina. The outer nuclear layer and the inner nuclear layer were of equal size at this stage. Cells in the outer nuclear layer are more organized close to the retinal pigment epithelium. Here the cells have better developed inner segments than E 29 retinas. In the inner nuclear layer, cells in its outer part are somewhat elongated, whereas those in the inner part are more rounded (fig. 4).

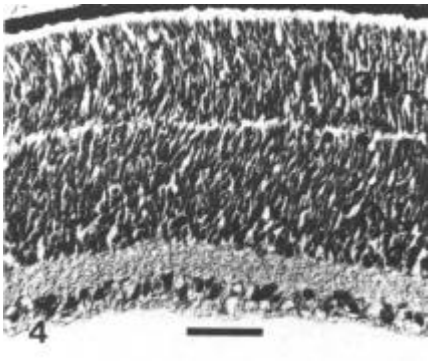

Fig. 4. A postnatal day 0 rabbit retina showing almost equal sized inner (INL) and outer nuclear layers (ONL). Inner segments (arrowheads) are still poorly developed. Haematoxylin and eosin. Bar = 50 µm.

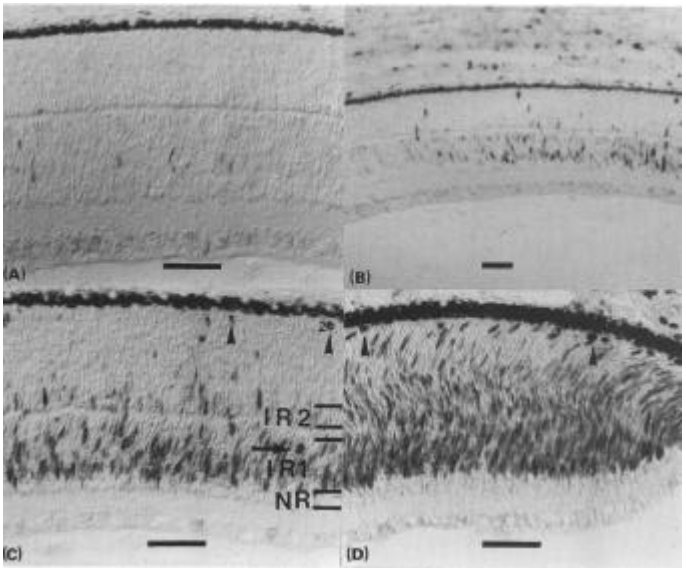

Fig. 5. A postnatal day 0 rabbit retina stained with the MIB-1 antibody at different eccentricities, central (5A), mid periphery (5B), periphery (5C) and retinal edge at the ora serrata (5D). The central area shows no immunoreactivity, but it gradually increases towards the periphery. The immunoreactivity is generally less than in comparable areas from 29 day retinas (fig. 3B). 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 (5C and D, arrowheads). Immunoeactive 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 (5C, arrow). Cells in the proximal and the distal parts of this layer are non-reactive (5C). In fig. 5D there are many more large and deeply stained cells (arrowheads) than in the more central retina. Bar = 50 µm.

An area essentially devoid of MIB-1 immunoreactive cells developed in the central retina (fig. 5A). The number of immunoreactive cells increased from this area towards the periphery (fig. 5A, B, C, D). In the peripheral part of the retina, 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. 5C and also seen in fig. 8B 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 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 parts than the central. 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. 5C). Immunoreactive cells could be seen on both sides of the outer plexiform layer. The reactivity in the inner nuclear layer was less in comparison with E29, and was confined to the

elongated spindle-shaped 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 (fig. 6). It was noted that some immunoreactive cells remained in the ganglion cell layer and the nerve fiber layer for a long time.

Fig. 6. A postnatal day 0 rabbit retina showing immunoreactivity, besides other layers, in the nerve fiber layer in the mylinated streak region (arrows). MIB-1 immunohistochemistry. Bar = 50 µm.

Postnatal stage P3

In the postnatal day 3 retina, the outer plexiform layer is clearly demarcated and has almost reached the periphery (see fig. 8B). The photoreceptor outer segments are not yet fully developed (fig. 7).

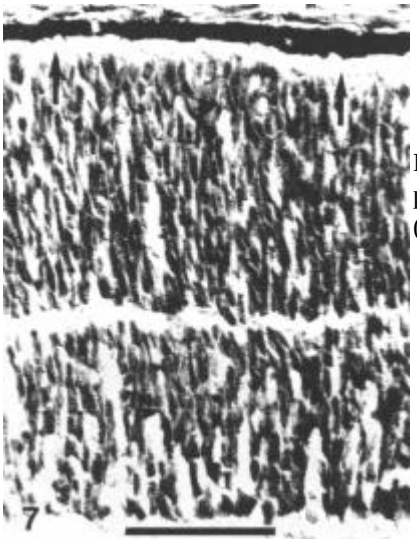

Fig. 7. A postnatal day 3 rabbit retina showing poorly developed photoreceptor outer segments (arrows). Haematoxylin and eosin. Bar = 50 µm.

The central area with no or few immunoreactive cells had 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. 8A). Cells in the inner and the outer parts of the inner nuclear layer were largely non-reactive. In

particular, there were no immunoreactive cells in the cell rows closest to the inner plexiform layer. In the outer nuclear layer, the immunoreactivity was much less than that in the inner nuclear layer, with most of the reactive cells being present close to the outer plexiform layer (fig. 8A). In other parts of the outer nuclear layer, scattered immunoreactive cells were present. Most cells in the outermost part of the outer nuclear layer were non-reactive, with some exceptions, including the deeply stained cells. In general, 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. 8B). Only few immunoreactive cells were present in the ganglion cell layer or the nerve fiber layer (fig 9), especially close to the optic nerve.

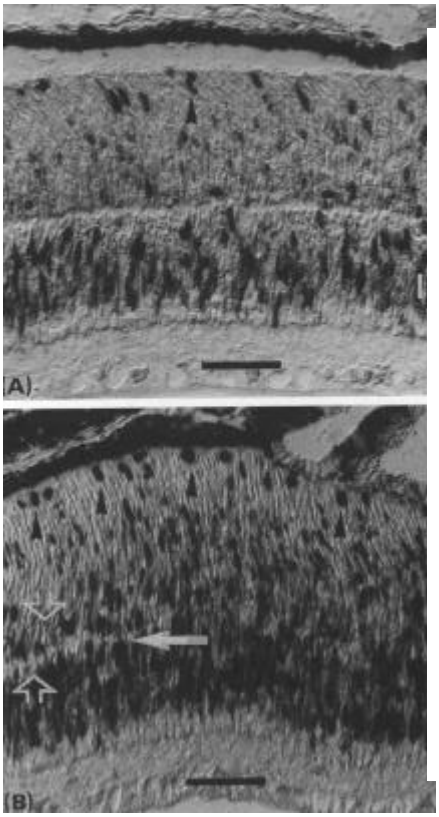

Fig. 8. A postnatal day 3 rabbit retina stained with the MIB-1 antibody. (8 A) Immunoreactivity in the retina slightly peripheral to the non-reactive central zone. Most of the reactivity is confined to the middle part of the inner nuclear layer (IR). The cells in the inner and outer parts of this layer 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 in distal parts of the outer nuclear layer (arrowheads). (8 B) Immunoreactivity in the extreme periphery of the retina. There are many more reactive cells than in the more central retina as seen in fig. 8A. 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.

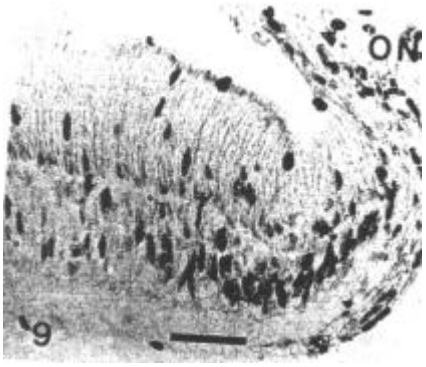

Fig. 9. A postnatal day 3 rabbit retinal region close to the optic nerve (ON) showing immunoreactivity, besides other layers, in the ganglion cell layer or the nerve fiber layer (arrows). MIB-1 immunohistochemistry. Bar = 50 µm.

Postnatal stage P5

On postnatal day 5, the photoreceptor outer segments are not developed yet, but otherwise the retina shows no special new features in routine stained sections.

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. 10). The central area with no or only few immunoreactive cells had enlarged. In the ganglion cell layer or nerve fiber layers, some cell were immunoreactive (fig. 11). In the peripheral parts of the retina, MIB-1 positive cells were occasionally seen in the outer plexiform layer.

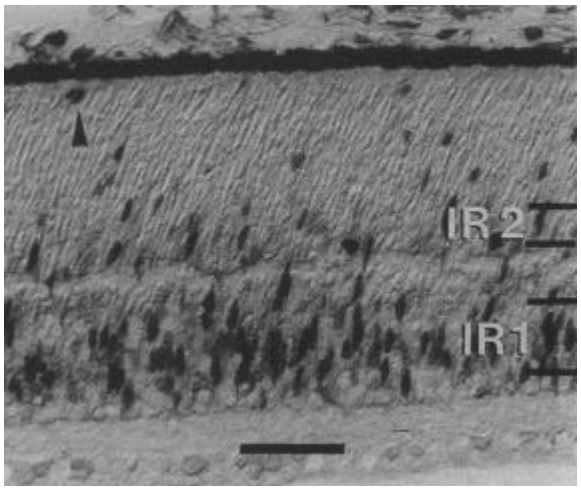

Fig. 10. A postnatal day 5 rabbit retina showing the peripheral region where most of the immunoreactive cells in the outer nuclear layer are situated close to the outer plexiform layer (IR2). Most cells in the distal part of the outer nuclear layer are non-reactive except the some large deeply stained cells (arrow head). The immunoreactivity in the inner nuclear layer is mostly in the middle regions (IR1). MIB-1 immunohistochemistry. Bar = 50 µm.

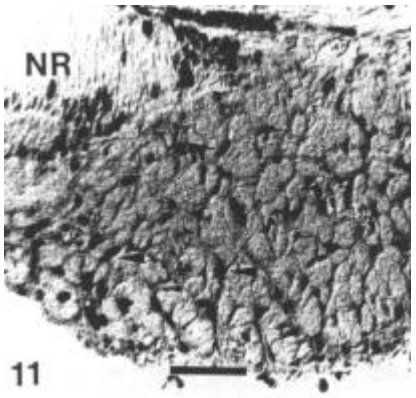

Fig. 11. A postnatal day 5 rabbit retina showing immunoreactive cells in the mylinated streak (arrowheads), close to the optic nerve. Some adjacent region of the retina (NR) are also visible. MIB-1 immunohistochemistry. Bar = 50 µm.

Postnatal stage P7

On postnatal day 7, the outer plexiform layer has developed all the way to the periphery. Further, the photoreceptor outer segments were better but not fully developed (fig. 12).

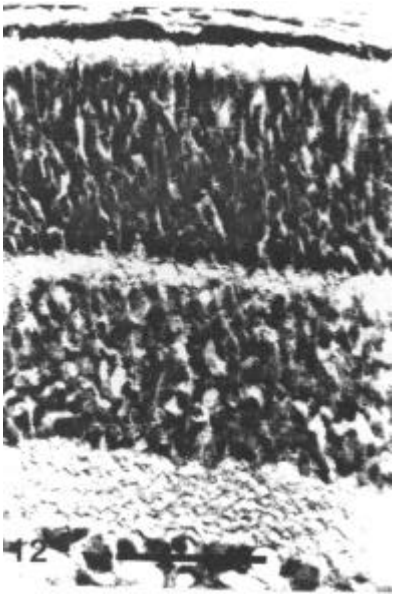

Fig. 12. A postnatal day 7 rabbit retina showing photoreceptor outer segments (arrows). Note that although the outer segments are better developed than shown in postnatal day 3 retina in (fig. 7), they are not fully developed. Haematoxylin and eosin. Bar = 50 µm.

There was a prominent center to periphery gradient in the number of immunoreactive cells, which were essentially absent in the central area. Towards the periphery, the distribution of immunoreactive cells progressively resembled the more immature stages described earlier (Fig. 13A 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. 13A 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 streak, with a gradient that increased towards the optic nerve (fig. 14).

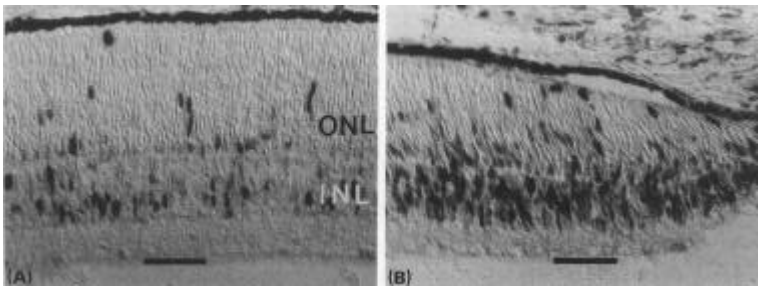

Fig. 13. A postnatal day 7 rabbit retina, MIB immunohistochemistry. (13 A) Immunoreactivity in the retina taken slightly peripherally of the non-reactive zone. Most of the immunoreactive cells are present in the middle of the inner nuclear layer (INL). Some cells in the outer nuclear layer (ONL) are reactive. (13 B) Immunoreactivity in the extreme periphery if the retina. Bar = 50 µm.

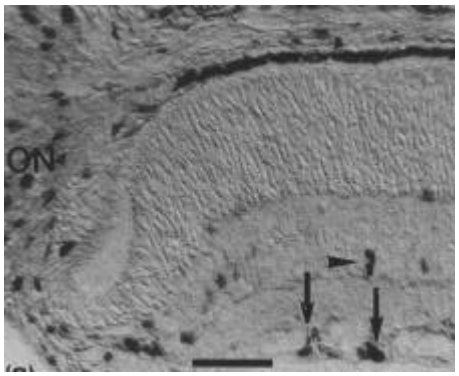

Fig. 14. A postnatal day 7 rabbit retina showing immunoreactive cells in the nerve fiber layer or the ganglion cell layer (arrows) close to the optic nerve (ON). Occasional cells in the inner nuclear layer are also reactive (arrowheads). MIB-1 immunohistochemistry. Bar = 50 µm.

Late stages:

At postnatal day 11 and 15, photoreceptor outer segments were well developed (fig 15). Only 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. 16). The optic nerve also showed immunoreactive cells (fig. 16). We did not attempt to find the age at which immunoreactive cells are no longer present in the optic nerve or the nerve fiber layer of the mylinated streak.

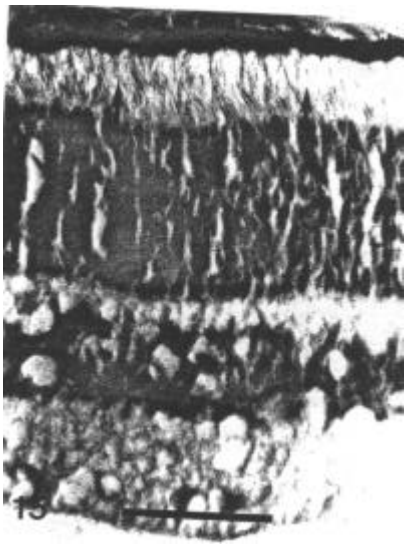

Fig. 15. A postnatal day 15 rabbit retina showing well developed photoreceptor outer segments (arrows). Haematoxylin and eosin. Bar = 50 µm.

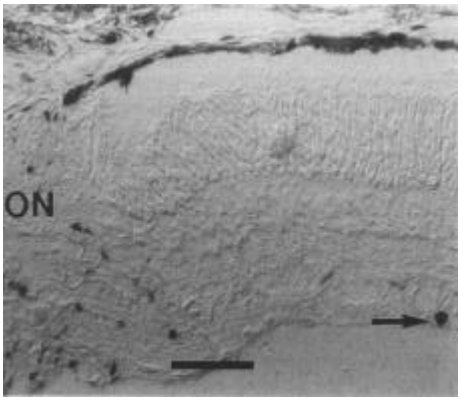

Fig. 16. A postnatal day 15 rabbit retina showing immunoreactive cell in the nerve fiber layer (arrow) close to the optic nerve (ON). MIB-1 immunohistochemistry. Bar = 50 µm.

Controls

When the slides were not incubated in the primary antibody, there was no staining of the neural retinas in any of the stages of developing retina.

Discussion

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). The cDNA encoding this protein has been cloned and sequenced (Schluter et al., 1993), and the gene for Ki-67 has been assigned to the long arm of the human chromosome 10 (10q25; Fonatsch et al., 1991). However, the function of the Ki-67 antigen is unknown. 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; Gerdes et al.). Immunostaining with this antibody is therefore a reliable tool of detecting proliferating cells. The MIB-1 antibody has previously been used for assessing proliferation in various neoplasms (Houmand, Abrahamsen and Tinggaard Pedersen, 1992; Bilous, McKay and Milliken, 1991).

Since MIB-1 labels cells in all the phases of cellular proliferation, it is able to demonstrate the details of the proliferative activity in different regions of retinal layers at different time points during development. Previously used methods have certain disadvantages in this respect. For example, when counting mitotic figures, the sample size becomes small and inaccurate because the observable mitoses constitute only a small fraction of all replicating cells. Further, the compact mitotic figures are difficult to differentiate from pyknotic nuclei and the problem may be worsened by delayed fixation (Donhuijsen et al., 1990). Autoradiography of tritiated thymidine is another method that has been used, but it involves a complex kinetic analysis which depends on various parameters that are difficult to standardize.

Development of retinal layers

At embryonic day 15, which was the earliest age studied, the retina was largely undifferentiated and comprised the neuroblastic cell mass and a thin anuclear layer. The neuroblastic cell mass contained the precursors of both neurons and glial cells. The differences in the thickness of retina and different retinal layers in the central and peripheral parts of retina have been reported earlier (Reichenbach et al., 1991). The lightly stained cells towards the anuclear

layer innermost correspond to the postmitotic cells and are thus likely to be future ganglion cells, as discussed below.

The inner plexiform layer was seen to separate the ganglion cell layer from the neuroblastic cell mass by embryonic day 25. Others have reported that the ganglion cell layer starts to form as early as E20 and by E24 it forms a distinct layer (Greiner and Weidman, 1982; Stone, Egan and Rapaport, 1985).

On embryonic day 29, some cells in the basal part of the neuroblastic cell mass could be identified as amacrine cells and some horizontal cells appeared at the outer one third and inner two thirds of the still undifferentiated neuroblastic cell mass. This is consistent with the findings of Greiner and Weidman (1982). The outer plexiform layer is also beginning to form at this stage.

The outer plexiform layer is apparent at the day of birth except at the extreme periphery. It was reported earlier (McArdle, Dowling and Masland, 1977; Noell, 1958) that the outer plexiform layer starts to develop on the temporal side of the optic disc and progresses peripherally reaching the temporal periphery by PN2 and nasal periphery by PN5 (Stone, Egan and Rapaport, 1985).

The photoreceptor inner segments began to form at E29 in the cells close to the retinal pigment epithelium in the neuroblastic cell mass. At this stage, the inner segments are highly undeveloped, appearing like small protrusions in the remains of optic ventricle. In retinas from this stage through to postnatal day 5, inner segments can be seen in different stages of development. It is not until the first postnatal week that the inner segments start to appear normal. Considerable inner and outer segment growth continues after the first post-natal week. The timings of the inner segment development in our study are consistent with that reported by Greiner and Weidman (1982) and Reichenbach et al. (1991).

Proliferation

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, Robinson and Stone, 1985; Rapaport, Robinson and Stone, 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 rounded and have large amount of chromatin. Other studies have also described mitotic figures in this region. (Greiner and Weidman, 1982). Dividing cells synthesize the DNA away from the ventricular surface (towards the anuclear layer or the developing inner plexiform layer) and then migrate through the whole thickness of neuroblastic cell mass to the ventricular surface for the completion of their division where the mitotic figures can be seen (Rapaport, Robinson and Stone, 1985). Since MIB-1 marks cell 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 that 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 in the previous study masked by the pigmentation of the cells. .

In the innermost part of the neuroblastic cell mass (distal to inner plexiform layer), there was a layer of non-proliferating cells, both at E25 and E29. These non-proliferating cells could not be differentiated 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, Polley and Fortney, 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, presumably the differentiating amacrine cells, and the number of proliferating cells decreased towards the distal retina, distinctly observable from E29 and onwards. In other words, there was an gradually increasing accumulation of postmitotic cells towards the distal retina. 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 on 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 the early phase of proliferation while the rods are born in the late phase (for review see Reichenbach and Robinson, 1995).

At the time of birth, the proliferation ceased in a small region in the central retina. This area of non-proliferation successively enlarged in PN3, PN5 and PN7 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 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, Egan and Rapaport, 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, Robinson and Stone, 1985) that the ventricular proliferation ceases with the development of the outer plexiform layer which was felt to act as a mechanical barrier (Rapaport, Robinson and Stone, 1984; Rapaport, Robinson and Stone, 1985) , preventing the proliferating cells to migrate to the inner retinal layers. A delay between the formation of the outer plexiform layer and the cessation of proliferation has been noted in rabbit (Stone, Egan and Rapaport, 1985) and was thought to be due to the interkinetic migration across the discontinuities in the outer plexiform layer. We noted that the proliferation of cells decreased more so 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 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, Robinson and Stone, 1985; Rapaport, Robinson and Stone, 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), where as 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).

The appearance 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, Robinson and Stone, 1985; Rapaport, Robinson and Stone, 1984). The cells in the nerve fiber layer are probably astrocytes (Schnitzer, 1990; Schnitzer, 1988) and were reported for up to at least 4 weeks after birth. In our 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.

Acknowledgements

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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Cell proliferation in retinal transplants

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Summary

To study the proliferation of cells in the transplanted retinal cells, MIB-1 antibody against a nuclear protein called Ki-67 was used. Fragmented donor tissue starts organizing in rosettes soon after transplantation. In the transplants, the pattern of proliferation strongly resembles that of normal developing retina. The dividing cells in metaphase line up in the luminal layers of the rosettes. Some 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 PN 2. Few cells in these regions proliferate for at least the equivalent age of PN 11 in transplants. There is a layer of non-proliferating, 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.

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 (26). Transplants survive at various transplantation sites (5,27,28), and they develop all the retinal layers and most of the cell types of the normal retina (7,11,12,24). Many of the essential proteins (16,17,28) and the neuro-transmitters (2,7) have also been found in the transplants. However, the first event in the process of development, proliferation, has not been much 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 which are still undergoing mitosis at the time of transplantation (29,30). Suitable markers for proliferating cells have recently

become 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 (29,30), and we now report the expression of the Ki-67 protein in transplanted retinal cells.

Materials and Methods

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. Ten minutes before the surgery, the right eye of the recipients was dilated with 1% cyclopentolate HCl (Cyclogyl®) and one drop of 10% phenylephrine HCl. The recipient rabbits were anaesthetized with Hypnorm® 1 ml/kg (fluanison 10 mg/ml and fentanyl 0.2 mg/ml). 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 2-4 mm behind the limbus. The capillary was advanced through the vitreous to the posterior pole of the eye, and the plastic capillary was then pushed into the subretinal space where its content was deposited by slowly injecting the fragmented donor tissue with the help of the precision microsyringe. Details of the procedure have been published (6).The retinal hole and the scleral incision were left unsutured. The animals were kept in a light/dark cycle of 12 hours each. 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 hours. 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. Non-specific 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 hours at 4°C in a humidified chamber. The slides were then rinsed with PBS and incubated for 1 hour in biotin conjugated horse-anti-mouse secondary antibody (Vector Lab. Inc. Burlingame CA,. USA) 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 minutes. The slides were then incubated in avidin-horseradish peroxidase (Vectastain® Elite ABC Kit; Vector Lab. Inc. Burlingame CA. USA) for 1 hour. 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,. USA). Adjacent sections were stained with hematoxylin and eosin.

At least two slides from each specimen were stained and in each experiment controls were obtained by omitting the primary antibody. The adult host retinas also served as internal controls since their nerve cells were not expected to divide.

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 neuro-retina, “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). The immunoreactive cells were observed scattered throughout the transplant. Certain large and deeply stained cells were homogeneously distributed throughout the transplant or occasionally formed small 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 (29,30). Immunoreactive and non-immunoreactive cells were homogeneously distributed throughout the transplant. On rare occasions, a few cells close to the host retina formed small, thin patches of small and pyknotic cells 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 and pyknotic. This same layer was devoid of immunoreactive cells (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 non-immunoreactive 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 non-reactive pyknotic cells.

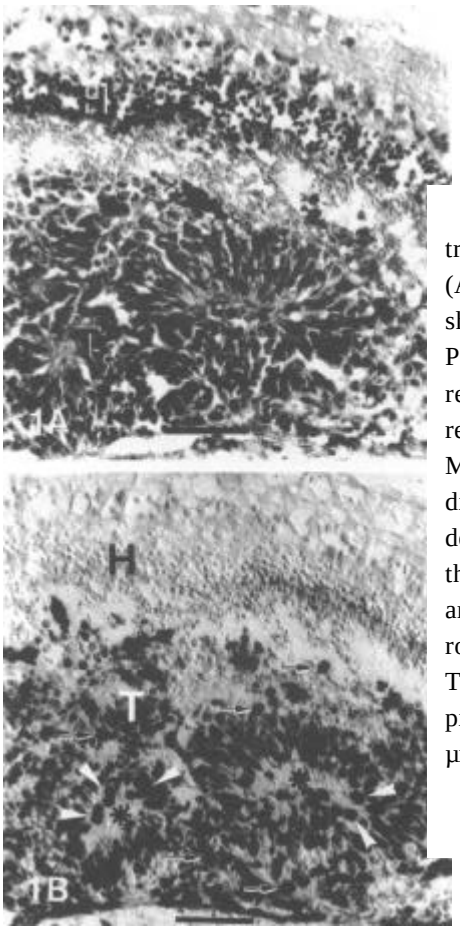

Fig. 1 Embryonic day 16 rabbit retinal transplant (T) one day after the transplantation. (A) Hematoxylin and eosin stained section showing the beginning of rosette formation. Pyknotic cells are visible both in the host (H) retina and a few in the transplant close to the host retina. (B) Immunohistochemical 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 (p). Bar = 50 µm

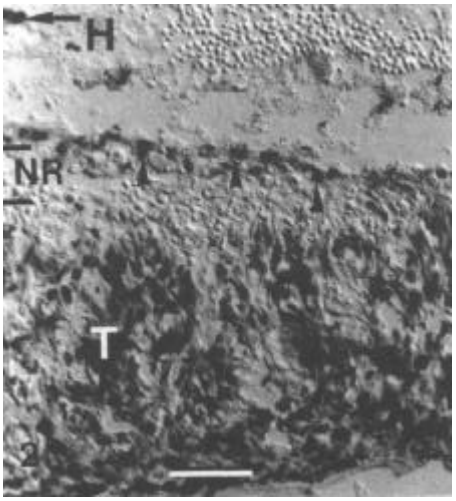

Fig. 2 Embryonic day 19 rabbit retinal transplant (T) showing a non-immunoreactive layer of cells (NR) close to the host retina (H). In the non-reactive layer there are a few cells at the host-graft interface (arrowheads), which are immunoreactive. One immunoreactive cell is visible in the host retina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

By embryonic age 21 or 22 days, the rosettes were more distinct. Small and pyknotic cells were seen close to the host retina as described earlier. Large, deeply immunoreactive mitotic cells were often found arranged in the innermost parts of the rosettes, close to the lumen (Fig. 3 A and B). Some cells in the innermost layers of the rosettes had become non-immunoreactive, but the deeply stained cells were still present. There were reactive cells in the outer layers of the rosettes and small clusters of non-reactive cells in between the rosettes. There were clusters of immunoreactive cells in between the rosettes as well as small clusters of cells that were not immunoreactive. A layer of non-reactive

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 non-reactive area (Fig. 3A). A small number of immunoreactive cells were still present in the host retina close to the transplant (Fig. 3B).

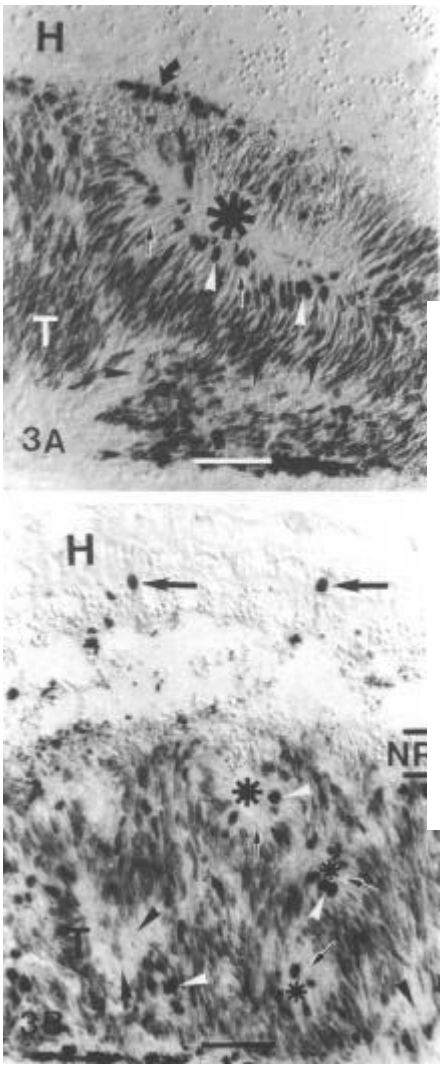

Fig. 3 (A and B) Embryonic day 21 rabbit retinal transplant (T). Some of the cells in the innermost layers of rosettes (lumen of rosettes marked with asterisks) are non-reactive (white arrowheads). There are also clusters of non-reactive cells in between the rosettes (arrowheads). In the layer of non-reactive 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 non-immunoreactive cells close to the host retina. Bar = 50 µm.

At embryonic age 26 days (Fig. 4), most cells in the inner layers of the rosettes were non-reactive, 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.

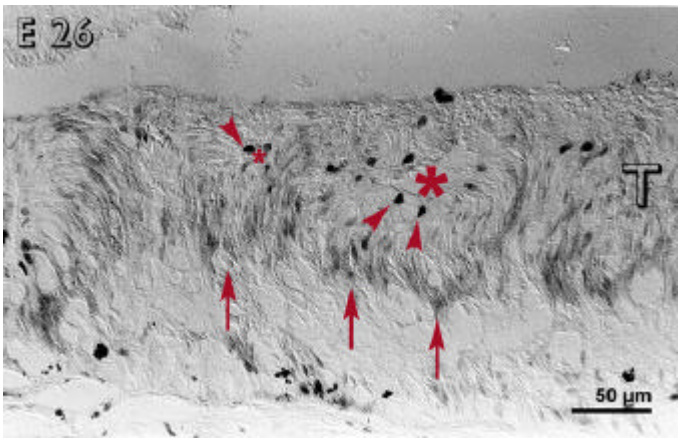

Fig. 4 Embryonic day 26 rabbit retinal transplant (T) showing rosettes (lumen marked with asterisks) with deeply stained cells (arrowheads) towards the outer limiting membrane. The inner layers of the rosettes are non-reactive whereas the outer layers are reactive (arrows). Immunohistochemical staining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

At embryonic age 29, cells in the inner layers of the rosettes were non-reactive, 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.

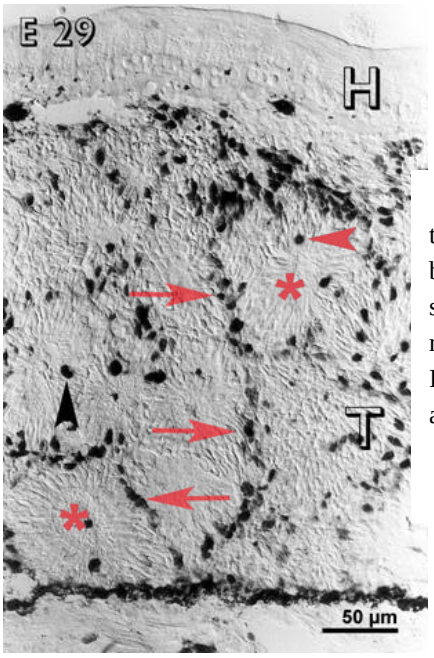

Fig. 5 Embryonic day 29 rabbit retinal transplant (T) showing reactive cells only in between the rosettes (arrows) and some deeply stained cells (arrowhead) towards the lumen of the rosettes (lumina marked with asterisks). Immunohistochemical staining with the MIB-1 antibody. H = host retina, Bar = 50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

At a transplant age corresponding to postnatal day 2, the cells in between the rosettes were still immunoreactive and there were immunoreactive cells in the host retina and host-graft interface (Fig. 6). At a transplant age corresponding to postnatal day 4, most of 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.

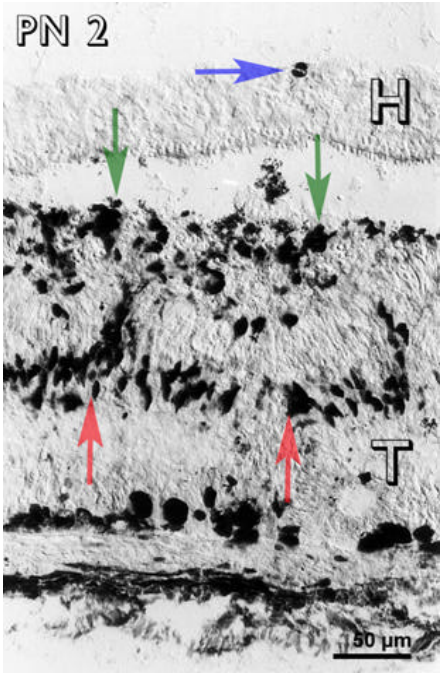

Fig. 6 Postnatal day 2 rabbit retinal transplant (T) showing immunoreactive cells in between the rosettes (arrows) and at the host-graft interface (green arrows). An immunoreactive cell is seen in host retina (blue arrow). H= host retina. Immunohistochemical staining with MIB-1 antibody. Bar = 50 µm.

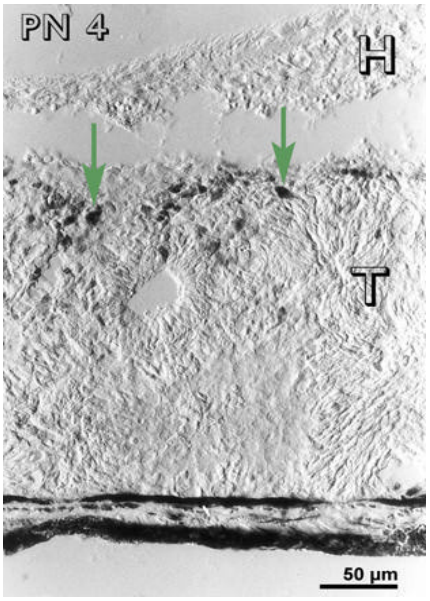

Fig. 7 Postnatal day 4 rabbit retinal transplant (T) showing immunoreactive cells at the host-graft interface (curved arrows). Immunohistochemical staining with the MIB-1 antibody. H= host retina, Bar = 50 µm.

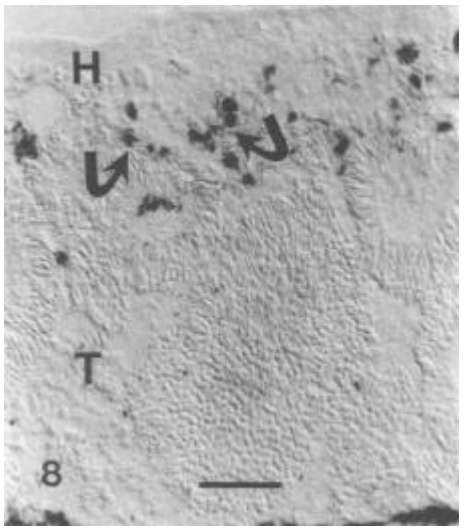

Fig. 8 Postnatal day 11 rabbit retinal transplant (T) showing immunoreactive cells at the host-graft interface (curved arrows). Immunohistochemical staining with the MIB-1 antibody. H= host retina, Bar = 50 µm.

Controls

When the slides were not incubated in the primary antibody, there was no staining of the neural retinas in any of the transplanted retinas.

Discussion

Proliferation and development of retinal transplants

One day after the transplantation, only minimal organization was observed in the transplanted cells. Four days after the transplantation (E19), the cells often appeared to be arranged in rosettes, and this became more and more prominent with increasing age of the transplant. Photoreceptors were present at the luminal side of the rosettes, and were surrounded by layers corresponding to the inner layers of normal retina. In developed transplants, the photoreceptor outer segments were protruding into the lumen of the rosettes, where at times an outer limiting membrane could be seen. The rosette formation and the lamination in retinal grafts has been well documented in previous studies (6,28,32). The transplants lack a pigment epithelium of their own, but it has been reported that the phagocytic function of the pigmented epithelium may be performed by macrophages in the lumen of the rosettes (4).

In the early transplants, the proliferating cells were randomly distributed, including the mitotic ones, which appear as large, rounded and deeply stained cells. This most likely reflects the random overall organization seen at this stage. In places, darkly staining mitotic cells appeared to cluster together to form the beginning of what would become a rosette.

Randomly distributed postmitotic cells were also seen, as might be expected from the observation that there are such cells already in E 15 retinas (29,30). It has been suggested that the best time for harvesting CNS donor tissue is before the cells undergo terminal mitosis (9,10). Since E 15 retinas already have

postmitotic cells, and since these postmitotic cells are likely to be the ganglion cells (29,30) this stage may not be the best time to harvest the donor tissue with respect to ganglion cell survival and development. Most transplants have previously been performed with tissue from E 15 or older fetuses, and this may partially explain why it has hitherto not been possible to see ganglion cells in the transplants.

As the transplanted cells reorganize themselves in rosettes (E19), the proliferating cells also adopt the general proliferation pattern seen in normal retinogenesis. It is interesting to note that the mitotic figures in the normal developing retina accumulate at the outer limiting membrane (the distal retina;) (29,30). 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 (29,30), 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 E 21 and more so at E 22, 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 non-proliferating cells in between the rosettes should be the postmitotic cells differentiating into amacrine and horizontal cells, since these cells are born early in ontogeny (20,21,29,30).

In E 26 transplants, the luminal-most layers of the rosettes were largely non-proliferating, 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 E 21 or E 22. The pattern of proliferation thus resembles that in normal development where at E 25 and E 29 most of the proliferating cells are in a region distal to the postmitotic amacrine cells. This fits very well with the distribution of immunoreactive cells in the transplants.

In E 29 and PN 2 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 (29,30).

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 (22,29,30). 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 E 19 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 non-proliferating 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 (6,28). 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 non-proliferating pyknotic cells was much smaller in small transplants. Since 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 (24). In the same transplants, intrinsic graft glial cells (mostly Müller cells) develop approximately according to their normal timetable, becoming partly reactive to GFAP after 3 weeks, and completely reactive 5 weeks 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 (25) and glial fibrillary acidic protein (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 E 25 and E 29 normal retinas, the proliferation of cells was much more than at comparable ages in transplants. For example, at E 29 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 (29,30). Further, proliferation in postnatal transplants was minimal, whereas significant proliferation persists up to day PN 7 to PN 11 in normal development. However, cell death is prominent in the transplants and may also be a factor retarding the development 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. (22). After the retinal cell proliferation ceases in the first postnatal week in rabbits (13,22,23,29-31) the retina passively stretches with the sclera increasing the surface area up to three fold in rabbits at the cost of retinal thickness (22). 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 (15,18,19). 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 (24). Hypertrophy and migration of Müller cells within the host retina was also noticed adjacent to the lesion site, commencing 2 days after the transplantation (24). This is in accordance with the proliferating cells being glial cells.

Acknowledgements

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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