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rd1 Photoreceptor Degeneration: Photoreceptor Rescue
and Role of Metalloproteases in Retinal Degeneration

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2005

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In the memory of
My mom Sudesh Ahuja
My grandmother Tara Devi Wadhwa
My mentor Natalia I Kudrishova

Papers included in this Study

This thesis is based on the following manuscripts, referred to by their roman numerals in the text:

Paper I

Caffe AR, Ahuja P, Holmqvist B, Azadi S, Forsell J, Holmqvist I, Soderpalm AK, van Veen T

Mouse retina explants after long-term culture in serum free medium.

J Chem Neuroanat. 2001 Nov; 22(4):263-73

Paper II

Ahuja P, Caffe AR, Holmqvist I, Soderpalm AK, Singh DP, Shinohara T, van Veen T

Lens epithelium-derived growth factor (LEDGF) delays photoreceptor degeneration in explants of rd/rd mouse retina.

Neuroreport 2001 Sep 17; 12(13): 2951-5

Paper III

P. Ahuja, A. R. Caffé, S. Ahuja, P. Ekström, T. van Veen

Decreased Glutathione Transferase Levels in rd1/rd1 mouse retina, Replenishment protects photoreceptors in retinal explants.

Neuroscience 2005; 131(4): 935-944

Paper IV

S Ahuja, P Ahuja, AR Caffé, P Ekström, M Abrahamson, T van Veen
rd1 Mouse Shows Imbalance in Cellular Distribution and Levels of TIMP-1/ MMP-9, TIMP-2/ MMP-2 and Sulphated Glycosaminoglycans.

Submitted to Ophthalmic Research

Other Related Papers

Paper I

Archer SN, Ahuja P, Caffè R, Mikol C, Foster RG, van Veen T, von Schantz M

Absence of phosphoglucose isomerase-1 in retinal photoreceptor, pigment epithelium and Müller cells

Eur J Neurosci. 2004 Jun; 19(11):2923-30.

Paper II

Hauck S, Ekström P, Suppmann S, Ahuja Jensen P, Paquet-Durand F, van Veen T, Ueffing M

Differential Modification of Phosducin Protein in rd1 Mice Detected by Proteomic Profiling

Manuscript

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I Abbreviations

AIF	Apoptosis Inducing Factor
Ask-1	Apoptosis signal Regulating Kinase-1
Apaf-1	Apoptosis protease activating factor
ARVO	The Association for Research in Vision and Ophthalmology
BAF	boc-aspartyl (OMe)-fluoro-methyl ketone
BDNF	Brain Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
CNTF	Ciliary Neurotrophic Factor
Cyt c	Cytochrome c
DD	Death Domain
DED	Death Effector Domain
DHA	Docosahexanoic Acid
ECM	Extracellular Matrix
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GDNF	Glial Neurotrophic Factor
GST	Glutathione S-Transferase
HDGF	Hepatoma Derived Growth Factor
JNK	c-Jun N-terminal kinase
HSE	Heat Shock Element
Hsp	Heat Shock Proteins
IRBP	Interphotoreceptor Retinoid Binding Protein
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
IPM	Interphotoreceptor Matrix
LEDGF	Lens Epithelium Derived Growth Factor
LEC	Lens Epithelial Cells
MAPK	Mitogen-activated Protein Kinase
MEKK1	Mitogen-activated Protein Kinase (MAPK) Kinase Kinase
MMPs	Matrix Metalloproteinases
NMDA	N-methyl-D-aspartate
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PARP	Poly (ADP- ribose) Polymerase
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline plus Triton X-100
PDE	Phosphodiesterase
PEDF	Pigment Epithelium Derived Factor
PN	Postnatal day
PVDF	Polyvinylidene difluoride
<i>rd1/rd1</i>	Retinal Degeneration 1
RdCVFs	Rod-dependent Cone Viability Factor
RCS	Royal College of Surgeons rats
RP	Retinitis Pigmentosa
RPE	Retinal Pigment Epithelium
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
STRE	Stress Related Regulatory Element
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline plus Triton X-100
TIMPs	Tissue Inhibitors of Matrix Metalloproteinases

II Aims of the Study

The overall aim of the studies is:

To evaluate the effect of different rescue factors in protecting the retina from degeneration using serum free *in vitro* organ culture system and to study the role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in retinal degeneration.

The specific aims of these studies are:

- To develop long term serum free retina organ culture system so as to mimic the *in vivo* retina. The organ culture has an advantage as it maintains the normal intracellular interaction in the retina. Efficacy of various substances can be studied by their addition to the culture medium.
- To identify and study molecules that could eventually be used as rescue factors viz. effect of Lens Epithelium Derived Growth Factor (LEDGF) and Glutathione S Transferase (GST) alpha and mu in rescuing the photoreceptors in *rdl/rdl* mouse; and to study the mechanism behind the rescue effect of LEDGF and GST.
- To study biochemical basis of retinal degeneration by evaluation of differences in levels of MMPs and TIMPs in the normal wild type retina and *rdl/rdl* retina undergoing degeneration.

III Introduction

Retinitis Pigmentosa (RP) is a heterogeneous group of inherited retinal degenerative diseases leading to blindness. The condition is characterised by progressive loss of rod photoreceptors followed by that of the cones. In the industrialised world RP affects 1 in 3000 people (Kalloniatis and Fletcher, 2004). Early stage of the disease, resulting from the loss of rods, is characterised by impaired adaptation to light/dark environment, night blindness and constriction of mid-peripheral visual fields. The loss of peripheral vision progresses slowly over many years until only central (tunnel) vision remains. Contrast vision and colour vision also deteriorate with time. In later stages patients typically show abnormal accumulation of pigments in the midperiphery of the retina, attenuated retinal vasculature and waxy type of optic disc atrophy.

RP is inherited as an autosomal dominant, autosomal recessive, X-linked, simplex and multiplex disease where autosomal recessive is the most common mode of inheritance (Kalloniatis and Fletcher, 2004). Inherited forms of retinal degeneration are largely caused by gene mutations within the photoreceptors or retinal pigment epithelium cells (RPE) and are caused by defects in activation or deactivation of the visual pigments or in proteins / enzymes important for functioning of photoreceptor or those involved in the visual phototransduction cascade. In all forms of RP the final common pathway leading to the loss of photoreceptors is apoptosis (Chang et al., 1993; Portera-Cailliau et al., 1994; Lolley, 1994; Lolley et al., 1994). At present, except for genetic counseling, no effective treatment is available to prevent or cure the disease. Some of the different approaches focused on finding a treatment for RP are as follows:

- Gene therapy: aimed at curing the specific genetic disorder (Bennett et al., 1996; Jomary et al, 1997, Pang et al., 2004) or to be used on secondary targets involved in the activation/inhibition of the apoptotic cascade (antiapoptotic gene therapy) (Bennett et al., 1998).
- Retina cell or tissue transplantation and stem cell transplantation: aimed at replacing the lost cells and / or as extrinsic providers of trophic factors (Gouras et al., 1992; Gouras et al., 1994; Ghosh and Ehinger, 2000; Mohand-Said et al., 2000; Lu et al., 2002; Gouras and Tanabe, 2003; Lund et al., 2003; Arai et al., 2004).
- Pharmacological neuroprotection of photoreceptors as a way of stabilizing the process of photoreceptor degeneration by stopping or retarding the process of degeneration by using (a) cytokines and growth factors e.g. Ciliary Neurotrophic Factor (CNTF), Brain Derived Neurotrophic Factor (BDNF) and other factors (b) rescue factors e.g. calcium channel blocker diltiazem; Rd-dependent Cone viability factor (RdCVFs); (c) antioxidants like vitamin A, coenzyme Q10 (Lavail et al., 1996; Cayouette and Gravel, 1997; Lavail et al.,

1998; Li et al., 1998; Frasson et al., 1999b; Bush et al., 2000; Ogilvie, 2001; Caffè et al., 2001b; Van Hooser et al., 2000; Takano et al., 2004; Leveillard et al., 2005).

Animal Model for Recessive form of Human RP

A number of natural animal models of RP are available displaying many of the gene mutations responsible for retinal degeneration in humans. Several of these animal models have been studied in order to elucidate the developmental progression of the disease and the molecular pathology of RP. As a consequence, the use of these animal models has led to a better understanding of the genetic and biochemical mechanisms resulting in photoreceptor death and also in devising approaches to rescue the photoreceptors. The *rdl/rdl* mouse is one of the most widely used animal model. It displays a mutation in the β subunit of cGMP phosphodiesterase (PDE), one of the genes also responsible for some forms of human autosomal recessive RP (Ulshafer et al., 1980; McLaughlin et al., 1993; McLaughlin et al., 1995). In the normal retina phototransduction is initiated when the rod rhodopsin absorbs a photon of light. Photo excited rhodopsin then complexes with rod G protein called transducin. The activated α subunit of transducin in turn activates PDE complex by releasing the inhibitory PDE γ subunit from the catalytic complex composed of PDE α and PDE β subunits. Activated PDE hydrolyzes cGMP and a decrease in cGMP levels leads to closure of cation channels in the cell membrane leading to hyper polarisation (Stryer, 1991). In the RP mouse model, *rdl/rdl* a mutation in the β subunit of the rod cGMP phosphodiesterase gene leads to an increase in cytoplasmic cGMP (Bowes et al., 1990). Increased cGMP concentration in cytoplasm results in permanent and continuous opening of cGMP-gated channels in the photoreceptor plasma membrane, allowing the excessive entry of extracellular ions, particularly calcium. Increase in the intracellular calcium (Fox et al., 1999) in turn causes a metabolic overload of the cells, eventually leading to rod cell death by apoptosis (Chang et al., 1993; Travis, 1998; Jomary et al., 2001). This increase in retinal cGMP precedes rod degeneration (Lolley et al., 1977).

Development and Degeneration in *rdl/rdl* Retina

In the *rdl/rdl* mouse, which shows rapid degeneration of rod photoreceptors, the development of retina is comparable to the normal wild type (*wt*, *+/+*) but it is somewhat slower until postnatal (PN) day 10. The separation of the inner nuclear layer (INL) and the outer nuclear layer (ONL) occurs two days later than in the normal wild type retina. The growth of inner segments is retarded and the inner segments at PN4 contain fewer mitochondria and ribosomes. Between PN6 and PN8 in the inner segments vacuole like structures can be seen as well as mitochondria showing disintegration of the mitochondrial matrix. The outer segments develop but are smaller and contain fewer discs than wild-type photoreceptors (Sanyal and Bal, 1973; Bowes et al., 1988; Bowes et al., 1989). At

PN10 the photoreceptors reach their maximum development in the *rd1/rd1*. After this age the outer segments become disorganized and a rapid degeneration of the rods begins (Sanyal and Bal, 1973). In the *rd1/rd1* mouse the degeneration of the rods in the ONL becomes visible after PN11 and in the adult PN14 there is a considerable decrease in the number of rods in the ONL. By PN21 all the rod photoreceptors have degenerated and the ONL consists only of a single layer of cones without inner and outer segments (Figure 1.). The numbers of cones also decreases with increasing age (reduced to 50% by PN28) and eventually they all

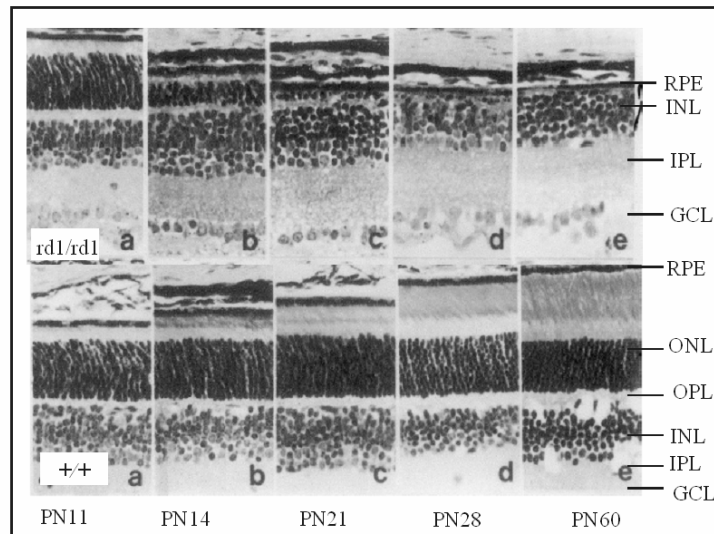


Figure 1. Comparison of the *rd1/rd1* and *+/+* retina at different ages in development

degenerate in the absence of rods. There are a number of hypotheses to explain the secondary loss of cone photoreceptors because they are not affected directly by the genetic mutation (Delyfer et al., 2004). Some of the hypotheses are mentioned below:

- Toxic by-products of rod cell death may be responsible for promoting the cone cell death.
- The loss of rods causes both structural and biochemical changes in the microenvironment of the cones.
- Rods secrete survival factor(s) that are essential for the cone viability. When the rods are lost the cones are deprived of such survival factor(s), and eventually degenerate.
- The secondary degeneration of cones may also be related to abnormal synaptogenesis and due to the loss of vasculature.

Mechanism of Apoptosis in *rd1/rd1* Retina

Apoptosis is possibly the mode of cell death in the *rd1/rd1* mouse retina and has been shown to be responsible for the loss of rods (Chang et al., 1993; Portera-Cailliau, et al., 1994) but the exact mechanism still needs to be elucidated. Apoptosis in the retina can take place via the caspase-dependent pathway or the caspase-independent pathway (Reme et al, 1998; Wenzel et al., 2005). It could also be possible that both these processes are involved as caspase inhibition offers only a transient protection.

It is widely accepted that in *rd1/rd1* mouse retina, apoptosis takes place via the caspase dependent pathway (Jomary et al., 2001; Kim et al., 2002; Sharma and Rohrer, 2004) but reports contrary to this also exist (Yoshizawa et al., 2002; Zeiss

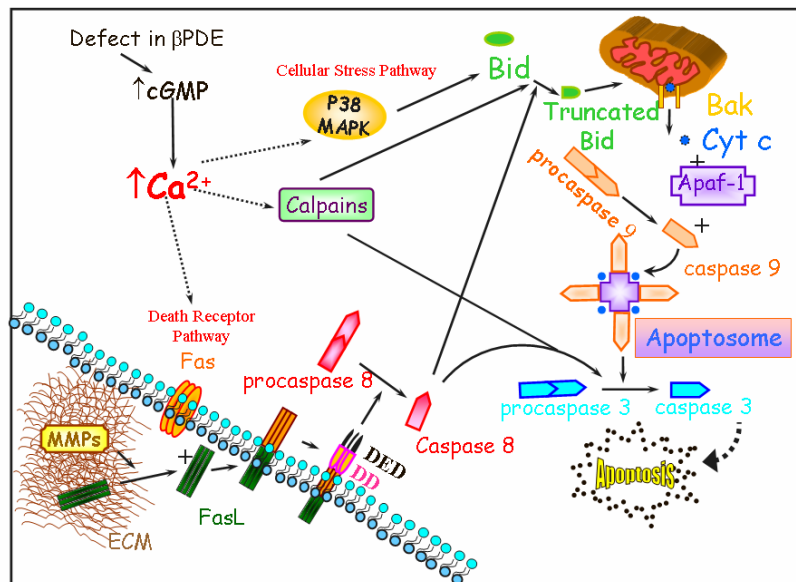


Figure 2. Apoptotic pathway in the *rd1/rd1* retina

et al., 2004; Doonan et al., 2003). According to Jomary et al., (2001) in *rd1/rd1* mouse retina the apoptosis involves the caspase dependant pathway comprising of the death receptor and cellular stress pathway (Figure 2.).

According to the cellular stress pathway, p38, a mitogen activated protein kinase can mediate the cleavage of Bid. Truncated Bid moves from the cytosol to the outer mitochondrial membrane where it interacts with Bak, a member of the Bax subfamily of the proapoptotic Bcl2 family. This interaction results in conformational change in Bak and formation of pores in the mitochondrial outer membrane, enabling the release of cytochrome c (Cyt c). Once free in the cytoplasm Cyt c promotes the assembly of apoptotic protease-activating factor (Apaf-1) and procaspase 9 into a tetrameric complex called the apoptosome. The

active caspase 9 in the apoptosome converts procaspase 3 to its active form, caspase 3. Caspase 3 activates DNA fragmentation factor, which in turn activates endonucleases and terminates in apoptosis.

The death receptor pathway begins with the binding of the death receptor Fas to its ligand Fas L. This causes rapid formation of a death inducing signaling complex involving death domains (DD) and death effector domains (DED). This complex activates pro caspase 8 to caspase 8 which in turn activates caspase 3 and cleaves Bid. Both of them can lead to the downstream processes of apoptosis.

Another recent report in favor of the caspase dependent apoptotic pathway has shown that in the *rd1/rd1* retina that Ca^{2+} dependent cysteine proteases, calpains, are activated by an increase in intracellular calcium levels. Calpains can cleave bid and/ or cleave procaspase 3 to form caspase 3 which initiates the whole process as described above (Sharma and Rohrer, 2004; Sanvicens et al., 2004)

In the *rd1/rd1* retina, the caspase independent pathway has not been described exactly and in detail. The key players to be investigated in such a pathway could possibly be non-caspase proteases like cathepsins, calpains, AP-24, granenzyme A and B or even MMPs. Also of interest are caspase independent death effectors like apoptosis inducing factor (AIF), endonuclease G and poly (ADP- ribose) polymerase (PARP) (Wenzel et al., 2005).

The Retinal Explant Organ Culture System

The retinal explant paradigm was developed in the laboratory of Dr Sanyal (Caffé et al., 1989). It involves careful dissection of the retina with the attached RPE and after dissection the tissue is placed on a membrane which is further cultured in the culture medium. The retina can be maintained *in vitro* for more than 4 weeks and this gives an opportunity to study the major developmental as well as degenerative processes both in the normal *+/+* and the *rd1/rd1* mouse model (Caffé et al., 1989; Soderpalm et al., 1994; Ogilvie et al., 1999). The retinal explant organ culture system has the following advantages:

- The retina in the organ culture is cultured as a whole organ with the RPE attached to it so that normal cell-to-cell connections and interactions are maintained. Photoreceptor cell-RPE-cell interactions are also maintained which are important for normal retinal and photoreceptor development. The *in vitro* explant organ culture system is a condition nearest to the *in vivo* situation.
- The organ culture system provides an excellent tool for studying retinal development and degeneration. Most of the histotypic and neurochemical characteristics develop at roughly equivalent ages when compared with the *in vivo* littermates.

- It allows study of the whole retina as the development and degeneration proceed from center to periphery.
- The retina is easily accessible for experimentation and the test substances can be added to the culture medium at different concentrations to study their effect.
- The cultured retina and spent medium can be collected and analyzed to study the changes in retina and molecules secreted by the retina.

The culture medium used in most of the culture systems contains serum. Serum is a complex emulsion of proteins (globulins, albumin), polypeptides (trophic factors and growth inhibitors), lipids and additionally organic and inorganic molecules. Even tissue inhibitors of matrix metalloproteinase-1 (TIMP-1) and TIMP-2 have been reported to be present in the serum. These constituents can stimulate growth (Wang et al., 2002). Another report shows that thrombopoietin which is present in the serum is proapoptotic (Ehrenreich et al., 2005). Thus serum can be attributed heterogeneity in its composition. Secondly serum components differ from batch to batch qualitatively. This limits the validity of the system and creates obstacle(s) for straightforward interpretation of results. Thus in the first paper (Paper I) we tried to establish a serum free retina explant organ culture.

In the retina explant organ culture, the *rdl/rdl* retina develops and degenerates similarly as compared to the *in vivo* age matched *rdl/rdl* retina but the degeneration is slightly slow. After four weeks in culture (PN28) the *in vitro* retina contains two to three rows of photoreceptors in the ONL as compared to one row of photoreceptors in the age matched *in vivo rdl/rdl* retina. An intriguing feature of the organ culture system is that green cones do not develop in culture (Soderpalm et al., 1994; Caffè et al., 2001a).

Oxidative Stress

Oxidative stress or changes in the redox state is the result of cellular production of reactive oxygen species (ROS) which are involved in the pathogenesis of neurodegenerative diseases like Alzheimer's and Parkinson's disease. It can also be defined as an imbalance between pro-oxidants and/or free radicals on the one hand, and anti-oxidizing systems consisting of enzymes like glutathione peroxidase, catalases on the other. ROS are also produced in the normal state of the retina (Marak et al., 1990; Yildirim et al., 2004) due to the following special conditions of the retina:

- As compared to any other tissue the oxygen supply and consumption of the retina is very high (Sickel, 1972).

- Retina is exposed to high levels of cumulative irradiation due to the extensive exposure to light
- The retina contains a large amount of photosensitizer molecules e.g. retinal, which on contact with oxygen generate singlet oxygen (Delmelle et al., 1978; Shvedova et al., 1982)
- The photoreceptor outer segment membranes contain a large amount of polyunsaturated fatty acids which can initiate a cytotoxic chain reaction e.g. docosahexanoic acid (DHA).
- The extensive and continuous process of phagocytosis of photoreceptors by the RPE also generates ROS

In the *rd1/rd1* retina the toxic byproducts of degeneration are also another factor that leads to the increase in ROS and thus oxidative stress. Additional evidence that ROS and oxidative stress are involved in retinal degeneration comes from the fact that many antioxidants have been shown to retard or inhibit the degeneration process (Ahuja et al, 2005; Lam et al., 1990; Rosner et al., 1992; Ranchon et al., 1999). Also in microarray experiments comparing retinas from *rd1/rd1* and wild type mice, it has been shown that in the *rd1/rd1* retina, genes of products that are related to providing protection against oxidative stress are downregulated and can be involved in secondary loss of cones (Hackman et al., 2004). As mentioned above the retina has a rich supply of oxygen and when the rods are lost due to the degeneration the requirement of oxygen decreases but the supply is unchanged and this results in excessive oxygen supply to the cones (Hackman et al., 2004). This hyperoxia in the cones over a long period can lead to their degeneration (Okoye et al., 2003; Yamada et al., 2001).

Oxidative stress and ROS can also lead to apoptosis in many ways (Carmody et al., 1999), both via the caspase dependent and caspase independent pathways. For example, calpains are involved in photoreceptor cell death via the caspase dependent pathway, but their activation can be prevented by scavengers of ROS (Sanvicens et al., 2004). ROS in turn can inactivate caspases by binding to thiol group in the active site of the enzymes. ROS may thus inactivate caspases but they in turn can by themselves lead to apoptosis via the caspase independent pathway (Carmody and Cotter, 2000). ROS induces expression of MMPs (Lorenzl et al., 2004) which can degrade IPM / ECM and lead to the detachment of photoreceptors from RPE. The detached photoreceptors are likely to undergo apoptosis (see below).

Different survival factors especially various antioxidants have been reported to protect the eye from the damaging effect of oxidative insult. Various substances like glutathione S-transferases (GSTs) (Maeda et al., 2005); DHA (Rotstein et al., 2003); melatonin (Liang et al., 2004); CNTF (Koh, 2002); BDNF (Okoye et

al., 2003); FGF2 (Yamada et al., 2001) and LEDGF (Singh et al., 1999; Sharma et al., 2000; Fatma et al., 2001; Matsui et al., 2001) have been shown to protect cells in the retina from oxidative stress.

Survival Factors

Faktorovich et al. (1990) were the first to show that basic fibroblast growth factor (bFGF) had survival-promoting effect on the photoreceptors of Royal College of Surgeons (RCS) rats. Since then a large number of substances with different modes of action have been investigated in different animal models of RP and one of these factors, CNTF has reached the stage of clinical trials. In the *rd1/rd1* retina a number of substances including various neurotrophins e.g. BDNF; cytokines e.g. CNTF; growth factors e.g. Glial neurotrophic factor (GDNF), Pigment epithelium derived factor (PEDF), (bFGF); inhibitors of apoptosis e.g. boc-aspartyl (OMe)-fluoro-methyl ketone (BAF) and calcium channel blockers e.g. diltiazem have been investigated and shown to have neuroprotective effect (Lavail et al., 1996; Lavail et al., 1998; Cayouette et al., 1999; Frasson et al., 1999a; Frasson et al., 1999b; Ogilvie, 2001; Caffè et al., 2001b). These neuroprotective substances prevent rod photoreceptor degeneration by stopping or slowing down the process of degeneration. They are thus involved in modulating the microenvironment of the photoreceptors, prevent cone loss and consequently stabilize vision. Some of these substances act by blocking apoptosis. In some preliminary studies it has also been shown that growth factors are also able to up regulate levels of other endogenous growth factors (Azadi et al., 2002). It has been shown in various studies that when growth factors are used in combination they have strong synergistic effect on rod survival (Caffè et al., 1993; Ogilvie et al., 2000; Caffè et al., 2001b). Substance with different modes of action can possibly be combined to reach a greater survival effect as when used alone. Another possibility is that different survival factors can be used in combination with other methods e.g. with transplantation to support the transplanted cells or with stem cell transplantation to help in differentiation of these cells. The advantages of the use of survival factors are that they can, with high probability, be used in the treatment of many different types of RP as they circumvent the tremendous genetic heterogeneity of RP and thereby provide treatments applicable to most of the inherited retinal degenerations (Delyfer et al., 2004).

Limitations of the use of these survival factors are that most of these substances are in the experimental stage and exact pathways of their mode of action remain unknown. Knowing the cellular and molecular mechanism underlining the rescue effect are a prerequisite for human trials. Secondly all substances do not have similar effect in different animal models of the same disease as well as different animal models for different diseases (Chader, 2002; Pearce-Kelling et al., 2001). Also there are many other obstacles to be overcome e.g. determination of the

mode of delivery, dosing regime, safety and side effects of the use of such rescue factors.

An important aspect concerning different survival factors is the determination of the most efficient mode of delivery to the retina. Traditionally most of the substances have been injected intravitreally (Lavail et al., 1998), subretinally (Faktorovich et al., 1990), intraperitoneally (Yoshizawa et al., 2002), retro-ocular (Lambiase and Aloe, 1996) and orally (Pearce-Kelling et al., 2001). Some novel methods of delivery of growth factors have been published recently. One of these methods is the adeno-virus mediated transfer of genes providing these growth factors (Cayouette and Gravel, 1997; Bennett et al., 1998). The other method is the use of encapsulated cells to deliver these substances (Tao et al., 2002). Tao et al. (2002) used polymer membrane capsules (1.0 cm in length and 1.0 mm in diameter), which were loaded with mammalian cells that were genetically engineered to secrete CNTF. The advantage of these two methods is that they allow a slow, sustained and continuous delivery of these substances (Cayouette and Gravel, 1997; Bennett et al., 1998).

Lens Epithelium Derived Growth Factor (LEDGF)

Singh et al. (1998) first reported about a novel growth, adhesive, differentiation and antiapoptotic factor cloned from a human lens epithelial cell (LEC) cDNA library. LEDGF belongs to a family of homologous proteins including hepatoma derived growth factor (HDGF) and HGDF-related protein-1 and -2 (Nakamura et al., 1994; Izumoto et al., 1997). It is a 60 kDa protein found in the nucleus at low levels in most of the actively dividing cells and long living cell types such as lens epithelial cells and neural cells (Singh et al., 1999; Singh et al., 2000; Kubo et al., 2000). LEDGF rescues many cell types under stress (thermal and oxidative) e.g. LECs, cos7 cells, fibroblasts and keratinocytes in cell culture. LEDGF has been shown to rescue embryonic chick photoreceptor cells from serum starvation and heat stress (Nakamura et al., 2000); rescues light damaged photoreceptor cells in Lewis rats and RCS rats (Machida et al., 2001) and protects rat retinal cells against cell death induced by NMDA (Inomata et al., 2003). During the present studies the effect of LEDGF supplementation on the rescue of photoreceptors in *rd1/rd1* mouse was studied and presented as paper II.

Glutathione S- Transferase

Glutathione S-Transferases (GSTs) constitute a family of cytosolic (microsomal) isoenzymes that are involved in the detoxification of electrophilic xenobiotics. GST conjugates reduced glutathione to a variety of electrophilic xenobiotics, toxicants and products of oxidative stress and thus has a role as antioxidant (Listowsky et al., 1988; Hayes and Pulford, 1995; Hayes and Strange, 1995). GSTs are classified into alpha, mu, pi, theta, zeta and omega classes with alpha, mu and pi being the predominant ones. Each class is further divided into

subclasses. The major sites of occurrence of various forms of GST in the rat retina are as follows: Y_{b1}-mu is present in the outer segments, outer plexiform layer (OPL), inner plexiform layer (IPL), müller cell body and end feet; Y_{b2}-mu is present in the IPL, müller cell body and end feet; Y_p-Pi is in amacrine cells, IPL, ganglion cells and Müller cell end feet; Y_a-alpha is present in the OPL, müller cell body and end feet (McGuire et al., 1996).

Recent studies have shown that supplementation of glutathione peroxidase, thioredoxin, superoxide dismutase (SOD), or catalase and their synthetic mimetics, to growth medium may protect neurons or RPE cells *in vitro* (Akeo et al., 1996; Castagné and Clarke, 2000). All of these are redox-regulating enzymes. However, their neuroprotective effect may be independent from their involvement in redox regulation. Such an example is GST. Intracellular π -GST interacts with c-Jun N-terminal kinase (JNK), whereas μ -GST and thioredoxin interact with and inhibit apoptosis signal-regulating kinase 1 (ASK-1), which modulates the two downstream JNK and p38 mitogen activated protein kinase (MAPK) apoptotic pathways and inhibits or deactivates them (Adler et al., 1999; Cho et al., 2001). The p38 apoptotic pathway has been linked directly to *rd1/rd1* retinal degeneration (Jomary et al., 2001). Several forms of GST isoenzymes interact with different protein kinases in stress-induced pathways thus indicating that GST isoenzymes might play an additional role at the level of cellular signalling and regulation. Usually intracellular GST levels change after trauma or during pathology (Mannervik and Danielson, 1988; Lovell et al., 1998), in agreement with reports that investigated this issue in retina (McGuire et al., 1996; McGuire et al., 2000). But, whether this also happens during *rd1/rd1* retinal degeneration is unknown. Therefore, we studied the following questions: (1) if tissue levels of GST change during *rd1/rd1* retinal degeneration and (2) whether exogenous GST can delay *rd1/rd1* photoreceptor loss *in vitro*. The results are described in paper III

MMPs and TIMPS

Matrix Metalloproteinases (MMPs) are a group of enzymes that are involved in the continuous maintenance of tissue architecture. This is controlled mainly by the coordinated activities of these enzymes with their endogenous inhibitors i.e. tissue inhibitors of MMPs (TIMPs). MMPs are a family of Zn²⁺-containing and Ca²⁺-requiring endoproteases capable of degrading elements of the extracellular matrix such as collagens, elastin, laminin, fibronectin, proteoglycans and glycoproteins in normal and pathological conditions. The expression and activity of MMPs are controlled at the transcriptional level, where MMP expression is regulated by growth factors, cytokines and free radicals (Beuche et al., 2000), activation after removal of inactivating peptide and by endogenous inhibitors. They are released from astrocytes, neurons and microglia as well as leukocytes and macrophages, and their target are compounds mentioned above (Lorenzl et al., 2003). In humans, the MMP family comprises 22 members that can be

classified into five subgroups, based on domain structures and substrate specificity: interstitial collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), membrane type-MMPs (MT1, -2, -3, -4, -5, and -6-MMPs) and other MMPs (Sternlicht and Werb, 2001). Because MMPs are produced in zymogen form (proMMP), they must be activated by the extracellular, or pericellular pathways to exhibit their proteolytic activities in the tissues. The activity of MMPs is dependent on activation of latent proforms, and additionally inhibition by endogenous TIMPs (Brew et al., 2000). TIMP-2 accelerates activation of proMMP-2 by functioning as a link protein for the interaction between proMMP-2 and MT1-MMP on the cell membranes and formation of tri-molecular complex proMMP-2/TIMP-2/MT1-MMP (Butler et al., 1998). MMPs involved in the degradation of the extracellular matrix can interfere with cell attachment and signalling leading to apoptosis. MMPs can also release cell associated Fas ligand (Figure 2), which may then mediate apoptosis (Yong et al., 2001). In an interesting review, Nelson and Melendez (2004) reported the role of ROS and oxidants which activate signalling kinases like MAPKs, PI3K and p38 (Woo et al., 2004) that can participate in driving MMP expression.

TIMPs family consists of four members that have been characterized as TIMP-1, TIMP-2, TIMP-3, and TIMP-4. These are secreted as multifunctional proteins that bind MMPs to form tight, non-covalent inhibitory complexes and inhibit MMP activity. TIMP-1 and TIMP-2 are capable of inhibiting the activities of all known MMPs and play a key role in maintaining the balance between extracellular matrix (ECM) deposition and degradation in different physiological processes (Gomez et al., 1997). TIMP-2 forms a complex that is important in the cell-surface activation of pro-MMP-2, whereas TIMP-1 forms a specific inhibitory complex with pro-MMP-9 (Mannello and Gazzanelli, 2001). TIMPs also exhibit a variety of additional cellular functions, which are independent of MMP-inhibitory activity e.g. TIMP-1 and -2 can act as growth factors (Hayakawa et al., 1994). TIMPs have both pro-apoptotic as well as anti-apoptotic function (Mannello and Gazzanelli, 2001) e.g. TIMP-3 is pro-apoptotic (see below) while TIMP-1 has been shown to suppress apoptosis and act as survival factor in B cells (Guedez et al. 1998). In the eye TIMP-3 has been shown to be involved in three different retinal degenerative diseases: simplex retinitis pigmentosa (Jones et al., 1994b; Jomary et al., 1995), Sorsby's fundus dystrophy (Weber et al., 1994) and age related macular degeneration (Leu et al., 2001). In simplex RP, the expression of TIMP-3 is increased and may cause restructuring of the ECM architecture or disruption of inter photoreceptor-matrix interactions which could lead to activation/ initiation of apoptotic cell death processes.

The TIMPs and MMPs are involved in the regulation of ECM metabolism (Murphy, 1991; Woessner, 1991) and imbalances of the MMPs-TIMPs system(s) may result in diseases with uncontrolled turnover of matrix. An excess of protease over inhibitor can lead to excessive tissue destruction. MMPs have been

associated with certain degenerative diseases e.g. stroke (Clark et al., 1997); multiple sclerosis (Leppert et al., 1998); Parkinson's disease (Lorenzl et al., 2002) and Alzheimer's disease (Peress et al., 1995). In the eye MMPs and TIMPs are also involved in the normal turnover of the ECM that surrounds the neural retina and imbalances in this system can be seen in eye diseases like diabetic retinopathy (Noda et al., 2003) as well as in age related macular degeneration (Plantner et al., 1998a). The presence of MMPs and TIMPs has been demonstrated in normal IPM (Plantner, 1992; Plantner and Drew, 1994; Plantner et al., 1998b). Potential roles for this IPM MMPs–TIMPs system(s) could be physiological remodelling of the neural retina–RPE cell interface and digestion of the shed rod outer segments, as well as in retinal disease processes (Jones et al., 1994a).

We initiated studies on the cellular distribution and levels of different proteinases and their endogenous inhibitors like cathepsins/ cystatin C, MMPs/ TIMPs, calpains/ calpstatins and serine proteinases/ serpins. The present studies on MMP-2, MMP-9, TIMP-1 and TIMP-2 are reported here giving emphasis on MMP-2, MMP-9, TIMP-1 and TIMP-2 was made because of the above described multifunctional role in not only maintenance of ECM but also for their role in oxidative stress; process of apoptosis and role in retinal degeneration. The MMPs and TIMPs systems have not been described earlier in the *rd1/rd1* retina. This work is presented in paper IV.

IV Material and Methods

1) Animals and Tissues

All animals were treated in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and the European Communities Council Directive (86/609/EEC). The Swedish National Animal Care and Ethics Committee approved the experiments.

Congenic wild type control (+/+) mice of the C3H strain and homozygous retinal degeneration 1 (*rd1/rd1*) were used for the studies. Day of birth was considered as postnatal day 0 (PN0). The PN2 and PN7 pups were sacrificed by decapitation, whereas older mice (PN14, PN21 and PN28) were sacrificed by asphyxiation on dry ice.

For immunocytochemistry using different antibodies, eyes were taken from both *rd1/rd1* and +/+ mice during different ages in development viz. PN2, PN7, PN14, PN21 and PN28. After enucleation, the eyes were fixed in cold 4% paraformaldehyde in Sørensen's buffer for 1-2 hours and cryoprotected in Sørensen's buffer containing increasing concentrations of sucrose. 8µm sections were obtained on a cryostat (MICROM HM 560, MICROM Laborgeräte GmbH, Walldorf, Germany) and stored at -20°C until used.

For western blots and ELISA studies eye were enucleated from the similar group of animals as for immunocytochemistry. After enucleating the eyes, the anterior segment, vitreous body, sclera and choroids were removed in the cold dissecting medium and retinas with RPE were frozen at -80°C until used.

2) The Culture System

a) Culture Method

After the animals had been sacrificed, the heads were removed and cleaned with 70% ethanol. The eyes were enucleated aseptically and were incubated in R16 basal medium supplemented with 0.12% proteinase K (ICN Biomedicals Inc., Aurora, Ohio, USA) at 37°C for 15 min. This was done in order to facilitate the removal of the neural retina and RPE from adjoining mesenchymal cell layers. To stop the enzyme action by protein dilution, the eyes were placed in 5ml of 10% fetal calf serum (FCS) and then dissected aseptically under the dissecting microscope (Olympus SZX9, Olympus in Europe, Hamburg, Germany) in a Petri dish containing R16 basal medium. The anterior segment, lens and vitreous body were removed together by cutting a little behind the limbus (Figure 3). Using the #5 forceps with rounded edges the sclera and choroid were carefully peeled off leaving the neural retina intact with the RPE attached. An effort was always made to remove the retina with the intact RPE. Four cuts perpendicular to the edges of

the retina were made with the Vanessa iridectomy scissors. With the #5 forceps the retina was gently lifted holding the remnants of the cloquets canal and (with the RPE) flat-mounted with the photoreceptor side down (Figure 3) on a piece of GN-4 Metrical cellulose filter paper with pore size 0.8µm (Pall Gelman Sciences,

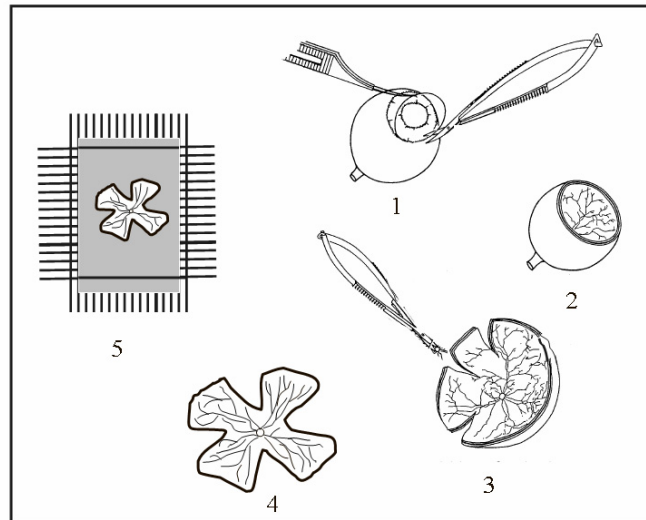


Figure 3. Culturing technique

Lund , Sweden) attached to a Monodur PA 56N polyamide grid (AB Derma, Gråbo, Sweden). The explants were placed in 6 well culture dishes containing 1.6 ml culture medium. The culture dishes were kept in a CO₂ incubator (HERA cell, Kendro Laboratory Products GmbH, Hanau, Germany) with 100% humidity and 5% CO₂ in air at 37°C.

b) Culture Medium

The culture medium used was the chemically defined serum free R16 medium (Cat. # 07490743A, Invitrogen Life Technologies, Paisley, Scotland). The R16 medium was originally used for culturing brain tissue (Romijn, 1988; Romijn et al., 1988). The R16 culture medium consists of ingredients (Table 1) that can be divided into three groups:

Group 1 Salts

Group 2 Amino acids except for the neurotoxic glutamate and aspartate

Group 3 Sugars, hormones and vitamins

To the R16 powder- Millipore water, NaHCO₃, biotin (0.1µg/ml), ethanolamine (1µg/ml) are added and this forms the basal medium. To the basal medium, 0.2% BSA and 19 other supplements (Table 2) composed of hormones and vitamins are added to form the complete R16 medium which is used for culturing. Cytidine 5'-

diphospho ethanolamine and cytidine 5'-disphospho choline were also added to the culture medium. The test substance was added to the R16 medium in the required concentrations. The medium in the culture dishes was replaced every other day with the same volume of fresh medium.

Table 1: List of Ingredients of R16 Culture Medium

Ingredient	mg L ⁻¹	Mol. Wt (kDa)	Molar Conc.	Ingredient	mg L ⁻¹	Mol. Wt (kDa)	Molar Conc.
L-Alanine	2.01	89.1	0.23 × 10 ⁻⁴	Glucose	3443.0	180.2	19.1 × 10 ⁻³
L-Arginine HCl	104.12	210.7	4.94 × 10 ⁻⁴	D(+)-Galactose	15.0	180.2	8.3 × 10 ⁻⁵
L-Asparagine H ₂ O	3.38	150.1	0.23 × 10 ⁻⁴	D(+)-Mannose	10.0	180.2	5.6 × 10 ⁻⁵
L-Cystine Na ₂	38.33	286.0	1.34 × 10 ⁻⁴	Choline chloride	6.07	139.6	43.5 × 10 ⁻⁶
L-Glycine	21.94	75.1	2.92 × 10 ⁻⁴	Pyridoxal HCl	2.72	203.6	13.4 × 10 ⁻⁶
L-Histidine HCl.H ₂ O	33.07	209.5	1.58 × 10 ⁻⁴	CaCl ₂ .2H ₂ O	188.74	147.0	1.28 × 10 ⁻³
L-Isoleucine	71.63	131.0	5.46 × 10 ⁻⁴	Fe(NO ₃) ₃ .9H ₂ O	0.068	404.0	0.17 × 10 ⁻⁶
L-Leucine	73.70	131.0	5.62 × 10 ⁻⁴	FeSO ₄ .7H ₂ O	0.19	278.0	0.68 × 10 ⁻⁶
L-Lysine HCl	106.90	182.5	5.85 × 10 ⁻⁴	KCl	320.34	74.5	4.29 × 10 ⁻³
L-Methionine	21.25	149.0	1.42 × 10 ⁻⁴	MgSO ₄ .7H ₂ O	168.27	246.5	0.68 × 10 ⁻³
L- Phenylalanine	45.67	165.0	2.76 × 10 ⁻⁴	NaH ₂ PO ₄ .2H ₂ O	95.38	156.0	0.61 × 10 ⁻³
L-Proline	7.78	115.0	0.68 × 10 ⁻⁴	Na ₂ HPO ₄	31.95	142.0	0.23 × 10 ⁻³
L-Serine	30.72	105.0	2.92 × 10 ⁻⁴	ZnSO ₄ .7H ₂ O	0.20	287.5	0.7 × 10 ⁻⁶
L-Threonine	66.94	119.0	5.62 × 10 ⁻⁴	Folic acid	3.0	441.4	6.79 × 10 ⁻⁶
L-Tryptophan	11.26	204.0	0.55 × 10 ⁻⁴	i-Inositol	8.78	180.2	48.7 × 10 ⁻⁶
L-Tyrosine	49.82	227.0	2.75 × 10 ⁻⁴	Nicotinamide	2.71	122.1	22.2 × 10 ⁻⁶
L-Valine	65.82	117.0	5.62 × 10 ⁻⁴	Hypoxanthine	0.92	136.1	6.75 × 10 ⁻⁶
Putrescine	16.11	88.2	0.18 × 10 ⁻³	Riboflavine	0.28	376.4	0.74 × 10 ⁻⁶
L-Carnitine	2.0	161.2	12.4 × 10 ⁻⁶	Thymidine	0.162	242.2	0.67 × 10 ⁻⁶
				NaCl	6030.0	58.5	103.0 × 10 ⁻³
Cytidine 5'-diphospho ethanolamine	1.28	446.2		D- Calcium pantothenate	2.75	476.6	5.77 × 10 ⁻⁶
Cytidine 5'-disphospho choline	2.56	488.3		Sodium phenol red	5.0	376.4	

Table 2. List of ingredients added to R16 Medium to Form Complete R16 Medium

	Components	Final concentrations	R16 medium volume required (ml)			
			100 ml	200 ml	300 ml	400 ml
1	Basal R16		80	160	240	320
2	BSA	0.2%	5	7	9	11
3	Transferrin	10µg/ml	0.1 each	0.2 each	0.3 each	0.4 each
4	Progesterone	0.0063µg/ml				
5	Insulin	0.2 µg/ml				
6	T3	0.002µg/ml				
7	Corticosterone	0.020 µg/ml				
8	Thiamine HCl	2.77µg/ml				
9	Vitamin B12	0.31 µg/ml				
10	Thioctic acid	0.045 µl/ml	0.2 each	0.4 each	0.6 each	0.8 each
11	Retinol/ Retinyl acetate	0.1 µg/ml/ 0.1 µg/ml				
12	DL-Tocopherol/ Tocopherol acetate	1µg/ml/ 1µg/ml				
13	Linoleic acid/ Linolenic acid	1µg/ml	0.1 each	0.2 each	0.3 each	0.4 each
14	L-Cysteine	7.09 µl/ml				
15	Glutathione	1µg/ml				
16	Sodium Pyruvate	50µg/ml	1	2	3	4
17	Glutamine + Vit. C	25µg/ml + 100µg/ml				
18	Distilled water		12.3	27.6	42.9	59.2
19	FCS	0%	0	0	0	0
20	Distilled water		5.3	10.6	15.9	21.2
21	FCS	10%	10	20	30	40

c) Test Substances Added to Culture Medium to Study the Rescue Effect

i) LEDGF: 10ng ml⁻¹ of LEDGF was added to the culture medium. LEDGF was provided by Prof. T. Shinohara, Department of Ophthalmology, Nebraska Medical Center, University of Nebraska Medical Center, Omaha, USA.

ii) Alpha GST (α -GST) and Mu GST (μ -GST): 10ng ml⁻¹ of α -GST or μ -GST were added to the culture medium. α -GST and μ -GST were purchased from Oxford Medical Research, Oxford, Michigan, USA.

d) Tissues taken for Retina Organ Culture

Retinas from homozygous *rd1/rd1* and congenic control *+/+* mice of the C3H strain at different ages namely PN2, PN7, PN11 and PN21 were used for culturing. These were cultured upto PN28 meaning that in the case of PN2 the retinas were cultured for 26 days; in case of PN7, PN11, PN21 these were cultured for 21, 17 and 7 days respectively.

To test the rescue effect of LEDGF and GSTs, retinas were taken from PN2 and PN7 mice. Two different ages were taken to determine the possible stage at which the treatment could be initiated.

As control both *rd1/rd1* and *+/+* tissue from both ages were cultured without LEDGF or GST.

e) Tissue Processing after Culturing

All explants were cultured till the age of PN28. After completion of culture period, the retinas attached to the nitro-cellulose membrane were fixed by immersion in 4% para formaldehyde for 1 hour, washed four times with 3% sucrose in Sørensen's phosphate buffer and subsequently put overnight in 25% sucrose in Sørensen's phosphate buffer for cryoprotection. The explants were vertically cut at 8 μ m thickness on a cryostat (MICROM HM 560, MICROM Laborgeräte GmbH, Walldorf, Germany). The cryosections were stained with hematoxylin/eosin (H/E) to study the morphology and for cell count purposes.

f) Cell Counts

A vertical column in the centre of the retinal explant was chosen for counting of rows of nuclei in the ONL. Four to five explants were counted in each category and the number of sections in each category was taken randomly.

g) Statistics

All data is presented as Mean \pm SEM. Cell count data was analysed using one way analysis of variance (ANOVA) at 5% significance level, followed by Fisher's protected least significant difference post-hoc comparisons. The difference between groups was regarded as significant at * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

3) Immunocytochemistry

Table 3. List of primary antibodies used for detection and cellular localisation of bio molecules

Primary antibody	For detection of	Dilution	Source
IRPB	Interphotoreceptor retinoid-binding protein	1:500	Kind gift from Dr.G.J. Chader and Dr. I. Grey, NIH, Bethesda, MD, USA
Arrestin	Arrestin	1:5000	
OS-2	Blue cone opsin	1:2000	Kind gift from Prof. A Szél Semmelweis University of Medicine, Budapest, Hungary
COS-1	Green cone opsin	1:500	
AO	Rod opsin	1:10000	
Calbindin	Horizontal cells and their processes	1:200	Sigma. St Louis, MO, USA
Neurofilament		1:3000	
Parvalbumin	Profils centered around the IPL	1:100	Chemicon, Temecula, CA, USA
Calretinin			
Glutamine synthetase	Müller cells	1:100	
α -GST	Alpha form of Glutathione-S Transferase	1:500	Oxford Medical Research, Oxford, Michigan, USA
μ -GST	Mu form of Glutathione-S Transferase		
MMP-2	Matrix metalloproteinase-2	1:100	Chemicon, Temecula, CA, USA
MMP-9	Matrix metalloproteinase-9	1:200	
TIMP-1	Tissue Inhibitors of metalloproteinase-1	1:50	Santa Cruz Biotechnology, Inc., California, USA
TIMP-2	Tissue Inhibitors of metalloproteinase-2		

List of primary antibodies used are mentioned in Table 3. The primary antibodies were diluted in phosphate buffered saline (PBS pH 7.2) containing 1% bovine serum albumin (BSA) and 0.25% Triton X-100 (PBST). Sections were pre-incubated with PBST for 30 min at room temperature followed by overnight

incubation with the diluted primary antibody at 4°C. In case of negative control sections, the incubations with primary antibodies were omitted. After washing with PBST for 3x5 min, bound primary antibody was detected by incubation with suitable secondary antibody conjugated to fluorescein isothiocyanate (FITC) from DAKO, Glostrup, Denmark. After washing with PBST for 3x5 min each, the slides were mounted with Vectashield anti-fade medium (Vector laboratories Inc. Burlingame, CA, USA).

For the MMP-1, MMP-2, TIMP-1 and TIMP-2 immunocytochemistry the eyes were enucleated and frozen directly on dry ice and stored at -80°C until sectioned. The frozen eyes were embedded in an albumin-glycerin medium, where after 8 µm thick sections were cut on a cryostat, assembled on glass slides and fixed for 10 min at 20°C in a cold mixture of methanol / acetic acid (3:1).

For double labelling, sections were initially incubated overnight with the first primary antibody followed by the first secondary antibody and then again overnight with the second primary antibody followed by the second secondary antibody. Washes with PBST were performed in between each step as described above.

Immunohistochemical labelling was examined and documented using an Axiophot photomicroscope (Zeiss, Oberkochen, Germany). Adobe Photoshop® was used for image processing. Only contrast and brightness of images was adjusted. In the double labelling experiments separate digital images of the fluorophores were superimposed on each other, resulting in a yellow or orange signal depending on the intensity in case of co-localisation.

4) Western Blots for GSTs

Two retinas of the same kind were pooled from each age group (PN2, PN7, PN14, PN21, PN28) and for each genotype (wild type *+/+* and *rd1/rd1*) and homogenised by hand in homogenising buffer (2% sodium dodecyl sulphate, 10% glycerol and 62.5 mM Tris [pH 6.8]), the homogenate was centrifuged at 10,000 rpm for 5 minutes. Protein concentration of the soluble particulate free supernatant was determined by using Plus One 2-D Quant Kit (Amersham Biosciences AB, Uppsala, Sweden). 5µg-10µg of protein was loaded in each well and fractionated in a discontinuous SDS-polyacrylamide gel (3% stacking gel, 12.5% separation gel) in a Mini-PROTEAN II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Molecular weight markers were of broad range biotinylated SDS-PAGE Standards (Bio-Rad Laboratories, Hercules, CA, USA).

In case of western blots for GST experiments, *E coli* produced human recombinant α-GST (62.5 ng) or µ-GST (50 ng) (Oxford Biomedical Research, Oxford, Michigan, USA) served as molecular weight markers and positive

reference protein, respectively. Proteins from the gel were transferred using a Semi-dry blotter (Model Sammy Dry, Schleicher & Schuell, BioScience GmbH, Dassel, Germany) on to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) using blotting buffer (48 mM Tris base, 39 mM glycine, 0.0375% SDS, 20% methanol). The membranes were blocked for 1hr in Tris buffered saline (TBS) (pH 7.2) containing 0.1% Triton X-100 (TBST) and 5% skim milk. This was followed by overnight incubation at 4°C with either of the antibodies (1) anti-rat GST Ya (goat polyclonal, 1:10,000) that detects α -GST or (2) anti-rat GST Yb (goat polyclonal, 1:10,000) that detects μ -GST (Oxford Biomedical Research, Inc, Oxford, Michigan, USA). After 3x5 min rinse the membrane was incubated for 60 min with 1:10,000 HRP conjugated donkey anti goat (SDS Biosciences, Falkenberg, Sweden) at room temperature followed by 3x5 min washes in TBST. The immune complexes were visualised by enhanced chemiluminescence (ECL, Amersham Biosciences AB, Uppsala, Sweden) on to X-Ray film (Hyperfilm ECL, Amersham Biosciences AB, Uppsala, Sweden). The intensity of each band was compared after semiquantification by optical densitometry (BIO-1D Software, Vilber Lourmat, France). Experiments were performed in triplicate.

5) RT-PCR for IRBP

PCR analyses were performed using the following pairs of primers, 5₋-CAG AGG ATG CCA AAG ACC GA (forward) and 5₋-GAA TCT CAA GTA GCC AAT GT (reverse). After an initial hot start at 94°C for 10 min the following PCR-conditions were used: denaturation 94°C for 30 s, annealing at 55°C for 30s, and extension at 72°C for 60 s, using Ampli Taq (Applied Biosystems, the Netherlands) in standard buffer and performing 35, 25, 20 or 15 cycles. Products were analyzed on 1% agarose gels.

6) ELISA for estimation of MMPs and TIMPs

Retinas from five animals from each age group (PN2, PN7, PN14, PN21, PN28) and each genotype (C3H wild type and C3H *rd1*) were homogenized in 50 mM HEPES (4-2-hydroxyethyl-1-piperazieethane sulfonic acid) buffer containing 4% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) (Gehring-Burger et al., 2005) and subsequently centrifuged at 10,000 rpm. The supernatants representing retinal extracts from each age group and background were analyzed for MMPs, TIMPs, and sGAG. Quantification of proteins was done by the same procedure as mentioned before.

Active and 4-aminophenylmercuric acetate (APMA) activated MMP-9 / MMP-2 in retinal extracts were estimated, before and after activation with APMA, quantified by immunocapture ELISA, using genetically engineered pro-urokinase containing sequences recognized by MMPs, according to protocols (except for increase in the period of incubation up to twenty three hours) supplied by

Amersham Pharmacia Biotech, Uppsala (Sweden). The t_0 absorbance (Abs) values were obtained at 405 nm, using an ELISA Plate Reader (Molecular Devices Corporation, USA or Multiskan Ascent®, Thermo LabSystems, Helsinki, Finland). MMP-9 activities were calculated according to: $\text{Abs } t_{ON} - \text{Abs } t_0 \times 1000 / h^2$ where h is hours of incubation.

The procedure for estimating active and total MMP-2 was as stated above, except that a 90 minutes pre-incubation step was excluded.

TIMP-1 and TIMP-2 were quantified by sandwich ELISA as per protocol supplied by R&D Systems (Minneapolis, USA). The absorbance was read at 450nm and corrected for 595nm (instead of 570nm), the absorbance of the plate material by using an ELISA Plate Reader.

The data obtained at different time points were analyzed by using one-way ANOVA and Fisher's protected least significant differences *post-hoc* comparisons to determine statistical differences in the values observed for *wt* and *rd1* respectively at different ages.

Lectin Blotting of Proteoglycans

Retinal extracts were fractionated (Lammeli, 1970) by SDS-PAGE using 12% acrylamide gels which were stained sequentially with Coomassie Blue R250 and Periodic Acid Schiff stain for proteins and glycoproteins / proteoglycans, respectively. The proteins fractionated on gels were blotted by semidry Western blotting and sequentially reacted with fluorescein labeled wheat (*Triticum vulgare*) germ agglutinin (WGA-Texas Red) and peanut (*Arachis hypogea*) agglutinin (PNA-fluorescein) to determine the age-dependent differences in the nature of saccharides associated with the proteoglycans. WGA reacts with N-acetyl β -D-glucosamine / sialic acid and PNA does so with D-galactose- β 1 \rightarrow 3 N-acetyl galactosamine.

Spectrophotometric estimation of Sulfated Glucosamin-glycans (sGAG)

The sGAG content of retinal extracts, were quantified by an Alcian blue (a positively charged dye which reacts with carboxyl and sulfate groups) binding assay (Gold, 1981) according to the protocol provided by Wieslab AB, Lund, Sweden. Absorbance was read at 600-620 nm using ELISA Plate Reader.

V Results and Discussion

Paper I Mouse retina explants after long-term culture in serum free medium

It is possible to culture the intact mouse retina, neonatal and late postnatal, in a serum-free medium for a considerable period of time

The aim of this study was to develop a retina organ culture system, where the *in vitro* retinal tissue corresponds to the *in vivo* counterpart in such a way that the explant can be accepted as an excellent model to assess this phenomenon of retinal biology. In our earlier studies when the culture system was newly developed, the culture medium used had high concentrations of serum as the nutrient requirement of individual cells of the retina was unknown. Secondly, However, Turner (1985) indicated that some retina cells may degenerate rapidly in serum-free culture conditions. But other reports show that neural tissue can also be cultured successfully in serum free conditions in medium containing commercially purified bovine serum albumin having traces of globulins (Romijn, 1988; Kivell et al., 2000). In the media supplemented with 2% or 10% serum the retinal explants survive equally well (Caffe et al., 2001b) but the successful use of the present serum-free medium marks a significant advance in retinal explant culture (Ahuja et al., 2001; Azadi et al., 2002; Ahuja et al. 2005). Isolated neurons of mouse retina from different ages have been studied in serum free cultures (Politi and Adler, 1988; Politi et al., 1988; Abrams et al., 1989; Politi et al., 1989) and show that PN2 photoreceptors survive for roughly 2 weeks *in vitro*, whereas those from PN5 and PN7 retina were viable only for approximately 7–8 and 4–5 days, respectively.

Whole mouse retinas from wild type ++ were dissected out at PN2, PN7, PN11 and PN21 and maintained for 26 (PN2+ div26), 21 (PN7+div21), 17 (PN11+div17) and 7 (PN21+div7) days *in vitro* (div), respectively. At PN28 *in vivo* mouse retina showed 13.6 ± 0.2 rows of cell bodies in the ONL. The PN2+div26 and PN7+div21 retinal explants cultured in serum-free medium acquire the characteristic histotypic lamination and maintain 7.9 ± 0.2 and 8.1 ± 0.3 rows of nuclei in the ONL, respectively. PN11+div17 explants show good preservation of retinal architecture with 8.0 ± 0.2 rows of nuclei in the ONL. PN21+div7 explants display 7.4 ± 0.3 rows in the ONL. The number of rows in the ONL of the PN28 *in vivo* tissue was significantly higher than those in the explants of all ages, but it shows statistically non-significant difference between explants. Hematoxylin and eosin staining of sections at the end of the culture period showed recognizable elongated outer segment-like structures by PN2+div26 to PN11+div17 photoreceptors whereas these structures are degenerating and form a debris zone in the PN21+div7 explants. Second, clumps of pigment-laden cells not observed in PN2+div26 to PN11+div17 tissue, and are traversing the subretinal space in PN21 + div7 explants. Thus, in our hands the

whole retina, even when obtained from the late postnatal mouse, can be kept viable much longer than previously reported for isolated cells.

Distribution pattern of photoreceptor-specific proteins in explants corresponds to the litter-matched *in vivo* retina

Expression pattern of the three photoreceptor-specific proteins namely rhodopsin, arrestin and IRBP by the retinas was studied and comparison was made between their distribution in the *in vitro* explants and litter-matched *in vivo* retinas. In the *in vivo* retinas strong rhodopsin labelling is seen in the rod outer segments while the PN2+div26 and PN7+div21 explants show this labelling in rod cell bodies and, when present, the outer segment-like structures. In PN11+div17 explants rhodopsin labelling is present in the outer segment which corresponds to litter-matched retina while in the PN21+div7 explants the labelling was very weak. In the *in vivo* retinas arrestin labelling is present in the outer and inner segments, photoreceptor cell bodies and spherules. In the *in vitro* explants at PN2+div26 to PN11+div17 labelling is only present in the photoreceptor segments when they are present. Similarly to rhodopsin, arrestin labelling in the PN21+div7 explants is very weak, if present. In the *in vivo* retina IRBP labelling is present exclusively in the IPM while in the explants, IRBP labelling is completely different. In PN2+div26 and PN11+div17 *in vitro* cultured retina do not show immunoreactivity for IRBP in the subretinal space and IRBP labelling is also totally absent from the IPM debris zone in PN21+div7 explants. However, RT-PCR performed on cultured retina and age-matched controls show no or little difference in IRBP gene expression.

Comparison between the present study using serum free medium and earlier studies with serum containing medium (Caffe et al., 1993; Caffe et al., 2001a), no difference is seen in the distribution of arrestin, rhodopsin, and IRBP immunoreactivity. Thus, serum does not influence the metabolic parameters and transport of these photoreceptor-specific proteins *in vitro*. When the issue of *in vitro* versus *in vivo* is considered, PN11+div17 cultures display rhodopsin and arrestin expression limited to the outer segments, exactly as *in vivo*. Therefore, culturing does not adversely affect production and transport of these proteins, provided outer segments are present in the tissue. This suggests that photoreceptor metabolic processes in the explants are apparently normal.

RT-PCR studies show similar amounts of IRBP message both in the *in vivo* and *in vitro* retinas, but the IRBP immunostaining in retinal explants is much weaker than that of age-matched *in vivo* controls. Normally IRBP binds to matrix proteoglycans in the IPM and with insoluble matrix components in the subretinal space (Uehara et al., 1990; Mieziwska et al., 1994; Mieziwska, 1996). The failure to detect IRBP in the subretinal compartment of retinal explants might be due to changes in the nature of the IPM components. It is also possible that the IRBP is secreted into the culture medium (Smith et al., 1992). IRBP has a half-

life of seven hours and this could be another reason for not detecting the protein in the IPM (Cunningham and Gonzalez-Fernandez, 2000). As metalloproteases are present in the IPM they may be responsible for their degradation.

Green cones are absent in explants taken at an early period

Green cones are known to be absent both in mouse explants (Soderpalm et al., 1994) and rabbit retinal transplants (Szel et al., 1994a). However, short wave cones are present. Explants used for this investigation were sectioned and labelled so that both the UV-, (ventral S-field) and the green cone zones (dorsal M-field) were included in the sections. The *in vivo* litter-matched retina showed a normal M-field. Green cones are completely absent in PN2+div26 explants. In the PN11+div17 explants a few green cones can be seen whereas in PN21+div7 explants many immuno-labelled elements persist in the sub-retinal debris zone containing the dorsal M-field. Control staining of PN21+div7 tissue displayed only sporadic pigment-laden profiles in the sub-retinal space, confirming that the immuno-positive labelling represented true green cone labelling. The dotted appearance of green cone elements, however, indicated degenerating outer segments and confirmed the observations made after the haematoxylin and eosin staining.

The mouse retinas consist of approximately 3% cones of two types, viz. green cones and UV cones which are sensitive to light of middle-long wavelength and short wavelength, respectively. The green cones also known as M cones form a majority and are located in the dorsal half of the retina. The UV- cones are also known as S cones and constitute 10% of all cones and are located all over, but show a higher density in the ventral part of the retina (Szel et al., 1992; Szel et al., 1993; Szel et al., 1994b). In the transition zone in the middle of the retina individual cones expressed both S cones and M cone opsins (Rohlich et al., 1994). Applebury et al., (2000) have shown by *in situ* hybridisation that co-expression of S and M cones is not limited to the transition zone instead the vast majority of cones co-expressed UV and M opsin. Only M opsin was detected in some cones in far dorsal retina while only UV opsin was detected in some cones, in middle and ventral retina and two gradients were observed. The frequency of cones expressing UV pigment decreased in a ventral-to-dorsal gradient, and the amount of M opsin protein and mRNA per cell appeared to decrease in a dorsal-to-ventral gradient. In the rat retina green cones develop from blue cones by a shift in spectral sensitivity as until PN9 all cones in the retina are blue and during the transition many cones express pigments for both the blue and green cones. It is only by PN20 that the normal ratio between the blue and green cones is established (Szel et al., 1994b). Also in the mouse the above mentioned differences are present i.e. S cones can be detected from PN4 while M cones start to appear only from PN11 (Szel et al., 1996). This gave rise to the hypothesis that by default, all rat cones initially express UV opsin, and in response to a later

developmental switch, most cones stop expressing UV opsin and begin to express M opsin.

It can be speculated that shift in spectral sensitivity does not occur in the explants and therefore we do not see any green cones. The reasons for the shift in spectral sensitivity and absence of expression of the green cones could be due to the fact that the culture medium lacks stimulatory factors needed for green cone expression or there are suppressive factors present in the medium but are absent in the *in vivo* retina or both. Liljekvist-Larsson et al., (2003) have shown that in the *in vitro* rat retinas the mRNA levels of M cone photoreceptors is four times lower and that of S cones is two times lower than the *in vivo* retina. This has also been seen in detachment studies where the surviving cones show similar pattern (Lindberg et al., 2001; Rex et al., 2002). It is also possible that the RPE in the explants has an inhibitory role as it has been shown that dissociated chick embryo retinal cells, which readily express visual pigments in homotypic re-aggregation cultures (retinospheroids), are inhibited from doing so when co-aggregated with RPE cells (Layer et al., 1997).

Several extrinsic factors have been reported to modulate visual pigment expression e.g. retinoic acid (Soderpalm et al., 2000); taurine (Altshuler et al., 1993; Wallace and Jensen, 1999), and growth factors such as FGF (Hicks and Courtois, 1992) and CNTF (Fuhrmann et al., 1995; Fuhrmann et al., 1998; Kirsch et al., 1996; Kirsch et al., 1998; Ezzeddine et al., 1997). An extrinsic factor modulating green cone expression during the late postnatal period could be thyroid hormone and their receptors (Rohlich et al., 1994; Kelley et al., 1995). In recent studies Ng et al., (2001) have shown that thyroid (T3) hormone and receptors are strong candidates for mediating the external signals. That these signals continue to act during the late postnatal period which follows from our observations that further green visual pigment biosynthesis is halted after the retina has been isolated for *in vitro* culture. Thus for determination of green cone identity, we postulate that external regulatory signal have an important role.

The calcium-binding protein markers expressed by the inner retina cellular elements are similar to age-matched *in vivo* retina

Antibodies directed against three calcium-binding proteins viz. calbindin, parvalbumin, calretinin were used to quantitatively study the inner retina cellular elements. Calbindin is a marker of the horizontal cells and their processes in the OPL, whereas parvalbumin and calretinin labelled cell profiles are centred around the IPL. In the *in vivo* retina at PN28 calbindin labels neurons in the ganglion cell layer and a mixed population of somata in the inner IPL forming three bands of processes in the IPL. Apart from this, typical horizontal cells and their dense network neurites that are confined within the boundaries of this structure in the OPL are strongly immunoreactive. In all retinal explants the horizontal cell bodies occupy a normal location and are of the same shape as encountered *in*

vivo. Most of their neurites are also located, as they should be, within the boundaries of the OPL in the cultured tissue. However, some aberrant sprouting, most evidently into the ONL, is also present. Some of these aberrant processes even traverse the entire ONL to reach the external limiting membrane. It can also be seen that, in the age-matched *in vivo* retina, the calbindin immunopositive neurons at the inner part of the INL are greater in number than those in the retinal explants. The parvalbumin antibody strongly reacts with INL cells lining the IPL and with a great number of cells in the ganglion cell layer of the PN28 *in vivo* mouse retina. Horizontal cells are not stained. In the IPL a dense network of processes is present, but clearly distinct layers are not produced. The OPL is not labelled. In retinal explants the staining pattern of parvalbumin is qualitatively the same. However, although no quantitative analysis was performed, the impression is that the number of profiles is reduced both in the INL and ganglion cell layer of the retinal explants. There is no evidence of parvalbumin immunoreactive neurites beyond the normal location. In the PN28 *in vivo* retina, calretinin labels numerous neurons of various types and sizes in the ganglion cell layer and very many cells in the inner part of the INL. Also cells located in the position of bipolar cells are stained. Three bands of calretinin immuno-positive processes are produced in the IPL. The *in vitro* retina maintains normal and comparable calretinin organization; however, the number of stained cells in the INL and ganglion cell layer of the retinal explants is reduced. Thus staining for calcium-binding proteins did not demonstrate clear and consistent differences between PN2+div26, PN7+div21, PN11+div17 and PN21+div7 retinal explants.

The results we have achieved in the normal retina are identical to those previously reported by Haverkamp and Wassle (2000). A comparison between *in vivo* and cultured *in vitro* tissue shows a qualitatively normal localisation of different calcium binding antibodies in the retinal explant, except for some sprouting of processes from the OPL into the ONL. Although the difference in the numbers of labelled cells was not verified statistically during this study, yet the knowledge of the normal distributions permitted us to conclude that the number of immunostained elements by all three markers is reduced in the mouse retinal explants. In the explants changes are not only seen when comparing presence and localisation of calcium binding proteins but also in the glial cell system (Caffe et al., 1993). In the rat retinal explants, after 2–3 weeks *in vitro* changes were seen in the cholinergic and nitrenergic transmitter systems (Johansson et al., 2000). Thus it appears that the entire inner retina circuitry seems to react to the explant preparation and these phenomena must be monitored during further development of the retinal explant culture system.

Paper II Lens epithelium-derived growth factor (LEDGF) delays photoreceptor degeneration in explants of *rd/rd* mouse retina

LEDGF rescues photoreceptor in the *rd1/rd1* retina

At the end of the culture at PN28, both the explants from PN2 and PN7 wild type *+/+* showed the normal histotypic lamination characteristic for PN28 while the *rd1/rd1* explants showed excessive loss of photoreceptor cells. PN2+div26 *rd1/rd1* explants not treated with LEDGF showed 3.1 ± 0.1 rows of nuclei in the ONL, whereas similar tissue treated with LEDGF exhibited 4.3 ± 0.1 rows in the ONL. PN2+div26 *+/+* explants not treated with LEDGF showed 7.1 ± 0.2 rows of nuclei in the ONL, whereas similar tissue treated with LEDGF showed 7.4 ± 0.2 rows in the ONL. PN7+div21 *rd1/rd1* explants without LEDGF treatment showed 2.4 ± 0.1 rows of somata in the ONL and with LEDGF treatment they showed 4.0 ± 0.0 rows. Wild type PN7+div21 without LEDGF showed 8.0 ± 0.2 rows of somata in the ONL and when treated with LEDGF they showed 7.6 ± 0.1 rows in the ONL.

The possible mechanism of action of rescue by LEDGF treatment is shown in Figure 4 and along with the possible caspase dependent mechanism of apoptosis in *rd1/rd1* mouse retina. Stress signals possibly activate the expression of

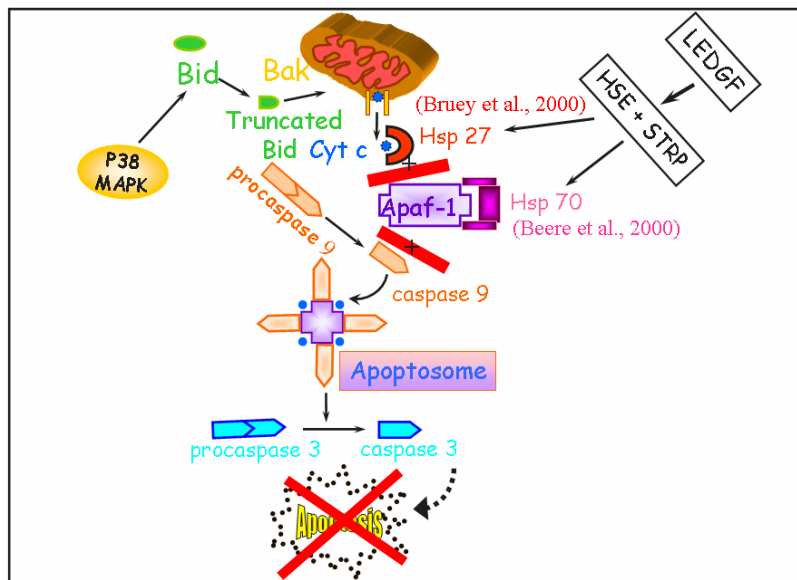


Figure 4. Possible mechanism of action of LEDGF

LEDGF gene, which results in the increase of levels of LEDGF in the cells (Sharma et al., 2000). LEDGF binds (Figure 4) to heat shock element (HSE) and stress related regulatory element (STRE) to activate the expression of stress

related genes namely heat shock protein 27 (Hsp 27), Hsp 70 and $\alpha\beta$ -crystallins (Singh et al., 2001). Both these Hsps promote the inhibition of apoptosome function but target different proteins. Hsp 27 binds to cytochrome c upon its release from the mitochondria and thus prevents a productive interaction with Apaf-1 (Bruey et al., 2000). Hsp 70 binds to Apaf-1 thus precluding the eventual recruitment of procaspase-9 to the apoptosome (Beere et al., 2000; Saleh et al., 2000; Li et al., 2000).

It has been shown that LEDGF highly promotes photoreceptor survival in light-damaged and RCS rats but not in transgenic rats showing P23H mutation (Machida et al., 2001). One explanation for discrepancy between the efficacies observed in the P23H rat and the *rd1/rd1* mouse retinal explants is the time of onset and duration of treatment. In the culture system the retinal explants are subjected to continuous exposure of LEDGF and from PN2 and PN7 age i.e. well before the onset of photoreceptor cell death and eventual induction of the apoptotic machinery.

LEDGF treatment did neither affect opsin, nor arrestin expression in the presence of RPE

Earlier Caffè et al. (2001b) showed that opsin and arrestin immunoreactivity was decreased in explants treated with CNTF and BDNF in the presence of RPE. To check if LEDGF treatment had similar effect on the expression of opsin and arrestin in the explants with RPE, immunolabelling with antibodies against opsin and arrestin was done. In both *rd1/rd1* and *+/+* explants at both PN2 and PN7, with or without LEDGF treatment a similar pattern of labelling was seen in the explants in the presence of RPE. Thus LEDGF treatment did not affect either the opsin or the arrestin expression.

Rescue effect is statistically significant and similar to that of BDNF

The statistical analysis of the results obtained from the wild type *+/+* with and without LEDGF showed no significant differences. In contrast, *rd1/rd1* explants treated with 10 ng/ml LEDGF significantly rescued the photoreceptors both at PN2 ($p < 0.001$) and the PN7 ($p < 0.001$) when compared to explants not treated with LEDGF, taken at the same age. When the rescue effect of LEDGF between retina of *rd1/rd1* at PN2 and PN7 ages was compared, no significant differences were obtained.

The results obtained from this study enable us to compare the rescue effect of LEDGF to other factors namely NGF, FGF2, CNTF or BDNF that have been tested in our laboratory before using the same *in vitro* conditions either alone or in combination viz. NGF and FGF2 or CNTF and BDNF. When NGF or FGF2 was used separately at a concentration of 50ng/ml they did not protect *rd1/rd1* mouse photoreceptors (Caffè et al., 1993) but have been reported to enable

photoreceptor survival at 20ng/ml FGF2 in a dissociated rat culture paradigm (Fontaine et al., 1998). When CNTF and BDNF were used separately then 10ng/ml CNTF and 10ng/ml BDNF showed protective effect. BDNF showed 3 to 4 rows in the ONL while CNTF showed maintenance of 5 to 6 rows in the ONL, of the PN28 *rd1/rd1* explants, respectively (Caffe et al., 2001b). In the *rd1/rd1* control, without the survival factors, the number of photoreceptor rows in the ONL was 2 to 3 by PN28. The combination therapy displayed different results. NGF + FGF2 combination treatment showed the survival of 5 to 6 rows in the ONL while CNTF + BDNF the number of rows in the ONL was 7 to 8 rows in the PN28 retinal explants (Caffe et al., 1993; Caffe et al., 2001b). As 10ng/ml LEDGF rescues 4 to 5 rows in the ONL, it can be concluded that the protective potency of LEDGF is similar in magnitude to that by BDNF.

LEDGF displays a greater rescue effect than either NGF or FGF2, but the effect is less than that of CNTF. Secondly the adverse side effect on photoreceptor protein expression exhibited by CNTF and BDNF is lacking. From this conclusion, that a combination therapy involving LEDGF and NGF or FGF2 rather than LEDGF and CNTF or BDNF is warranted.

Paper III Decreased Glutathione Transferase Levels in *rd1/rd1* mouse retina, Replenishment protects photoreceptors in retinal explants

***α*-GST levels are lower in *rd1/rd1* mouse retina**

Western blots analysis was done for the detection and semi-quantification of α -GST and μ -GST in the retinal extracts from both *rd1/rd1* and wild type *+/+* at different ages in development viz. PN2, PN7, PN14, PN21 and PN28. In the blot with α -GST a doublet band at ~35 kDa co-migrating with the positive control was considered for quantification. Densitometry of this band for semi-quantification showed that in *rd1/rd1* the levels of α -GST increased up to PN7 and afterwards there was a decline in the levels of α -GST up to PN 28. The level of α -GST at PN7 in the *rd1/rd1* retina was significantly higher than that at PN21 and PN28 ($p < 0.01$). In the wild type *+/+* mice retina the levels of α -GST also increased until PN7 and after that the levels remained relatively stable and showed non-significant differences. From PN14 onwards α -GST levels were significantly reduced in *rd1/rd1* retina compared to *+/+* counterparts.

In the μ -GST blot several positive protein bands were seen at ~17 kDa, ~31 kDa, ~37 kDa and ~60 kDa but the doublet ~37 kDa was taken into consideration as it was in the same level as the positive control. In the *rd1/rd1* retinal extracts after PN7, the levels of μ -GST have a tendency to decrease while in the *+/+* the levels are relatively stable during the whole period. Statistically non-significant differences were seen.

In our studies we observed that levels of retinal GST increase, first in the early stages of the degenerative process and then decrease as the degeneration progresses in the *rd1/rd1* retina. This supports the notion that GSTs behave in a bimodal fashion, first the GSTs levels increase to cope with the oxidative stress of degeneration causing insult and later on with the progress of the disease the levels are lower possibly due to change in the status of oxidative stress and takeover of degeneration by other factors. Strunnikova et al. (2004) reported that in the RPE cells exposed to non-lethal oxidative stress the expression of GST gene(s) is up regulated along with those of thioredoxin, Hsp 70, caspase3, -6, -9 and MMPs. However, after recovery from oxidative stress, all these genes, except that of MMP-3 are down regulated. The observed decreased level in GST is not an artefact of photoreceptor cell loss since these cells do not express the GST protein.

α - and μ -GST are localised in Müller cells and horizontal cell fibres of *in vivo* retina

Immunohistochemistry was carried out to study the localisation of α -GST and μ -GST in the retinas from both *rd1/rd1* and wild type *+/+* at different ages in development viz. PN2, PN7, PN14, PN21 and PN28. Both α -GST and μ -GST had similar pattern of localisation. At PN2 both in the *rd1/rd1* and *+/+* GST labelling was seen in the GCL. At PN7 in both the genotypes labelling was seen in the GCL region, Müller cell endfeet and weak-labelling was seen in the developing horizontal cells. At PN14 both α - and μ -GST are now clearly detectable in Müller cell endfeet, radial fibres of Müller cells and fibres coursing horizontally in the OPL. In the PN21 to PN28 retinas α - and μ -GST labelling is seen in the Müller cell endfeet and fibres in OPL

Retinas were double-labelled using antibodies against GST M1-1 combined with either GS or neurofilament to confirm its localisation in Müller cells and horizontal cell fibres. The results demonstrated co-localisation of μ -GST and GS, thereby confirming presence of GST in Müller cell endfeet and radial processes. This showed co-localisation of μ -GST and neurofilament, thereby confirming presence of GST in fibres extending from horizontal cells.

The localisation of various types of GSTs in the retina have been investigated in the rat retina and the place of localisation of μ -GST in Müller cells and photoreceptor outer segments while that of α -GST is Müller cells (Singh et al., 1984; Naash et al., 1988; McGuire et al., 1996). Labelling of μ and α -GST was seen in the Müller cells but also in large calibre horizontal cell fibres. Both Müller cells and horizontal cells contribute to the formation of the inner blood-retinal barrier composed of the deep capillary layer of retina at the OPL and the superficial retinal capillary layer at the inner limiting membrane (Knabe and Ochs, 1999; Yu and Cringle, 2001). This anatomical localisation suggests that these GSTs act as detoxifying agents. It is also possible that GSTs are secreted by

the Müller cells (Mukherjee et al., 1999) and interact with GSH and proteins present in the Müller cells (Pow and Crook, 1995) as well as on the surface of photoreceptors and influence their survival.

α - and μ -GST treatments rescue photoreceptors in PN2 *rd1/rd1* mouse retinal explants

At the end of the culture at PN28, both the explants from PN2 and PN7 wild type *+/+* showed the normal histotypic lamination characteristic for PN28 while the *rd1/rd1* explants showed excessive loss of photoreceptor cells. PN2+div26 *rd1/rd1* explants not treated with GSTs showed 2.6 ± 0.1 rows of nuclei in the ONL, whereas similar tissue treated with α -GST and μ -GST showed 4.6 ± 0.2 and 4.7 ± 0.2 rows of nuclei in the ONL, respectively. In both treated cases a rescue effect was found that was significant at $p < 0.01$. PN2+div26 *+/+* explants not treated with GSTs showed 7.3 ± 0.2 rows of nuclei in the ONL, whereas similar tissue treated with α -GST and μ -GST had 7.1 ± 0.1 rows of nuclei and 7.2 ± 0.1 rows of nuclei, respectively. No statistically significant difference was seen. At PN7+div21 *rd1/rd1* both α -GST and μ -GST rescued photoreceptors but the effect was less pronounced. PN7+div21 *rd1/rd1* explants without GSTs treatment showed 2.3 ± 0.2 rows of nuclei in the ONL, whereas similar tissue treated with α -GST showed 3.1 ± 0.1 rows of photoreceptor and that treated with μ -GST showed 2.7 ± 0.2 . Only treatment with α -GST was significant at the $p < 0.05$ level when compared to the untreated one. Wild type PN7+div 21 *+/+* explants without GSTs treatment showed 8.0 ± 0.2 rows of somata in the ONL while α -GST treated showed 7.1 ± 0.1 rows of nuclei and μ -GST treated explants showed 7.2 ± 0.1 rows of nuclei in the ONL. The rescue effect by α -GST and μ -GST on *rd1/rd1* explants at PN2+div26 was significantly higher than the effect at PN7+div21.

The results obtained from this study enable us to compare the rescue effect of GSTs to other factors namely LEDGF, NGF, FGF2, CNTF or BDNF that have been tested in our laboratory before, by using the same *in vitro* conditions either alone or in combination namely NGF and FGF2 or CNTF and BDNF. The GSTs rescue effect was similar to the rescue effect of LEDGF (Ahuja et al., 2001).

The possible ways by which GST could rescue photoreceptors are

- Uptake through the plasma membrane to act intra-cellularly, however the evidence that GST can cross the cell membranes is lacking.
- Binding of GSTs extra-cellularly to trans-membrane receptors- no trans-membrane receptors that recognize GST are known, although it has been reported that other redox regulating enzyme, i.e. extra-cellular superoxide dismutase, can bind to the cell surface via heparan sulfate proteoglycans (Karlsson et al., 1988).

- Modulation of the extra-cellular environment- exogenously added GST is likely to exert its rescue effect by modifying the extra-cellular culture environment. This mode of action is not novel since other redox regulating enzymes like superoxide dismutase, catalase, and glutathione peroxidase or their synthetic mimetics exert either neuro-protective or neuro-destructive effects when added to culture media (Oury et al., 1992; Bonfoco et al., 1995; Ricart and Fiszman, 2001). Our hypothesis is that exogenously added GST, may also function as a peroxidase (Saneto et al., 1982; Singhal et al., 1999), thereby acting as an extra-cellular antioxidant similar to its function within the cell cytoplasm.

Hypothetical explanation of the mode of action of GST mu: Cytotoxic stress, reactive oxygen species (ROS) and growth factor withdrawal can lead to the activation of Apoptosis signal-regulating kinase (Ask-1). Ask-1 is a MAP kinase kinase that activates the JNK and p38 mitogen-activated protein (MAP) kinase cascades in response to the above mentioned and these can further relay

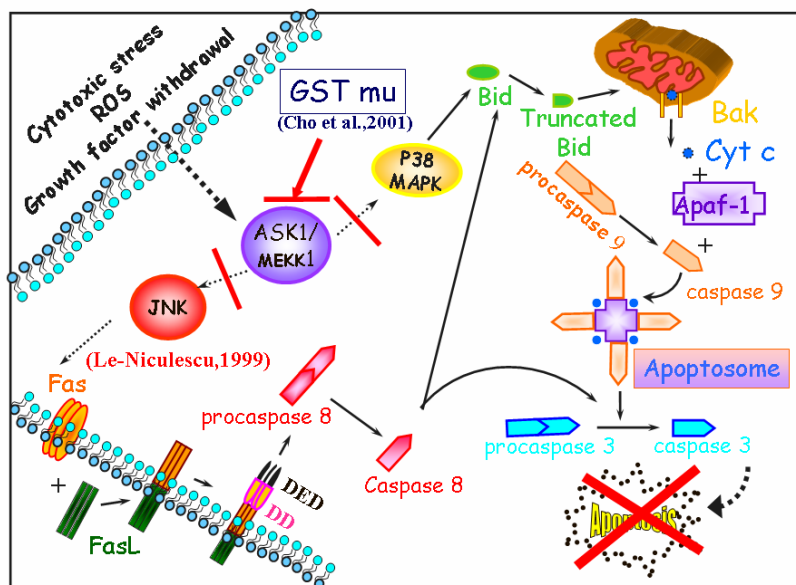


Figure 5. Possible mechanism of action of GST mu

these signals and induce apoptosis (Tobieme et al., 2001; Matsuura et al., 2002) (Figure 5). GST mu monomer interacts with Ask-1 and results in the suppression of Ask-1 activity as well as Ask-1 dependent apoptotic cell death (Cho et al., 2001).

The GST rescue effect was more so in the explants taken at PN2 than in explants taken at PN7 and this difference may be explained to the age related decline in cellular response to oxidative stress (Holbrook and Ikeyama, 2002). Secondly during maturation, physical changes like establishment of new neural circuits and

activation-dependent events take place that induce functional changes like specialization of initial dual action receptors (Joseph et al., 1998; Puopolo et al., 2001). It is plausible that such maturational changes also occur in redox-sensitive receptor systems in the *rd1/rd1* retinal explants leading up to the differential response of PN2 and PN7 tissue to GST treatment.

It was also seen that α -GST rescue effect was more potent than that of μ -GST and this could be due to the fact that α -GST has more reductive power compared with μ -GST (Hayes et al., 1995).

Paper IV *rd1* Mouse Shows Imbalance in Cellular Distribution and Levels of TIMP-1/ MMP-9, TIMP-2/ MMP-2 and Sulphated Glycosaminoglycans.

MMP-2, MMP-9, TIMP-2 and TIMP-1 are present in the IPM and OPL of both *rd1/rd1* and *+/+* while only MMP-2 and TIMP-2 are present in the Müller cells in the *rd1/rd1* retina

During development MMP-2, MMP-9, TIMP-2 and TIMP-1 have similar localization both in the *rd1/rd1* and *+/+* retinas. MMP-2, MMP-9, TIMP-2 and TIMP-1 immunoreactivity was seen in the developing subretinal space at PN2 in both the genotypes. At PN7 both in *rd1/rd1* and *+/+* subretinal space MMP-2, MMP-9, TIMP-2 and TIMP-1 staining becomes more pronounced and weak labeling also appears in the developing outer plexiform layer. At and after PN14 differences in the genotypes become visible. At PN14 in the *rd1/rd1* when retinal degeneration is well underway, MMP-2, MMP-9, TIMP-2 and TIMP-1 staining remains in the collapsing subretinal space, but in addition MMP-2 and TIMP-2 immunoreactivity is seen in the radial Müller fibers. This immunopattern was still present at the age of PN21 and PN28, when rod degeneration is essentially complete. In the *+/+* at PN14 the retina displays immunoreaction for MMP-2, MMP-9, TIMP-2 and TIMP-1, which was limited to the inner segment part of the IPM. This immunoreactive pattern was also seen in PN21 and PN28 retinas. Thus, the retinal distribution of TIMP-2 and TIMP-1 in both the genotypes of mouse was similar to those of MMP-2 and MMP-9 respectively.

The main source of MMP-2 and MMP-9 is considered to be the RPE and these are preferentially secreted by the apical surface of the RPE, which interacts with the photoreceptors and borders the IPM (Padgett et al., 1997; Leu et al., 2002). In our studies we observe labelling for MMPs and TIMPs in the IPM but not in RPE. The presence of MMPs and TIMPs in the IPM has been reported by others also (Jones et al., 1994a; Plantner et al., 1998b). It is possible that these molecules diffuse from the RPE into the IPM after secretion. Secondly, histological distribution of MMP-2 and MMP-9, as well as TIMP-1 and TIMP-2 in the retina seems to depend on the studied species and antibodies employed e.g. Agapova et al. (2003) found that normal control monkey retinal ganglion cells expressed MMP-2, TIMP-1, and TIMP-2. After optic nerve transection, they could show

that reactive astrocytes express these proteins along with MMP-9. The immunopatterns of MMP-2 and TIMP-2 were similar in *+/+* and *rd1/rd1* with high intensity of staining in the IPM during development, but expression in the Müller cells was noticed only during the *rd1/rd1* degenerative process. MMP-9 and TIMP-1 staining intensity in the IPM declined during maturation while expression in Müller cells was absent. As MMP-2 requires TIMP-2 and cell surface MT1-MMP for its activation it is possible that MT1-MMP associated with cell surface may be provided by Müller cells and other retinal cells. The presence of MMP-2 and TIMP-2 in the Müller cells also suggests that they may have a role in the maintenance of the retinal integrity (Limb et al., 2002).

The *rd1/rd1* retina has less of core proteins but more of desialylated, galactosylated sulfate rich sGAG

In the *+/+*, retinal protein content increases significantly with age while in the *rd1/rd1* the protein content increases up to PN14 and then decreases significantly up to PN28. The PN21, PN28 *rd1/rd1* retinal extract had significantly lower levels of proteins as compared to corresponding *+/+* extracts.

The level of sGAG per mg proteins in *rd1/rd1* retinal extracts was significantly higher than that in *wt* extracts. The levels of sGAG decreases significantly with increasing age in the *+/+* retinas while in the *rd1/rd1* the levels first decrease up to PN7 and then increase significantly at PN14, PN21 and PN28. The sGAG content of PN14, PN21, and PN28 *rd1/rd1* retinal extracts was significantly higher than that of corresponding *+/+* extracts.

Thus at different time points the protein content of *rd1/rd1* extracts was significantly lower and sGAG was significantly higher compared to that of *+/+* extracts and these differences increase with age.

Presence of sGAG in *+/+* and *rd1/rd1* retinal extracts was confirmed by Coomassie Blue and the presence of glycoprotein / proteoglycan was confirmed by Periodic Acid Schiff stain. Bands of sGAG / proteoglycans in *rd1/rd1* retinal extracts were weakly stained for associated core protein(s) as compared to those in *+/+* extracts. White unstained halo representing non-stainable sulfate groups of sGAG was seen around the core protein part of sGAG / proteoglycan band. In *rd1/rd1* extracts compared to those in *+/+* extracts, a relatively more prominent halo of sulfate groups and less intensely stained core proteins was noticed. sGAG in *+/+* retinal extracts show maximum WGA (sialic acid-specific lectin) and PNA (galactose-specific lectin) binding fluorescence both in PN2 and PN7 retinal extracts, whereas sGAG in *rd1/rd1* extracts show WGA and PNA binding in PN7 and PN14 extracts but the maximum fluorescence is seen in PN14 extracts

Elevated levels and / or degree of sulfation of sGAG / proteoglycans are suggested by the increase in the sGAG content of *rd1/rd1* retinas. It is known that

the degree of sulfation and distribution of sulfate groups in sGAG varies in an organ / age specific manner and also that higher sulfation decreases the affinity of sGAG binding to FGF in non-retinal tissues (Feyzi et al., 1998). The binding of growth factors to the IPM / ECM may be modified in the *rd1/rd1* as these retinas show higher amount and degree of sulfation of sGAG / proteoglycans. Higher amount and degree of sulfation of sGAG / proteoglycans can also increase the degradation of IPM / ECM due to the increased level of active proteinases like MMPs, cathepsins and serine proteases. The above mentioned may thus in turn also contribute to retinal degeneration in the *rd1 /rd1* retinas.

In the *rd1/rd1* retina sialylation of proteoglycans is either low or the biosynthesis of sialylated conjugates is delayed and this leads to higher galactosylation. The above is not seen in the *+/+* retina. The higher galactolysation in *rd1/rd1* can also be a result of removal of sialic acid residues by sialidase activity. Terminal galactose in laminins serves as binding site for MMP-9 and thus increases the possibility to degrade laminin and prevent MMP-9 diffusion in the ECM (Van den Steen et al., 2002). So the deficiency of WGA binding sites in the PN2 and PN7 *rd1/rd1* retinal sGAG proteoglycans suggests a lack of sialic acid residue containing protein and this makes the core proteins susceptible to degradation by enhanced binding of MMP-9 to the galactosylated laminins. These changes in structure of the IPM, ECM, and properties of the associated proteins possibly modify the ECM and initiate the process of retinal degeneration.

Active MMP-9, MMP-2, and TIMP-1 in *rd1/rd1* retinas is disproportionately higher

The age dependant changes in the levels of active MMP-2, APMA activated MMP-2 and TIMP-2 in *+/+* retinal extracts are similar. Same was true for *rd1/rd1* extracts. Active MMP-2, APMA activated MMP-2 and TIMP-2, all show peaks at PN2. Levels of APMA activated MMP-2 decrease with increasing age after PN2 both in the *rd1/rd1* and *+/+*. Levels of active MMP-2 both in the *rd1/rd1* and *+/+* decrease from PN2 with increasing age but the decrease in the *rd1/rd1* extracts is less and levels are significantly higher than age matched *+/+* retinas. The levels of TIMP-2 level in the *rd1/rd1* retinal extracts increases significantly after PN7 while its level in the *+/+* extracts increases non-significantly with increasing age. Ratio of active MMP-2 to TIMP-2 in *rd1/rd1* retinal extracts is elevated.

The age dependant changes in the levels of active MMP-9, APMA activated MMP-9 and TIMP-1 in *+/+* retinal extracts are similar. Same is true for *rd1/rd1* extracts. In *+/+* retinal extracts the levels of active MMP-9, APMA activated MMP-9 and TIMP-1 show peaks at PN2 and then decrease with increasing age. While in the *rd1/rd1* extracts peak was also observed at PN2 and then the levels of active MMP-9 and TIMP-1 decrease till PN7 and levels of APMA activated MMP-9 decrease till PN14. After this decrease an increase in

their levels was observed at PN21 and PN28 in the *rd1/rd1* retinas. At PN28 and PN21 the respective levels of active MMP-9/ TIMP-1 and APMA activated 9 were significantly higher in *rd1/rd1* extracts, as compared to *+/+* extracts. The ratio of active MMP-9 to TIMP-1 in *rd1/rd1* retinal extracts appears to be elevated.

The ratio of active MMP-9 to active MMP-2 in *+/+* retinal extracts is almost constant but increases with age in *rd1/rd1* extracts, being significantly higher at PN21. However, the ratio in extracts from both genotypes shows statistically non-significant differences.

At different ages the constant ratio of active MMP-9 to MMP-2 in *+/+* and an increasing ratio in those of *rd1/rd1* extracts suggest that with increasing age there is an increase in active MMP-9 relative to that of MMP-2 in *rd1/rd1* extracts. Thus it is possible that there is relatively higher involvement of MMP-9 in degenerating *rd1/rd1* retina. Also significantly elevated but disproportionate levels of active MMP-9, MMP-2, and TIMP-1 in the *rd1/rd1* mouse retina suggest a greater activation of MMP-9 and MMP-2. The level of MMP-9 and TIMP-1 in *+/+* retinal extracts decrease with age, possibly due to decrease in biosynthesis and / or increase in degradation or due to decreased requirement for development of ECM. The higher ratio of active MMP-9 and active MMP-2 to TIMP-1 suggests that differential increase in active MMP-9 and MMP-2 results in degradation of ECM which itself is known to regulate the levels of MMPs and TIMPs (Kaczmarek et al., 2002). The degeneration of IPM / ECM may lead to detachment of photoreceptors from the RPE and initiate the process of their apoptosis (Limb et al., 2002). It has been reported by Zhang et al. (2004), that up regulation of MMP-9 promotes retinal degeneration and loss of photoreceptors, irrespective of the cause and species, and increases synthesis of heparin sulfate proteoglycans and HNK-1 glycoproteins (Landers et al., 1994). It has also been reported that laminin is most likely degraded by MMP-9, leading to ganglion cell death and also possibly other pathological changes in the retina. Weak staining of core proteins in the sGAG / proteoglycans band in the ECM / IPM of *rd1/rd1* retinal extracts, suggests degradation of core proteins leading to a decrease in viability of photoreceptors (Lazarus et al., 1993).

VI Conclusions

Paper I Mouse retina explants after long-term culture in serum free medium

The main conclusions from this work are that it is possible to culture the intact mouse retina both neonatal and late postnatal, in a serum-free medium for a considerable period of time i.e. 26 days. Isolating the retinal explant at different developmental ages shows that green cone visual pigment differentiation will not proceed if the tissue is taken before the moment this process is initiated *in vivo*. Therefore, the green identity of cones is linked to signals present outside the cone cell body and perhaps even from molecules entering the retina from outside. Except for soluble proteins like IRBP, the distribution pattern of photoreceptor-specific proteins in explants corresponds to litter-matched retina. The calcium-binding protein markers expressed by the inner retina cellular elements are equivalent to the *in vivo* retina, but some aberrant sprouting of neurites may occur in the former.

Paper II Lens epithelium-derived growth factor (LEDGF) delays photoreceptor degeneration in explants of *rd/rd* mouse retina

LEDGF rescues photoreceptor cells in the *rd1/rd1* mouse retinal explants, and it has no effect on the differentiation of rod photoreceptors in the presence of RPE as judged by the expression of opsin and arrestin. The protective efficiency of LEDGF is similar to that of BDNF treatment but not as effective as the combination of CNTF and BDNF treatment.

Paper III Decreased Glutathione Transferase Levels in *rd1/rd1* mouse retina, Replenishment protects photoreceptors in retinal explants

From the second postnatal week onwards, the levels of the most abundant forms of GSTs, α -GST and μ -GST are decreased in *rd1/rd1* retinal degeneration. Both the GSTs were present in Müller cells (particularly in the endfeet) and horizontal cell fibres in both *rd1/rd1* and *+/+* retina during different ages of development i.e. PN2, PN7, PN14, PN21 and PN28. This anatomical localisation suggests that these GSTs act as detoxifying agents. α -GST and μ -GST are efficient rescue factors, and that the treatment is more effective when provided at an early neonatal age, compared to retina from older mice. α -GST is more effective than μ -GST. The neuroprotection by GSTs may be mediated by reduction of tissue oxidative stress by acting as exogenous sources of antioxidants.

Paper IV *rd1* Mouse Shows Imbalance in Cellular Distribution and Levels of TIMP-1/ MMP-9, TIMP-2/ MMP-2 and Sulphated Glycosaminoglycans

We have shown that increased activities of proteinases, MMP-9 and MMP-2 and imbalance with their endogenous inhibitors, TIMP-1 and TIMP-2 could also be

involved in retinal degeneration in the *rd1/rd1*. MMP-9, MMP-2, TIMP-1 and TIMP-2 are present in the IPM of both the genotypes. In *rd1/rd1* retina, undergoing extensive degeneration, MMP-2 and TIMP-2 are also present in the Müller cells and may be involved in the maintenance of the retinal integrity. The levels of TIMP-1, active MMP-9 and active MMP-2 in *rd1/rd1* retinal extracts are significantly increased but the disproportionately lower level of TIMP-1 cannot totally inhibit the activated MMPs. The relative increase in activities of MMP2 and MMP9 degrades IPM / ECM and increases the sGAG content and / or elevates biosynthesis of sGAG with higher sulfation. The involvement of other proteinases like cathepsins is not ruled out.

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IX Populärvetenskaplig sammanfattning

Näthinnan, retina, är den ljusmottagande delen av ögat. Ljuset fångas av s.k. fotoreceptorer (stavar för mörkerseende och tappor för färg- och skarpseende) och informationen om detta vidarebefordras därefter till hjärnan via ett antal olika nervceller. *Retinitis pigmentosa* tillhör en grupp av ärftliga retinala degenerationsjukdomar och leder till fotoreceptordöd och synförlust. I den industrialiserade delen av världen drabbas en på tretusen av denna sjukdom. Patienternas synhandikapp består av tilltagande besvär med försämrat mörkerseende, bländningsfenomen och ringskotom. Detta utvecklas ganska snart till försämringar i förmågan att se vad som händer i utkanten av synfältet och, oftast i ett längre tidsperspektiv, till nedsättning av det centrala seendet, där vår egentliga syn ligger. I vissa fall kan nedsatt synskärpa vara ett tidigt symptom, medan kontrastseendet och färgseendet försämras så småningom. Detta mönster av synpåverkan, där många delar av synfunktionen försämras, innebär att synförmågan rent praktiskt ofta är betydligt mer nedsatt än vad man kan tro av mätningar av enbart synskärpan. Sjukdomen är ärftlig, och utöver klinisk genetisk rådgivning finns det för närvarande inte någon fungerande möjlighet att förebygga eller bota densamma. Några av de behandlingsinriktningar som studeras är transplantation av hela eller delar av retina, stamcellstransplantation, genterapi samt behandling med antioxidanter och tillväxtfaktorer. För att experimentellt kunna studera hur degenerationen av retina går till och hur man kan skydda mot detta, finns det flera djurmodeller tillgängliga, exempelvis den s.k. rd1-musen.

Helhetssyftet med undersökningarna har varit 1) att analysera effekten av olika substanser som kan tänkas ha en skyddande funktion på degenererande retina i rd1-möss, och 2) att studera vilken roll en särskild typ av proteinnedbrytande enzymer (metalloproteinaser; MMPs) och deras hämmare (TIMPs) har i retinadegenerationen.

De specifika syftena var att:

- Utveckla ett odlingssystem utan serum och som använder sig av hel retina, för att kunna efterlikna situationen i det levande djuret. Fördelen med att använda sig av hel retina i ett odlingssystem är att man då behåller både vävnadens normala uppbyggnad och dess cellulära interaktioner. Samtidigt kan man studera effekten av olika substanser genom att tillsätta dessa till odlingsmediet.
- Studera effekterna av olika räddningsfaktorer på näthinnan, som till exempel Lens Epithelium Derived Growth Factor (LEDGF) och Glutathione S Transferase (GST) alpha och mu.

- Studera retinadegenerationens biokemi genom att analysera nivåerna av MMPs (MMP-2 och MMP-9) och TIMPs (TIMP-1 och TIMP-2) i retina från såväl normala djur som från *rd1*-möss.

För experimenten användes möss av typ C3H *rd1/rd1*, som lider av samma typ av retinadegeneration som kan finnas hos människor, samt normala, friska C3H *+/+* möss som kontroll. Hel retina dissekerades från ögonen från antingen två eller sju dagar (PN2, PN7) gamla möss och odlades sedan med sitt pigmentepitel (RPE) i tjugosex respektive tjugoen dagar, med eller utan tillsats av den tillväxtfaktor som skulle undersökas. Medium byttes varannan dag och efter avslutad odling blev retinan fixerad, fryssnittad och färgad. För att se om behandlingen gav en skyddsseffekt räknades raderna av överlevande fotoreceptorceller i det skikt som kallas outer nuclear layer (ONL). Andra metoder som använts har varit immunohistokemi och western blotting, som kan ge information om var, och i vilken mängd, ett visst protein finns i retinan.

I det första arbetet visades att det är möjligt att odla hel retina från nyfödda djur i mer än fyra veckor utan serum. Den odlade näthinnan hade under sådana förhållanden en vävnadstypisk utveckling. Dessutom var proteinuttrycket i de odlade fotoreceptorerna i princip jämförbart med situationen i levande djur av motsvarande ålder.

I det andra arbetet beskrevs hur LEGDF kan rädda de annars döende fotoreceptorerna i *rd1/rd1*-retina. Det gick också att visa att LEDGF inte påverkade differentieringen av stavarna, eftersom uttryck av två nyckelproteiner för detta, opsin och arrestin, inte påverkades av LEDGF-behandlingen.

Det tredje arbetet fokuserade på GST alpha och mu. GST kunde påvisas i s.k. Müller-celler och horisontalceller i retinan och nivåerna av såväl GST alpha som mu var lägre i *rd1/rd1*-retina än i den normala, friska vävnaden. Den lägre halten av GST kan ha påskyndat *rd1*-fotoreceptorernas degeneration, eftersom tillskott av GST fick dessa celler att överleva bättre under odlingen, oavsett om behandlingen påbörjades vid PN2 eller PN7. Dock var effekten större när den påbörjades vid PN2 (se bilden till höger). Fynden visar att det finns en förändring i redoxstatus i näthinnan hos *rd1/rd1* möss när den jämförs med näthinnan från de friska djuren.

I arbete fyra visades att en ökad aktivitet av två metalloproteinaser, MMP-2 och MMP-9, och en obalans mellan dessa och deras hämmare, TIMP-1 och TIMP-2, skulle kunna vara inblandat i degenerationen i *rd1/rd1*-retinan. MMP-2, MMP-9, TIMP-1 och TIMP-2 fanns i retinas interphotoreceptor matrix (IPM), d.v.s runt omkring de yttersta delarna av fotoreceptorerna, och förmodligen har dessa proteiner utsöndrats från celler i RPE. MMP-2 och TIMP-2 fanns också hos Müller-cellerna i *rd1/rd1*-retinan efter det att degenerationen hade påbörjats, och de skulle här kunna vara inblandade i att skydda den degenererande retinan. Mätningar visade att aktiviteten hos såväl TIMP-1, MMP-2 och MMP-9 var betydligt högre i *rd1/rd1*-retinan än i den friska motsvarigheten. Dock var

ökningen av TIMP-1-aktiviteten mindre än den för MMP-2 och MMP-9, vilket gjorde att den sannolikt inte fullständigt skulle kunna hämma dessa båda proteaser. Således har *rd1/rd1*-retinan en proportionellt sett ökad aktivitet av MMP-2 och MMP-9, vilket skulle kunna degradera IPM och därmed förvärra degenerationen.