THE UNIVERSITY OF CALGARY

A ROLE FOR THE EXTRACELLULAR MATRIX
IN RETINAL REGENERATION

BY

THOMAS NAGY

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DEGREE OF MASTERS OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

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ABSTRACT

Regeneration of the amphibian retina provides a rare example of regeneration within the vertebrate central nervous system (CNS). Studies on Urodeles have demonstrated two sources for new neurons in the regenerating retina. Those neurons located at the periphery of the retina arise from increased proliferation at the ora serrata, which normally provides neurons to the growing retina, while those neurons arising within the central retina originate from the transdifferentiation of retinal pigmented epithelial (RPE) cells (Stone, 1950a;b, Hasagawa, 1958; Keefe, 1973a;b;c). However, until recently transdifferentiation of RPE cells into neurons in Anurans has only been demonstrated through transplantation of RPE cells into the posterior chamber of lensectomized eyes of the same species (Loposhov and Sologub, 1972; Sologub, 1977). The work presented within this theses explores the process of retinal regeneration in the Ranid Anurans. It demonstrates that Rana tadpoles regenerate their retinas in a manner similar to Urodeles; with new neurons arising form both the ora serrata and transdifferentiation of RPE cells. Further, it provides evidence that at least some of the factors regulating the process of retinal regeneration may be similar to those factors regulating normal retinal development.

One factor found to regulate retinal regeneration is the extracellular matrix. During the process of regeneration, prior to RPE transdifferentiation, the RPE cells detach from their normal location adjacent to Bruch’s membrane and form a strong association with the vascular membrane. We have found the vascular membrane to be extremely rich in laminin immunoreactivity relative to Bruch’s membrane. Further, we have demonstrated that a laminin rich substrate will induce RPE to neuron transdifferentiation in cultured RPE cells. Finally, we have shown that transdifferentiation in vivo can be blocked through injection of the Inhibitor of Neurite Outgrowth (INO) monoclonal antibody (directed against the laminin-heparin sulphate proteoglycan complex) (Matthew and Patterson, 1983) into regenerating Rana tadpole eyes. These results strongly support a role for the extracellular matrix in regulating regeneration in the amphibian CNS and may provide insights into similar development or regenerative processes in other vertebrate systems.
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<tr>
<td>BCIP</td>
<td>5-Bromo-4-Chloro-3-Indoyl Phosphate</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CGN</td>
<td>Central Germinal Neuroepithelium</td>
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<td>CH</td>
<td>Choroid Layer</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>DR</td>
<td>Differentiated Retina</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EGZ</td>
<td>Early Germinal Zone</td>
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<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm</td>
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<tr>
<td>EHS-BM</td>
<td>Engelbreth-Holm-Swarm Basement Membrane Extract</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>FN</td>
<td>Fibronectin</td>
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<tr>
<td>GAM</td>
<td>Goat Anti-mouse</td>
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<tr>
<td>GR</td>
<td>Germinal Retina</td>
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<tr>
<td>HSPG</td>
<td>Heparin Sulfate Proteoglycan</td>
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<tr>
<td>INL</td>
<td>Inner Nuclear Layer</td>
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<tr>
<td>INO</td>
<td>Inhibitor of Neurite Outgrowth</td>
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<td>IPL</td>
<td>Inner Plexiform Layer</td>
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<td>LGZ</td>
<td>Late Germinal Zone</td>
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<td>MAB</td>
<td>Monoclonal Antibody</td>
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<td>MCG</td>
<td>Microgram</td>
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<tr>
<td>MCL</td>
<td>Microliter</td>
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<tr>
<td>NCAM</td>
<td>Neuronal Cell Adhesion Molecule</td>
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<td>NPZ</td>
<td>New Proliferative Germinal Neuroepithelium</td>
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<td>NR</td>
<td>Neural Retina</td>
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<td>ONL</td>
<td>Outer Nuclear Layer</td>
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<td>OPL</td>
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<td>OPZ</td>
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<td>Polyacrylamide Gel Electrophoreses</td>
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<tr>
<td>PMSF</td>
<td>Phenyl-Methylsulfonylic Acid</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
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<td>PR</td>
<td>Photoreceptors</td>
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<td>RGC</td>
<td>Retinal Ganglion Cells</td>
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<td>RPE</td>
<td>Retinal Pigment Epithelium</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine Isothiocyanate</td>
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<td>VM</td>
<td>Vascular Membrane</td>
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Transdifferentiation is a term which describes a switch in the differentiated status of a cell from one specialized state to another. Many previous studies have documented transdifferentiation of Retinal Pigment Epithelium (RPE) cells into a variety of ocular tissues, particularly lens and retina (for review see Reyer, 1977; Eguchi, 1979; Okada, 1980; Yamada, 1982; Eguchi, 1986). Although well studied in the case of transdifferentiation to lens, far less is known concerning the transdifferentiation of RPE to retina. The purpose of this study is to determine the inductive signal responsible for initiating the transdifferentiation of RPE cells into retina.

BACKGROUND

Origin of the Eye

During development of the vertebrate eye, the optic vesicle develops as a lateral evagination of the neural folds as they draw together to form the neural tube (Fig.1) (Reyer, 1977). The apical region subsequently invaginates into the proximal region to form the optic cup (Reyer, 1977). Formation of the optic cup gradually obliterates the cavity of the optic vesicle leaving the proximal and apical layers in very close apposition (Hopper and Hart, 1980). It is thought that contact of the proximal layer with surrounding mesenchyme, as well as its close proximity to developing blood vessels, influences it to thin and acquire pigment granules (Reyer, 1977; Hopper and Hart, 1980). This layer eventually develops into the densely pigmented Retinal Pigment Epithelium (Fig.2). At the same time the inner layer
segregates into the \textit{pars optica retinae} and the \textit{pars caeca retinae} (fig.1) (Hopper and Hart, 1980). The thinner, nonnervous, \textit{pars caeca retinae} represents the circumferential region of the eye that eventually forms the Ciliary Margin and Iris (Hopper and Hart, 1980). This region is separated from the \textit{pars optica retinae} by a depression, the \textit{ora serrata} (Hopper and Hart, 1980). Cells within the \textit{pars optica retinae}, through the process of inner kinetic migration, continue to proliferate (Reyer, 1977; Hopper and Hart, 1980). As the \textit{pars optica retinae} thickens it differentiates to form the retina. First the ganglion cell layer is formed, followed by the inner plexiform layer, the inner nuclear layer, the outer plexiform layer, and finally the outer nuclear layer (Reyer, 1977). Proliferation first ceases in the fundic region; however, at later embryonic stages, larval stages, or in some adult amphibia new neurons are continually added along the periphery via proliferation within the \textit{ora serrata} (Reyer, 1977).

While the retina and RPE arise from neural tube the lens originates from the overlying ectoderm (Hopper and Hart, 1980). Early in development when the optic vesicle approaches the surface the overlying ectoderm is induced to thicken and form a lens placode (Hopper and Hart, 1980). As the optic cup forms, the lens placode invaginates into it forming a lens vesicle. Shortly there after the lens vesicle closes and breaks away from the epithelial surface to form a rudimentary lens. Gradually this rudimentary lens vesicle fills and differentiates to form
the lens proper (Hopper and Hart, 1980).

**Retinal Regeneration**

Regeneration of the retina following surgical removal (retinectomy) or devascularization is well documented in a number of vertebrate species including amphibians, avians, and mammals (Stroeva, 1962; Coulombre and Coulombre, 1965; Reyer, 1977; Eguchi, 1979; Okada, 1980). Although primarily restricted to larval or embryonic stages of development in most vertebrates, retinal regeneration in adult Urodeles is well documented. Studies by Stone (1950a,b) in *Triturus v. viridescens* initially suggested transdifferentiation of RPE cells as the sole source of new neurons in the regenerating retina following retinectomy. Hasegawa (1958), however, through surgical removal of the RPE as well as the neural retina (NR) in *Triturus pyrrhocaster*, demonstrated increased proliferation at the *ora serrata* as an additional source of new retinal neurons. Subsequent autoradiographic studies using $^3$H-Thymidine incorporation in *Triturus pyrrhocaster* have supported dual sources for new neurons (Keefe, 1973). Although rare, transdifferentiation of RPE to NR has also been demonstrated in clonal cultures of Newt RPE cells (Okada, 1980).

Studies on retinal regeneration in anurans have been less conclusive regarding the role of transdifferentiation in this process. Early studies of adult *Bombinator* suggest that the retina fails to regenerate following lens and retinectomy in this species (Stroeva, 1956), while other studies of this phenomenon
in *Bufo virides*, *Rana esculenta*, and *Rana temporaria* tadpoles suggest that regeneration occurs only via proliferation of the inner unpigmented layer of the iris and not by RPE transdifferentiation (Dabagyan *et al.*, 1967). However, transdifferentiation of *Rana temporaria*, *Rana esculenta* or *Xenopus* RPE to NR has been observed through culture of NR between sheets of RPE or following transplantation of RPE pieces into the anterior chamber of lensectomized eyes of the same species (Loposhov and Sologub, 1972; Loposhov, 1977). When small pieces of RPE are transplanted into the anterior chambers of larval frogs, complete new retinas form suspended in the vitreous. The aim of this work is to examine retinal regeneration in the Ranid Anurans in hope of eliciting the factor responsible for inducing RPE to neuron transdifferentiation *in vivo*. 
FIGURE LEGENDS

Figure 1. Schematic diagram illustrating the origins of the various tissues within the vertebrate eye (from Hopper and Hart, 1980).

Figure 2. Cross section of vertebrate eye later in development delineating the locations of the various ocular tissues involved (from Hopper and Hart, 1980).
CHAPTER TWO

A Possible Role for the Vascular Membrane in Retinal Regeneration in *Rana catesbeinna* Tadpoles.
INTRODUCTION

Regeneration of the vertebrate retina following retinectomy or devascularization of the eye has been well documented in urodeles. Studies by Stone (1950 a,b) first suggested that this regeneration arose from the metaplasia (or transdifferentiation) of retinal pigment epithelial cells (RPE) in the Salamander, Amblystoma punctatum. More recently, regeneration of retina in the newt, Notophthalmus viridescens, has also been extensively described (Keefe, 1973 a-d; Reyer, 1977; Gaze and Watson, 1968). Although these studies have generally confirmed the earlier work, showing that RPE transdifferentiation is a source of new retinal neuroblasts, they also demonstrated that an additional source of new cells was the ora serrata or ciliary margin of the retina, by an increase in proliferation of the cells that normally account for the retinal growth in larva and adult amphibians (Hollyfield, 1968; Straznicky and Gaze, 1971; Beach and Jacobson, 1979; Reh and Constantine-Paton, 1983).

Studies of retinal regeneration in other amphibians, particularly anurans, however, have not been as conclusive. For example, regeneration has not been observed in Bombinator, Bufo, or several species of Rana tadpoles following retinectomy (see Reyer, 1977 for review). However other studies have found that transplantation of RPE sheets to the posterior eye chamber of Rana temporaria, Rana esculanta, or Xenopus did result in metaplasia and subsequent production of new retina (Loposhov and Sologub, 1972; Sologub, 1977).
Therefore, in this report we have examined whether the *Rana catesbeinna* retina will regenerate following devascularization. We have found that retinal regeneration does occur in this species and, like the newt, the regeneration occurs from both increased cell proliferation at the ciliary margin, and transdifferentiation of RPE cells. In addition, using a recently developed monoclonal antibody raised in our laboratory against frog nervous system and neuroblasts, (Reh and Nagy, 1988) we have been able to follow the process of RPE transdifferentiation more accurately than was previously possible. Consequently, we have discovered a new step in the process; there is a high degree of association of migratory pigment cells with the vascular membrane at the time of the initial pigment cell transdifferentiation to retinal neuroblasts.

**METHODS**

*Rana catesbeinna* tadpoles at midlarval (TK/X-XV) stages were obtained from Nasco Scientific (Ft. Atkinson, WI, USA) or Kons Scientific (Janesville, WI USA), kept in 5 gallon tanks at 23 C, and fed on Tetramin. The devascularization was performed under 2-phenoxyethanol anaesthesia, by completely removing the eye from the orbit and then reimplanting it. This procedure insured that all blood supply, and the optic nerve, would be severed. The animals were then revived in oxygenated water and sacrificed at weekly intervals. The eyes of the animals were then fixed in either Bouins' solution for subsequent plastic embedding or in a 2% paraformaldehyde solution for cryostat sectioning. For
plastic embedding, the eyes were dehydrated and embedded in a methacrylate resin (Fisher, Edmonton, Alta Canada) and sectioned serially at 4 microns on a Sorval microtome; cryostat sections were taken on a LKB cryostat at 8-10 microns. Plastic sections were stained with toluidine blue.

The monoclonal antibodies used in this study were produced as follows. A mixture of larval and adult Rana catesbeinna retinas was homogenized in 2 ml 0.32 M sucrose with 25 strokes of a Dounce Teflon-glass homogenizer and a membrane fraction was prepared by standard differential centrifugation procedures. The final pellet was resuspended in 200 mcl of 0.32 M sucrose and assayed for protein concentration using the Bio-Rad (Richmond, CA, USA) dye binding micro-protein assay. This was then used as an antigen stock solution.

Balb/c mice were initially immunized by intraperitoneal injection of 1 mg antigen in Freunds complete adjuvant. Mice were then boosted intraperitoneally with 1 mg antigen (no adjuvant) at 2 to 3 week intervals for a total of 4 boosts. Five days after the final boost mice were sacrificed via dry ice asphyxiation; the spleens were then removed, and their cells were harvested (average: 2 x 10^5 cells/spleen) and fused with SP2 myeloma cells (typically 4 x 10^7) according to the method of Galfre et al (1976). The fused cells were resuspended in 125 mls of HAT media and plated onto six 24-well tissue culture plates. Cell growth was microscopically visible within 1-2 weeks. The hybridomas were screened by indirect immunofluorescence on both
fresh and 2% paraformaldehyde fixed cryostat sections of larval Rana catesbeinna retina. Positive wells were cloned by limiting dilution. Antibodies were considered monoclonal when dilution in a 96 well plate produced clones in less than one third of the wells.

Immunohistochemistry was carried out on the cryostat sections of regenerating retinas using the following protocol. Sections were first incubated with either 2% skim milk powder or 5% fetal bovine serum in frog Ringer’s for 30 min. Following removal of the blocking solutions, adjacent sections were incubated with culture supernatant from hybridoma 2D3 or 1C2. After a one to two hour incubation, the slides were washed twice with Ringer’s and incubated in a goat-anti-mouse antibody conjugated to rhodamine. (Sigma, St. Louis, MO, USA). Following a final rinse the sections were mounted in glycerol (with 2% n-propyl gallate added) and viewed with a Zeiss fluorescent microscope. The same region of adjacent sections from each of the two and three week cases (n=9) were located and drawn with a camera lucida and the length of correspondence of the two labels was measured from the drawings on a planimeter (Bioquant) and expressed as a percentage of total measured length (6.3 mm) in Table I. In another set of sections from the same animals, individual sections were double-labelled with a rabbit antiserum to fibronectin (Calbiochem, San Diego, CA, USA) followed by incubation with a goat-anti-rabbit antibody conjugated to fluorescein (Sigma, St. Louis, MO, USA), to label the vascular
membrane, and subsequently incubated with the 2D3 monoclonal, which had been directly conjugated to tetramethylrhodamine isothiocyanate (Molecular Probes, Eugene, OR, USA) by the method of Goding (1976). The rabbit anti-human fibronectin reliably labelled the vascular membrane and the scleral tissue surrounding the retina, but binding was never present within the nervous system. The specificity of the antiserum for fibronectin was checked by immunoblotting and by preabsorption with fibronectin. Camera lucida drawings were again made from these double-labelled sections and the length of contact between the vascular membrane and the 2D3 labelled cells could be determined directly; this is also expressed as a percentage of total measured length (9.2 mm) in Table I.

RESULTS

The normal retina of Rana catesbeinna tadpoles consists primarily of fully differentiated areas, similar to those observed in the adult frog (Fig. 6a). These regions display the same laminar pattern of other vertebrate species, ie, a retinal ganglion cell layer (RGC), an inner plexiform layer (IPL), an inner nuclear layer (INL), an outer plexiform layer (OPL), an outer nuclear layer (ONL), and a layer of photoreceptor outer segments. In addition, at the ora serrata or ciliary margin, there is an unlaminated, undifferentiated, proliferating neuroepithelium. This zone is responsible for the continued retinal growth that occurs during larval development, in a manner similar to that observed in other species of amphibians and
teleosts. (Hollyfield, 1968; 1971; 1972; Beach and Jacobson, 1979; Stranznicky and Gaze, 1971; Easter et al, 1977; Johns, 1977; Reh and Constantine-Paton, 1983). Adjacent to the neural retina on the vitreal surface is the retinal vascular membrane, while on the scleral surface the photoreceptor outer segments are in contact with the cells of the RPE. On their scleral surface the RPE cell contact a thick basement membrane, known as Bruch’s membrane, which is interposed between the RPE and the choroid, a zone of neural crest derived pigment cells that are in direct contact with the thick collagenous sclera that surrounds the eye.

The normal staining patterns of the monoclonal antibodies used in this study are shown on sections of control Rana tadpoles in figures 1 a-d. The neuron specific antibody 2D3 stains the entire differentiated retina, with the exception of the photoreceptor outer segments, as well as the zone of neuroblasts at the ciliary margin (Figs 1 a,b). In another study (Reh and Nagy, 1988) we have shown that this antibody is directed against a cell surface protein that is present only on neurons, and on all neurons and their processes. In addition, we have found that this antigen is present on all germinal neuroepithelial cells in Rana embryos and tadpoles. Preliminary immunoblot analysis indicates that the 2D3 antibody recognizes a 180-200 kD glycoprotein, similar to N-CAM, that is specific to Ranids. The 2D3 antigen is found throughout the nervous system of mid-larval staged R. catesbeinna and R. pipiens, but not Xenopus laevis or Bombina orientalis (Reh and Nagy, 1988). Although in the chick
N-CAM, has been found on non-neuronal tissues during embryonic development (Rutishauser, 1983; Edelman, 1983) a recent study in Xenopus, (Jacobson and Rutishauser, 1986) indicates that this molecule is restricted to the nervous system in embryonic frogs. Also, we have examined Rana embryos and tadpoles at many embryonic stages, and have found the 2D3 antigen restricted to the nervous system in all cases (Reh and Nagy, 1987). Therefore, it is likely that the 2D3 antibody can be used to reliably identify transdifferentiated neuroblasts in the regenerating retina. The second antibody used in this study, 1C2, was raised against the vascular membrane of the retina, and appears to stain all vasculature, as well as other connective tissue elements in the frog (Fig 1 c,d). Although we know much less about the molecular nature of the antigen recognized by 1C2, preliminary characterization suggests that it is likely to be localized extracellularly or on the cell surface.

One week following devascularization, all eyes examined show extensive degeneration of the neural retina. In most cases the trilaminar retinal structure is still recognizable, with the RGC and ONL surviving longer than the cells of the INL. However,
is still some faint 2D3 staining in the degenerating retina, but not on the pigment cells. Also, it is noteworthy, that the vascular membrane that normally covers the retinal surface, remains intact at this time, and in all subsequent times examined (Fig. 2 a-c). This can be seen as well in sections stained with MAb 1C2. At the ciliary margin, the zone of neuroblasts that normally accounts for retinal growth in tadpoles remains intact, (Fig 2a) and 2D3+ throughout the degenerative process. In some cases this zone even expands in response to the degeneration of the differentiated retina; however, this zone is always confined to the most peripheral tenth of the retina, and never extends into central, regenerating retinal regions.

By the end of the second week, virtually all of the degenerating retina and its associated dim 2D3 staining, has been removed. The pigment cell delamination, from Bruch’s membrane, first observed after one week is even more widespread at this time (Fig 2c) and in some cases the migratory pigment cells form large aggregates. No obvious regeneration of central retina has occurred at this time; however, some of the pigment cells are beginning to associate with the vascular membrane (Fig 3 a,b). When the cells reach this surface, many lose their pigment and adhere to the membrane. It is at this point that the first new 2D3+ cells appear in the central, regenerating retina. These largely depigmented cells stain intensely with the 2D3 antibody (Fig 4 a-b). All of the 2D3+ cells observed at this time are associated with the vascular membrane. Labelling of adjacent
sections with the two antibodies (2D3 and 1C2) demonstrate this close association (Fig 4 a-c). Although many clusters of RPE cells were present that had delaminated from Bruch’s membrane, only those cells that had made contact with the vascular membrane, were 2D3+. This close association of 2D3+ cells and the vascular membrane is further demonstrated in Table I, where this relationship is quantified from adjacent sections of the two and three week post-operative cases.

In addition, in five cases single sections were double labelled with rabbit antiserum to fibronectin, followed by a fluorescein-conjugated second antibody and a rhodamine conjugate of the 2D3 monoclonal, (see Methods). The antifibronectin antibody binds to the vascular membrane and the scleral tissue surrounding the retina in the normal tadpole and throughout the period of regeneration. No cells were ever found to contain both labels. However, as was observed using adjacent sections, cells that were positive for 2D3, were nearly always adjacent to fibronectin positive cells.

Figure 5 shows another example of this correspondence between the 2D3+ cells and the vascular membrane. In figures 5 a,b,c the distribution of the labels can be seen in a section of normal tadpole retina, while figures 5 d,e,f demonstrate the two labels are in a section of regenerating retina, three weeks following devascularization. The degree of adjacency of 2D3 labelling and the vascular membrane (now labelled with the antifibronectin antiserum) was also determined for these double
labelled sections as described in the Methods, and is also shown in Table I. Overall, the two methods of labelling, by adjacent sections, or double labelled single sections are in good agreement. There is a high degree of association between the 2D3+ cells and the vascular membrane at the very earliest stages of retinal regeneration. Table I also shows that at times, pigmented, 2D3 negative cells were found to be adjacent to the vascular membrane, however, 2D3 positive cells were rarely not in contact with this structure (3-4%).

The pigment cells continue to associate with this membrane in the next two weeks, adding additional neuroblasts to the regenerating retina (Fig. 3 b,c). By three weeks, many cases already showed evidence of a typical germinal neuroepithelium, (Fig. 3b) and by four weeks this central proliferative zone often joined with that at the ciliary margin to form a continuous hemisphere of proliferating cells. The presence of large numbers of mitotic figures demonstrates that the cells are undergoing proliferation at this time (Fig. 3c).

The first evidence of differentiated retina in the central visual area also occurs at the fourth week; however, differentiation is more commonly observed five weeks after devascularization. Although in some areas the ganglion cell layer and inner plexiform layer are becoming distinguishable much of the retina is composed of pigment cells and dividing neuroblasts.

Differentiation of the retinal laminae occurs in most cases
from the fifth to the eighth week postoperatively. While there is some interanimal variability in the precise time course of this differentiation, the sequence is invariant (Table II). The retinal ganglion cell and innerplexiform layers are the first to appear, (Fig. 3d) typically by the fifth week followed by the inner nuclear layer, at the sixth week. Next the outer plexiform layer and the outer nuclear layer, develop, such that by eight weeks after the devascularization, retinal regeneration is essentially complete; all cases have well laminated retinas with fully formed outer segments in central retina (Fig. 6 a-d). The remaining RPE cells are now only found in their normal location, adjacent to Bruch’s membrane. At this time, it is usually still possible to discern the relative contribution of the new retina from the ora serrata and the central RPE cells, since as in urodeles (Stone, 1950a), a discontinuity in the normal retinal structure is frequently present between them (Fig. 6c). In some cases, a new ora serrata-like proliferative zone is generated in the central retina, (Fig. 6d) resulting in a new germinal cone forming in the central retina.

DISCUSSION

Two main conclusions can be drawn from this study. First, in Rana catesbeinna tadpoles, retinal regeneration follows a time course and pattern similar to that observed in several urodeles. Second, there is a strong association of the retinal pigment epithelial cells with the vascular membrane at the time the pigment cells are undergoing transdifferentiation into retinal
germinal neuroepithelium. Figure 7 summarizes these results in diagrammatic form. The initial stage of retinal degeneration (B) and phagocytosis of retinal cells by migratory RPE cells is followed by a phase in which the pigment cells associate with the vascular membrane (C). Next these cells loose their pigment (D) and proliferate to form a germinal neuroepithelium (E) which differentiates into all retinal layers (A).

As mentioned in the Introduction some studies have failed to detect any regeneration in anurans following complete retinectomy (Lopashov, 1949, 1963; Sologub 1968). In some cases, where the iris was left intact, regeneration was at times observed (Reyer, 1977). These results suggested that the new retina arose, not from transdifferentiated pigment cells, but rather from an enhanced proliferation of the normally mitotically active germinal neuroepithelium at the ora serrata or ciliary margin. However, in light of the present results, it is likely that a significant amount of the new retina is generated from transdifferentiation of RPE cells following devascularization. In addition, transdifferentiation can occur in RPE sheets, transplanted into the posterior chamber of Rana temporaria tadpoles (Sato, 1953; Lopashov and Sologub, 1972), also consistent with the hypothesis that anurans do exhibit retinal regeneration in a manner similar to that found in urodeles; ie, via both continued addition at the normal ora serrata growth zone and by transdifferentiation of posterior retinal pigmented epithelial cells.
Although it has generally been accepted in urodeles that the new retina is derived from transdifferentiating RPE cells, (Reyer, 1977; Keefe, 1973 a-d; Stone 1950 a,b) recent evidence in the goldfish suggests that the slowly dividing stem cell population of rod precursors within differentiated retina may give rise to other retinal cell types following retinal cell damage by neurotoxins (Johns, personal communication). We do not believe that this is the source of the new retina in Rana catesbeinna for several reasons. First, there is no evidence for rod precursors in differentiated Rana tadpole retina despite extensive H³-thymidine labelling experiments at all stages (Reh and Constantine-Paton, 1983). Second, there is typically complete degeneration of all cells in the differentiated retina prior to the appearance of a germinal neuroepithelium and 2D3 positive cells at the vascular membrane. Third, transitional stages of depigmenting RPE cells are usually seen to be in contact with the vascular membrane. Lastly, clonal cell cultures of RPE cells from Rana tadpoles can give rise to 2D3+ and tetanus toxin positive neuronal cells, like those generated in similar cultures of urodele RPE (Reh et al, 1987).

The factors that induce RPE cells to transdifferentiate into the cells of the germinal neuroepithelium have not yet been identified. It has been suggested that mitotic division or loss of pigment granules are necessary steps in the process (Okada, 1980; Yamada, 1982; Eguchi and Itoh, 1981) however, direct tests of these hypotheses have not been conclusive (Masuda and Eguchi,
1982, 1984). What has been clear from earlier descriptive studies, as well as transplantation experiments, is that the RPE cells must detach from Bruch's membrane if they are to undergo the transition to neuroblasts (Loposhov and Sologub, 1972). In the present study, we have also observed that contact with the vascular membrane is highly correlated with RPE transdifferentiation, and may be important in the process. These results suggest that some components of the vascular membrane and Bruch's membrane may play a role in the regulation of the differentiated state of the RPE cells. One possible mechanism by which these structures might influence the differentiated state of RPE cells is through secretion of factors into the extracellular matrix. In vitro studies also support a role for the ECM in the transdifferentiation of RPE cells. High density culturing (Itoh and Eguchi, 1986) and culturing on collagen (type I) both inhibit RPE transdifferentiation to lentoid bodies (Yasuda, 1979). Along these lines we have recently found that certain ECM molecules, added to *Rana catesbeinna* clonal cultures, have profound effects on the morphology and biochemistry of pigment cells (Reh et al. 1987); in these cultures, laminin, a component of most basal lamina, induces RPE to neuronal transdifferentiation.

There is considerable evidence from other epithelial cell types that elements of the ECM have influences on the state of differentiation (see Hay, 1984 and Sabatini et al, 1983 for review). Differentiation of lens corneal epithelium (Hay, 1984),
as well as morphogenesis of salivary gland (Bernfield and Banerjee, 1978), testicular seminiferous tubules, (Hadley et al, 1985) metanephric duct, (Toole, 1981) and, mammary gland epithelium have been shown to involve interactions with ECM components. In addition, laminin and fibronectin have been shown to enhance neurite outgrowth as well as other aspects of differentiation in a variety of neuronal cell types (Acheson et al, 1986; Manthorpe et al, 1983; Edgar et al, 1984). Therefore, it may be that the ECM of the vascular membrane influences the transdifferentiation of RPE cells to germinal neuroepithelial cells during retinal regeneration in amphibia.
Figure 1. The immunohistochemical localization of the antigens recognized by the two monoclonal antibodies used in this study is shown in paired photo micrographs of normal *Rana catesbeinna* retinal sections. In A (fluorescent) and B (phase), the 2D3 antibody is present on both the mitotically active germinal neuroepithelial cells at the *ora serrata* (arrow) as well as those cells in the differentiated retina. No label is present on the pigment epithelial cells, the sclera, or on the cells of the vascular membrane. In C and D the 1C2 antibody binds an antigen that is present on cells of the vascular membrane (black arrow head), the sclera, and Bruch's membrane (white arrow head). Magnification: 187X A-D. Abbreviations: vascular membrane, vm; choroid, ch; retinal ganglion cell layer, rgc; inner nuclear layer, inl; outer nuclear layer, onl; retinal pigment epithelium, rpe.
Figure 2. Photomicrographs documenting the extensive retinal degeneration present in the first two weeks following devascularization (A and B). The retinal layers can still be seen, but they are primarily composed of darkly-staining, pycnotic nuclei. However, at the ora serrata, the germinal neuroepithelium survives intact (arrows, A) and the vascular membrane remains, and even expands (arrowheads, A,B). Two weeks following devascularization (C), the cells of the RPE have undergone extensive migration, while little remains of the retina. Magnification: 83X, A; 129X, B; 123X, C. Abbreviations: degenerating retina, DR; retinal pigment epithelium, rpe; choroid, ch; ora serrata, os.
Figure 3. Photomicrographs of regenerating retina at two (A), three (B), four (C), and five (D) weeks following devascularization. All four micrographs are from the most central area of retina. In A, the RPE cell are just beginning to associate with the vascular membrane. In B, pigmented cells (arrows) along the vascular membrane form a continuous layer with the unpigmented, presumptive germinal neuroepithelial cells. In C, the germinal neuroepithelium is well formed and many mitotic figures (arrows) are present. By five weeks (D), the retinal ganglion cell layer, and inner plexiform layers have begun to appear. Magnification: 535X, A; 234X, B; 365X, C; 375X, D. Abbreviations: vascular membrane, vm; retinal ganglion cell layer, rgc; retinal pigment epithelium, rpe; inner plexiform layer, ipl.
Figure 4. Adjacent 10 micron cryostat sections labelled with 2D3 in A and 1C2 in C; B is a phase micrograph of the section in C. The first 2D3 positive cells of the regenerating retina were always flat cells (arrow), closely adherent with the 1C2 positive vascular membrane (arrow head). Typically, these early 2D3 positive flat cells were lightly pigmented. Magnification: 204X, A-C.
Figure 5. Fluorescent and phase photomicrographs demonstrating double labelling in a normal retinal section (A-C) and a regenerating retina (D-F). Sections were labelled using the rabbit antifibronectin antibody (FITC) to label the vascular membrane (A and D), and the 2D3 mouse monoclonal antibody (TRITC) to label retinal neurons and neuroblasts (C and F). FITC labelled cells are adjacent to TRITC labelled cells, but are non-overlapping. Magnification: 2.44X for A-F.

Abbreviations: retinal ganglion cell layer, rgc; inner nuclear layer, inl; outer nuclear layer, onl; vascular membrane, vm; retinal pigment epithelium, rpe.
Figure 6. The final stages of retinal regeneration, showing sections of nine (A,B, and C) and fourteen (D) week regenerate animals. All retinal layers are present (A), as well as the proliferative cells at the ora serrata (B). Typically, there is a discontinuity (arrow) between the retina derived from transdifferentiation of RPE cells, and that from the germinal cells at the ora serrata (C). Occasionally, a new zone of proliferative cells is formed at the center of the eye (D). These animals then have double retinas that grow from the center of the eye as well as at the ora serrata. Magnification: 89X, A; 94X, B; 176X, C; 57X, D. Abbreviations: retinal ganglion cell layer, rgc; inner nuclear layer, inl; outer nuclear layer, onl; vascular membrane, vm; retinal pigment epithelium, rpe; choroid, ch; original and new proliferative germinal neuroepithelial zones, opz and npz, respectively.
Figure 7. Schematic diagram of the sequence of retinal regeneration observed in Rana catesbeinna. The initial stage of retinal degeneration (B) and phagocytosis of retinal cells by migratory RPE cells is followed by a phase in which the pigment cells associate with the vascular membrane (C). Next these cells lose their pigment (D) and proliferate to form a germinal neuroepithelium (E), which differentiates into all retinal layers (A).
TABLE I

DEGREE OF CORRESPONDENCE OF NEURON-SPECIFIC AND VASCULAR MEMBRANE ANTIBODY LABELLING

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<td>24%</td>
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<td>1C2-</td>
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<td>FN+</td>
<td>78%</td>
<td>18%</td>
</tr>
<tr>
<td>FN-</td>
<td>4%</td>
<td>-</td>
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Correspondence of the two labels measured as percentages of length of contact on adjacent sections when using the 1C2 antibody (total measured length 6.3 mm in 9 cases at 2 and 3 weeks after devascularization) and on the same sections when using the fibronectin antibody (FN)(total measured length 9.2 mm in 5 cases at 2 and 3 weeks after devascularization).
TABLE II

WEEKS FOLLOWING DEVASCULARIZATION

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<th>7</th>
<th>8</th>
<th>&gt;9</th>
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<td>5/5</td>
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<tr>
<td>NEUROBLASTS</td>
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<td>5/5</td>
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n = 43

These numbers reflect those cases of the total in which the particular cell type noted was observed. Abbreviations: Retinal Ganglion Cell Layer (RGC); Inner Nuclear Layer (INL); Outer Nuclear Layer (ONL).
CHAPTER THREE

Characterization of Rana Germinal Neuroepithelial Cells in Normal and Regenerating Retina
INTRODUCTION

Although restricted to fetal or embryonic stages in higher vertebrates (Coulombre and Coulombre, 1965; Stoeva, 1960), regeneration of the adult retina following damage or removal has been well documented in a number of Urodele species. Several studies have also demonstrated two sources for the new neurons which arise within the regenerating retina. Neurons arising at the periphery of the retina arise via enhanced proliferation at the ora serrata which is normally present throughout development. New neurons within the central retina arise from the transdifferentiation or metaplasia of retinal pigment epithelial (RPE) cells (Stone, 1950a; 1950b).

While early studies on Anurans, i.e. Bombinator, Bufo, and Rana, failed to demonstrate significant retinal regeneration (see Reyer, 1977 for review), more recently studies on Xenopus and Rana, when RPE pieces were transplanted into the anterior chamber of lensectomized eyes of the same species, have demonstrated that RPE can transdifferentiate to neural retina (Loposhov and Sologub, 1972; Loposhov, 1977). Retinal regeneration has also been reported in Rana catesbeinna tadpoles following devascularization, with new neurons arising from both the ora serrata and transdifferentiation of RPE cells (Reh and Nagy, 1987). In addition, we have shown that Rana RPE in vitro can give rise to neuronal cells (Reh et al., 1987). In the present study we have used a panel of neural specific monoclonal antibodies to show that this central germinal zone, derived from
the transdifferentiation of RPE cells, is very similar to the normal germinal neuroepithelial zone at the ora serrata. This supports the hypothesis that similar mechanisms regulate neurogenesis in regeneration and normal development.

MATERIALS AND METHODS

Animals

*Rana catesbeinna* tadpoles at midlarval (TK/X-XV) stages were obtained from Nasco Scientific (Fort Alkinson, WI) or Kons Scientific (Janesville, WI), kept in 5 gal. tanks at 23°C, and fed on Tetramin. Balb/c mice were obtained at six weeks of age from Charles River Laboratories (St. Constant, Quebec, Canada).

Monoclonal Antibody Production

Production of the monoclonal antibodies used in this study has been described previously (Reh and Nagy, 1987). Briefly, antigen was prepared from a mixture of adult and larval *Rana catesbeinna* retinas homogenized in .32M Sucrose and fractionated through a standard differential centrifugation protocol. The membrane fraction was collected and used as an antigen stock solution.

Balb/c mice were initially immunized by intraperitoneal injection of 1mg antigen in Freund's complete adjuvant. The mice subsequently received 4 boosts at 2 to 3 week intervals of 1mg antigen each (no adjuvant). Five days following the final boost the mice were sacrificed. The splenocytes were harvested and fused with SP2 myeloma cells according to the method of Gafee et al (1977). The fused cells were resuspended in 150mls of HAT
media and plated into six 24-well tissue culture plates. Hybridomas were screened using indirect immunofluorescence on both fresh and 2% paraformaldehyde fixed cryostat sections of larval *Rana catesbeinna* retina. Positive wells were cloned by limiting dilution.

**Immunohistochemistry**

Following a 20 minute preincubation with either 2% skim milk powder or 2% Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO), cryostat sections of *Rana catesbeinna* retina were incubated with the appropriate monoclonal antibody for two hours. The antibodies used were either from conditioned hybridoma supernatant or ascitic fluid diluted fifty times in Ringer’s solution. The sections were subsequently washed twice with Ringer’s solution and incubated for an additional hour with rhodamine conjugated goat anti-mouse antibody. Following two final washes the sections were mounted with glycerol and viewed using a Zeiss fluorescent microscope. For antibody characterization the *Rana* retina cryostat section was treated with either neuraminidase (100 units/ml; 15 min.), trypsin (0.1%; 15 min.), periodate (0.01% in acetate buffer; 30 min.), or chloroform/methanol (2:1,v:v; 30 min.) prior to blocking.

Retinal cultures were stained by overnight incubation in monoclonal antibodies which had been previously purified with DEAE Affi-Gel Blue chromatography (Bio-Rad, Richmond, CA). Following two washes in 10% Frog Culture Media (10% PBS, 100 mM L-Gln, 200 units/ml Penicillin, 2mg/ml Streptomycin in 0.5X L-15
media), the cultures were incubated in rhodamine conjugated goat anti-mouse antibody (Sigma, St. Louis, MO) for three hours. The cultures were given a final wash and viewed using a Zeiss inverted fluorescent microscope. Retinal cultures were produced by digestion of Rana retina with 200 units/ml Collagenase (Sigma, St Louis, MO) followed by trituration and culture in 10% Frog Culture Media.

**Immunoblotting**

Various tissues from larval Rana tadpoles were homogenized in distilled water containing 0.5mM phenylmethylsulfonylic acid (PMSF) (Sigma, St. Louis, MO). The protein concentration was determined using the Bio-Rad dye binding assay. Samples were boiled for five minutes in 8% SDS sample buffer, centrifuged 5 min. at 10,000xg, and resolved by SDS-PAG Electrophoresis on 7.5 or 10% nonreducing polyacrylamide gels. For endoneuraminidase pretreatment samples were digested 15 min. with 140 units/ml endoneuraminidase according to the method of Rutishauser et al (1985).

Following electrophoresis the samples were electroblotted onto nitrocellulose paper and probed with antibody. Blots were incubated in wash solution (500mMNaCl, 20mMTrisHCl, pH7.4) with 2% BSA for forty minutes. The wash solution was then replaced with primary antibody, either mouse monoclonal ascites diluted twenty five times or polyclonal rabbit antisera, and incubated overnight. The following day the blots were washed and incubated with the appropriate goat antibody (anti-mouse or anti-rabbit
IgG) conjugated to alkaline phosphatase (Sigma, St. Louis, MO). The blots were washed and immunoreactive bands visualized using 5-bromo-4-chloro-3-indoyl phosphate (BCIP) (Sigma, St Louis, MO) as a substrate (.5mg/ml,1M TrisHCl, pH8.8).

**Regeneration Experiment**

Eyes from *Rana catesbeinna* tadpoles were devascularized under 2-phenoxy ethanol anesthesia. The eyes were completely removed from their orbits and reimplemented to ensure that all blood supply and the optic nerve was severed. The animals were then revived in oxygenated water and sacrificed at weekly intervals. Following removal the eyes were fixed in 2% paraformaldehyde solution (2 hrs), dehydrated in 10% sucrose Ringer's, and embedded in OCT compound (Miles Scientific, Naperville, IL) for cryostat sectioning. Eyes were serially sectioned at 8-10 micron intervals on an LKB cryostat.

**RESULTS**

**NCAM specific monoclonal antibodies**

Two monoclonal antibodies(2D3 and 1C6) were found which stained all neural retina with the exception of photoreceptor outer segments. Staining includes both central (CNS) and peripheral(PNS) nervous system neurons as well as germinal neuroepithelial cells found within the ora serrata or within the neural tube. Monoclonal antibody (Mab) 2D3 (Fig.1) is *Ranid* specific and effectively labels retinal neurons in culture however, Mab 2D3 leaves glial cells unstained (Fig.1C). Western blots of Mab 2D3 probed against PAGE electrophoresed *Rana* CNS.
demonstrate reactive bands at 180 and 200kD (Fig.3A). This pattern is identical to that found using anti-chicken NCAM antibodies obtained as a gift from Urs Rutishauser (Fig.3A). Further evidence for the identity of the 2D3 antigen as NCAM is that sample pretreatment with endoneuraminidase, produces a tightening of the immunoreactive bands, typical of NCAM (Fig.3C) (Rutishauser et. al., 1985). Immunohistochemical analysis of whole tadpole cryostat sections (data not shown) as well as Western blot analysis of a variety tadpole tissues (Fig.3B) demonstrate that Mab 2D3 immunoreactivity is strictly confined to nervous tissue. Mab 1C6 demonstrates similar staining and blotting patterns (Fig.3D) to Mab 2D3. However, Mab 1C6 also effectively stains *Xenopus* as well as rat nervous tissue (Fig.2).

**A Monoclonal Antibody Specific to Early Germinal Neuroepithelium and Differentiated Neurons**

A third nervous tissue specific Mab characterized displays a distinctive staining pattern within the retina (Fig.4). Mab 2C6 stains all differentiated neurons as well as a population of germinal neuroepithelial cells at the extreme retinal margin. However, this antigen is not present on the majority of germinal neuroepithelial cells in the marginal zone. This antibody is active on both fixed and fresh CNS or PNS tissue as well as fixed retinal neurons in culture. Cryostat section immunoreactivity is sensitive to periodate, neuraminidase, chloroform/methanol, and detergent treatment suggesting a lipid carbohydrate complex;
possibly a sialic acid containing ganglioside. In addition, we have found the antigen is insensitive to trypsin, and cannot be immunoblotted.

Monoclonal antibodies specific to glial cells

Two new antibodies used in this study recognize antigens present in retinal Mueller cells, as well as retinal germinal neuroepithelial cells (Fig. 5). This antigen recognized by RAG 5 is restricted to the nervous system. Immunoblot analysis of frog CNS probed using RAG 5 reveals an antigen of 66kD. In addition, staining of glial cells in culture suggests that the RAG 5 antigen is an intracellular cytoskeletonally associated protein (Fig. 5G-J). The antigen recognized by antibody RAG 2 however, is distinct from that recognized by RAG 5 since RAG 2 also stains a small population of cells within the orbit as well as the retinal Mueller cells (data not shown).

Characterization of the Central Germinal Zone

The morphology of the central germinal region, which arises from transdifferentiation of RPE cells, is similar to that exhibited at the ora serrata. This single layer of cells thickens to form a pseudostratified epithelium which is continuous with the differentiated retina. Antibody staining patterns were compared between the two germinal regions. The NCAM (data not shown), as well as the glial specific antibody display similar staining patterns within the ora serrata and the central germinal zone. The NCAM antibodies label all germinal cells as well as differentiated retina. However, while RAG 5
labels all germinal cells, in the differentiated retina RAG 5 only labels Mueller cells (Fig. 6C, D). Further, Mab 2C6 displays the same unique staining pattern in both regions. In both the ora serrata and the central germinal zone the early germinal neuroepithelial cells as well as differentiated neurons are labelled while the intermediate germinal neuroepithelial zone remains unstained (Fig. 6A, B).

DISCUSSION

The CNS originates from the germinal neuroepithelial cells of the neural tube, which, in the process of forming the CNS proper, subsequently proliferate to form various germinal neuroepithelial zones. The amphibian retina provides an ideal system to examine normal CNS neurogenesis, since virtually all stages of development are represented at the ora serrata at all tadpole stages. This marginal zone provides a spatial representation of the temporal events which normally occur in CNS development. The most peripheral region of the germinal zone consists of a single layer of cells. This layer thickens to form a pseudostratified columnar epithelium, and eventually laminates as the retinal neurons differentiate. Therefore, the peripheral germinal zone may be divided into two regions: a unilayered peripheral region and a continually thickening region which becomes continuous with the differentiated retina.

Characterization of the Peripheral Ora Serrata

In Xenopus, NCAM expression is observed even at the neural tube stage and remains confined, with the exception of
adult liver (Levi et al., 1987), to neural tissue (Jacobson and Rutishauser, 1986; Levi et al., 1987). In *Rana* a similar pattern is observed. NCAM antibodies label all germinal cells within the *ora serrata* as well as all neurons within the differentiated retina. Both NCAM antibodies in this study recognize two bands upon Western blot analysis. However, Mab 1C6 also recognizes NCAM from *Xenopus* and rat suggesting that the epitope recognized by this antibody is evolutionarily well conserved.

However, while all peripheral germinal zone cells are NCAM positive, two lines of evidence suggest heterogeneity in this zone. Reh and Radke (1988) have demonstrated in *Rana* retinal slice cultures that $^3$H-Thymidine incorporation in the peripheral region is much slower than in the intermediate zone that is adjacent to differentiated retina. This slower incorporation suggests a slower cell cycle in the most peripheral germinal cells. Further, Mab 2C6 labels only the peripheral germinal neuroepithelial cells at the *ora serrata*, and the cells of the differentiated retina, but not the most actively dividing intermediate germinal cells. Whether this differential expression is specific to a particular developmental stage or a consequence of different cellular activities, i.e. mitotic activity, remains unclear. However, both lines of evidence do suggest heterogeneity within the germinal cells.

Previous reports have suggested such heterogeneity in the neurogerminal cells of chick embryos. Fujita (1986) has
postulated a temporal heterogeneity in germinal cells starting with a Stage I germinal cell capable of producing both neuroblasts and ependymoglioblasts. These cells proceed to Stage II where they proliferate to produce neuroblast cells. In Stage III the germinal cells change their developmental pathway to produce ependymoglioblasts. Thus as the nervous system develops the germinal cells go through a temporal sequestration of their developmental fates. While changes in the developmental fate on neurogerminal cells with time are well documented (Hinds and Hinds, 1974; 1978; 1979) with Mueller cells the last to develop within the retina (Turner and Cepko, 1987), recent lineage data indicates that neurons and Mueller cells share a common precursor as late as their final division.

**Comparison of the Central Germinal Zone to the Peripheral Germinal Zone**

Morphologically the central germinal zone and peripheral germinal zone appear very similar. Both posses a single layered germinal region which is continuous with a thickening (apparently proliferating) region of mitotically active neural and glial precursors. As in the peripheral germinal zone, the central germinal zone also exhibits a dichotomy in Mab2C6 binding. Only those germinal cells within the unilayered region are labelled. Therefore, it is likely that the central germinal zone also exhibits two populations of germinal cells; an early germinal region and a more mitotically active intermediate region.

In *Xenopus* the expression of NCAM has been correlated
closely with neural induction (Jacobson and Rutishauser, 1986; Levi et al., 1987). This association is so close that expression of NCAM has been postulated as a marker for neural induction (Jacobson and Rutishauser, 1986). This is consistent with the expression of NCAM on the earliest germinal cells within the ora serrata. Interestingly, in the regenerating retina, NCAM expression is acquired early in the transdifferentiation process. Soon after contact with the vascular membrane, RPE cells begin to lose their pigment and express NCAM, suggesting that they have undergone an inductive process.

Previously we have demonstrated that following establishment of a germinal neuroepithelium the regenerating retina differentiates in the inside-out manner similar to that seen in normal development (Reh and Nagy, 1987). In both normal development, and regeneration, the ganglion cell layer is formed first, followed by the inner nuclear layer and finally the outer nuclear layer. Here we have shown that the germinal cells formed by transdifferentiating RPE cells are very similar to those normally found at the ora serrata. Both germinal regions are morphologically similar and display identical antibody staining patterns. Further, labelling by Mab2C6 suggests that both regions are composed of two heterogeneous cell populations. These similarities, taken together with the possibility of a neural inductive process during transdifferentiation, suggest that while the source of neuroblasts is unique, the differentiation process in the regenerating retina closely mimics
the normal process of retinal development.
Figure 1. Monoclonal antibody 2D3 recognizes an antigen present on *Rana* neurons but not glia. A.B. Phase and fluorescent micrographs of a cryostat section from a *Rana pipiens* retina stained with Mab 2D3 and TRITC-GAM (secondary antibody) (A,B: 300X). Antigen is restricted to the neural retina. C.D. Phase and fluorescent micrographs of Mab 2D3 staining *in vitro*; a clump of retinal neurons binds the antibody, while flat glial cells do not (arrows) (C,D: 130X).
Figure 2. Monoclonal Antibody 1C6 Recognizes Xenopus and Rana NCAM. A.B. Phase contrast and fluorescent micrographs of Xenopus retina cryostat section stained with Mab 1C6.(A: 365X; B: 342X) C.D. Phase contrast and fluorescent micrographs of Rana retina cryostat section stained with Mab 1C6.(C;D: 319X)
Figure 3. Monoclonal antibody 2D3 recognizes NCAM. A Immunoblot of Rana tadpole CNS electrophoresed on a 7.5% non-reducing PAGE gel. Samples were loaded using a 26 gauge needle adapted to a Gilson P 20 Pipetman (10mcg/lane). Lanes 1 and 2 were probed using anti-chick NCAM antiserum (a gift from Dr. Urs Rutishauser) while lanes 3 and 4 were probed using Mab 2D3. Distribution of the 2D3 antigen. Various Rana tadpole tissues were run on a 7.5% non-reducing gel and blotted with Mab 2D3. Lane 1: Brain; 2: Eye; 3: Liver; 4: Muscle; 5: Brain; 6: Heart; 7: Skin. C. Immunoblot of Mab 2D3 as in A but lane 1 sample was pretreated with neuraminidase. D. Immunoblot of Rana tadpole CNS as in A, but probed using Mab 2D3 in lane 1 and Mab 1C6 in lane 2. Both antibodies recognize the same antigen.
Figure 4. Monoclonal Antibody 2C6 recognizes an antigen present on Rana differentiated retina, but its staining shows a distinctive pattern in the marginal proliferative zone. The antibody delineates two regions of cells in this area: An early germinal zone (egz) which bind Mab 2C6 strongly, and a late germinal zone (lgz) which shows markedly reduced levels of the antigen. A.B. Phase contrast and fluorescent micrographs of peripheral Rana retina showing binding pattern of Mab 2C6 in germinal zone (A: 347X; B: 394X) C.D. Phase contrast and fluorescent micrographs of central Rana retina. (C: 319X; D: 347X)
Figure 5. Monoclonal antibodies RAG 2 and RAG 5 recognize antigens common to Rana and Xenopus retinal Mueller cells and germinal neuroepithelial cells. A-D. Phase contrast and fluorescent micrographs showing binding of RAG 5 on marginal germinal cells (A.B.) and central retinal Mueller cells (C.D.). E.F. Phase and fluorescent micrographs of Central Rana retina showing binding of RAG 2 to Mueller Cells. G-I. Fluorescent Micrographs of glial cells in vitro obtained from Rana retinal cultures and stained with RAG 5. J. Immunoblot of Rana CNS probed using RAG 5. (A: 230X; B: 210X; C: 280X; D: 260X; E: 169; F: 210; G: 128; H: 128; 115)
Figure 6. The central germinal neuroepithelium of the regenerating retina resembles the peripheral germinal region present in the normal retina. A.B. Phase contrast and fluorescent micrographs of the central region of 8 week regenerate Rana cryostat sections stained using Mab 2C6. Staining pattern resembles that seen in peripheral retina (shown previously) with strong staining in differentiated retina (dr) and early germinal zone, but poor staining in the late germinal zone. (A: 174X; B: 194X) C.D. Phase contrast and fluorescent micrographs of regenerating retina as above, but stained using RAG 5. As shown previously in the peripheral germinal neuroepithelium, RAG 5 stains strongly within the central germinal neuroepithelium(cgn). (C: 161X; D: 226X). E.F. Phase contrast and fluorescent micrographs as above but stained using Mab Rag 2. RAG 2 shows a similar staining pattern to RAG 5 with strong staining in both the peripheral and central germinal neuroepithelium. (E: 206X; F: 277X)
CHAPTER FOUR

Retinal Pigmented Epithelial Cells Induced To
Transdifferentiate To Neurons By Laminin.
INTRODUCTION

Although the regeneration of nervous tissue in the vertebrate is very limited, there are a few remarkable examples of the process (Jacobson, 1978). Understanding the factors that regulate CNS regeneration in those areas where it occurs, will doubtless, provide generally applicable, essential information about the process. Regeneration of the amphibian retina following its destruction is well documented (Stone, 1950a, 1950b; Hasagawa, 1958; Keefe, 1976). Transplant studies (Stone, 1950a; Stone, 1950b; Loposhov and Sologub, 1972), confirmed later by in vitro experiments (Okada, 1980), have shown that one source of new neurons in the regenerating retina is the retinal pigmented epithelium. RPE cells can transdifferentiate to either neurons or lens cells in culture (Okada, 1980; Yamada, 1982), but little is known about the factors that regulate this process. We have provided evidence that the association of RPE cells with the retinal vascular membrane is an important step in transdifferentiation in vivo (Reh and Nagy, 1987). The vascular membrane and Bruch’s membrane represent the only sources of basement membrane within the amphibian retina. During regeneration the RPE cells detach from the extracellular environment present at Bruch’s membrane and move to the extracellular environment present at the vascular membrane before the onset of transdifferentiation. Here, we explore the possibility that it is the extracellular matrix present in the environment of the vascular membrane which is responsible for
inducing RPE to neuron transdifferentiation.

MATERIALS AND METHODS

Midlarval stage (TK/X-XV) tadpole *Rana catesbeinna* were purchased from either Nasco Scientific (Fort Atkinson, WI) or Kons Scientific (Janesville, WI), kept in 5-gal at 23°C, and fed tetramin.

Primary cultures were made by removing the eyes from 5 *Rana catesbeinna* tadpoles and microdissecting away the sclera, lens and retina in sterile Ringers’ solution. Only the posterior two-thirds of the RPE was used; the anterior RPE and Iris were excluded. The RPE was treated with 200 units/ml collagenase (Sigma, St. Louis, MO) for 15 min. followed by 2.5 min. in a 0.1% trypsin solution (Sigma, St. Louis, MO). The cells were then trituated, suspended in 10ml of a Frog Culture medium (0.5X HEPES buffered L-15, 10%FBS, 2mM l-glutamine, 200U/ml Penicillin, 200mcg/ml Streptomycin) and plated on 60mm tissue culture dishes (Falcon, Oxnard, CA). To ensure that no neurons or germinal neuroepithelial cells were present, the primary cultures were examined by phase contrast microscopy or by indirect immunofluorescent staining with neuron specific monoclonal antibody 2D3. Counts of all cells in low-density cultures showed that 99.4% of the cells in primary cultures were RPE cells; the remaining 0.6% of the cells were either choroid pigmented cells or fibroblasts: primary cultures that contained any 2D3 positive cells were not used in the experiments. Following three to five days, primary RPE cultures were plated onto 24-well tissue
culture plates (Falcon, Oxnard, CA) which had been precoated with one of the following: 10-100mcg laminin; 10-100mcg fibronectin; 10-100mcg polylysine; 0.1 mg/ml collagen type 1 and tissue culture plastic. To subculture primary RPE cells were rinsed twice with EDTA Ringers solution and incubated in EDTA Ringers with 100U/ml collagenase (Sigma, St. Louis, MO) for 10 min. Three to twelve weeks following subculture wells were immunohistofluorescently screened using either tetanus toxin, Mab 2D3, or a monoclonal antibody directed against the 200kD neurofilament subunit. For tetanus toxin immunoflorescence, wells were incubated with tetanus toxin (20mcg/ml) followed by a rabbit anti-toxin serum (both gifts of Wellcome Research Labs, Beckenham, England) and Tetramethylrhodamine isothyanate (TRITC) conjugated goat anti-rabbit antiserum(Sigma, St. Louis, MO). For monoclonal antibody immunohistoflorescence, wells were incubated with the 2D3 monoclonal antibody( hybridoma supernatant or DEAE-cellulose purified from ascites) or the neurofilament monoclonal antibody(Boeringer, W. Germany) and subsequently incubated with a goat anti-mouse TRITC conjugate. Wells were screened for neuronal cells both by phase microscopy and 2D3 or tetanus toxin immunoflourescence on a Zeiss III RS Fluorescent microscope. In order to detect the presence of RPE to lens transdifferentiation, an anti-crystallin antiserum raised in rabbit against newt gamma-crystallin (gift from D.S. McDevitt, Univ. Pennsylvania) was used to stain cultures, followed by a goat anti-rabbit FITC conjugate.

Tritiated thymidine incorporation was assessed at two weeks
following subculture onto the substrates. Wells were incubated 48 hours in 1mcl/ml isotope (20Ci/mmol) and precipitated using trichloroacetic acid (TCA). Cells were washed in normal frog Ringer’s three times, incubated for 10 min in 100mcl/well distilled water, and frozen and thawed to lyse the cells. Lysate (50mcl) was applied to Whatman No.1 filter squares. The paper was washed three times in 200ml of 10% TCA (4°C) and then washed in ethanol and acetone. Scintillation fluid was added and the mixture counted on a LKB Beta-counter.

Western blot analysis was used to assess the composition of various tadpole basement membranes. Basement membranes were dissected with the aid of a freeze-thaw regime and homogenized in a 0.5M NaCl solution with phenyl-methyisulphonic acid (PMSF) and EDTA. The homogenate was centrifuged for 10 min. at 10,000g and the pellet was resuspended and extracted by sonication for 12 hours at 23°C in 8M urea, with 2%(v/v) mercaptoethanol, and PMSF. The samples were then dialyzed for 24 hours at 4°C, protein concentration was assayed (Biorad, Richmond, CA) and equal amounts (10mcg/lane) of each sample electrophoresed on a 5% SDS-polyacrylamide gel. Gels were then electroblotted, and incubated in anti-laminin (BRL, Ontario, Canada) or anti-fibronectin (Calbiochem-Behring, CA) antisera. Following incubation with an alkaline phosphatase conjugated goat anti-rabbit antibody (Sigma, St.Louis, MO) the immunoreactive band was visualized by reacting with 5-bromo-4-chloro-3-indoyl phosphate (Sigma, St. Louis, MO).
RESULTS

When subcultured on plastic, the RPE cells gradually became less pigmented; however, after six weeks in vitro, most cells still possessed some pigment, and frequently reestablished the cuboidal array present in the primary culture. Many wells also developed structures similar to the "lentoid bodies" previously reported in cultures of newt and chick RPE (Yamada, 1982; Yasuda, 1979; Itoh and Eguchi, 1986) and characterised by clusters of elongated depigmented cells. The control cultures developed other morphologically distinct cell types, but no cells resembling neurons were observed. On two occasions (out of 19), we observed colonies of lightly pigmented cuboidal cells that stained with the 2D3 antibody in long-term (3 month) high-density control cultures, which may have represented early neuronal precursors like those found in vivo at the peripheral retinal margin. No other evidence for a neuronal phenotype was observed. Although neuronal transdifferentiation has been reported in dissociated newt RPE cells cultured on plastic substrates, it developed in only a very few cases (-0.3%) (Okada, 1980); as a result, it is not surprising that we failed to observe any neuronal-like, tetanus toxin/2D3 positive, cells in our control cultures.

When cultured on polylysine, collagen type 1 or fibronectin, the cells followed much the same course as cells plated on plastic. Occasionally, 2D3-positive cells were observed, but no other morphological features of neuronal cells were seen. But
when the cells were plated on laminin, the cells underwent remarkable morphological changes. Within one to three days after subculture on laminin the RPE cells began streaming towards one another to aggregate into clusters. Initially, these aggregates took the form of networks (Fig. 1b) which frequently went on to form spherical clusters. At this time there was extensive depigmentation, and the cells formed compact spheres of small cells by 3-4 weeks. Soon after this, the cells extended fine processes resembling neurites, with growth cones at their tips (Fig. 1c). Indirect immunofluorescent staining of these cultures using either tetanus toxin or the 2D3 monoclonal antibody, showed that these clusters were positive for both markers (Fig. 1d), indicating that the RPE cells were now expressing a neuronal phenotype. Also, about one-third of the cells in these aggregates, as well as all of the neuritic processes, contained neurofilaments (fig. 1e and f). The fact that only a fraction of cells stain for neurofilament is interesting in light of reports that only retinal ganglion cells, and not other retinal cell types, normally possess neurofilaments in vivo (Cohen et. al., 1986).

Another substrate that was particularly effective in promoting neural transdifferentiation was a commercial preparation of EHS basement membrane (EHS-BM)(Timpl et. al., 1979). Cells plated on this shared the same morphological changes that were observed for laminin substrates (table 1): because EHS-BM contains considerable quantities of laminin (Timpl
et al., 1979), it is likely that the laminin was also the active component in this substrate.

Cultures screened using anti-crystallin antisera demonstrated that transdifferentiation to lens was considerable on a plastic or collagen type 1 substrate. However, this transdifferentiation was greatly reduced when RPE cells were plated onto laminin or EHS-BM (Table 1). Further, fewer lentoid bodies were observed on the laminin or EHS-BM substrates. Tritiated thymidine incorporation, as assessed using TCA precipitation, suggests that the levels of RPE cell proliferation are similar on all substrates (Table 1).

Immunoblot analysis of various tadpole basement membranes indicate that the vascular membrane contains far more laminin than the other basement membranes examined (Fig. 2). However, immunoblots run in parallel, but assayed instead for fibronectin, show a very different pattern of staining, with the highest levels found in Bruch's membrane and lens, and much less in the vascular membrane. Densitometry of the blots show a ratio of laminin/fibronectin of 1.8:1.0 for the vascular membrane and 0.3:1.0 for Bruch's membrane.

DISCUSSION

From these results it seems that laminin containing substrates induce dramatic morphological and biochemical changes in RPE cells in vitro, promoting their transdifferentiation to neurons. However, the laminin does not merely promote non-specific transdifferentiation of RPE cells, because
transdifferentiation to lens cells seems to be suppressed on laminin or EHS-BM substrates. Rather than having a direct effect, laminin may act by promoting proliferation of RPE cells. It has been proposed that RPE cells need to undergo several rounds of cell division before neural transdifferentiation in vivo (Okada, 1980; Eguchi et al., 1974; Eguchi and Itoh, 1981). However, although $^3$H-thymidine incorporation and cell counts demonstrated proliferation on laminin- and EHS-BM-containing wells, there was also comparable cell proliferation in those wells with other substrates. It is possible that we failed to identify neurons on non-laminin containing substrates, because they do not provide as favorable surface for neurite outgrowth; however, polylysine and collage support neurite outgrowth from tadpole retinal cells within one week after dissociation (unpublished observations). Also the identification by tetanus toxin, 2D3 and neurofilament antibodies is not dependant on the presence of neurites (though the antibody staining is nearly always coincident with process-bearing aggregates of small phase-bright cells). Finally, it is also possible that laminin exerts its effect by inducing tight clustering of the RPE cells. This is unlikely because clusters and aggregates frequently form on other substrates such as fibronectin and collagen, which do not promote transdifferentiation to a neuronal phenotype.

We have recently shown by an immunofluorescent analysis of regenerating Rana retina in vivo, that there is a high degree of association of migratory RPE cells with the retinal vascular
membrane at the time the first new retinal neuroblasts appear (Reh and Nagy, 1987). Therefore, we proposed that contact with the retinal vascular membrane may be an important step in the process of retinal regeneration in amphibians. These results support and extend this hypothesis, by indicating that the relevant factor in the vascular membrane may be laminin. Laminin, a large glycoprotein present in basement membranes (Eguchi et al. 1974), influences many cellular processes including cell adhesion, growth, morphology and differentiation in a variety of epithelial cell types (Kleinman et al., 1985). Analysis of the retinal vascular membrane using immunoblot and immunohistochemical (not shown) techniques demonstrate the presence of several components normally found in basement membranes including laminin, fibronectin, and heparin sulphate proteoglycan. However, consistent with our hypothesis, the relative amount of laminin present in the vascular membrane greatly exceeds the amount found in Bruch’s membrane which RPE cells normally contact.

These results indicate that the process of retinal regeneration in amphibians is regulated, in part, by the extracellular matrix. Differences in the composition of Bruch’s membrane and the vascular basement membrane are likely to be important in the control of this process; the small amounts of laminin in Bruch’s membrane are consistent with the maintenance of the RPE phenotype, whereas, when these cells come in contact with the higher laminin levels in the vascular membrane, the
neuronal phenotype is favoured. Such basement membrane heterogeneity is involved in regulating the morphogenesis of several other developing tissues (Ekblom et. al., 1981; Ekblom et. al., 1980; Ekblom, 1983; Banerjee et. al., 1977), including mouse salivary gland branching and kidney nephron formation. Moreover, basement membranes obtained from various cell lines can profoundly influence the morphology and biochemistry, of several cell types in vitro, including kidney tubule epithelial cells (Gospodarowicz et. al., 1984;), corneal epithelial cells (Gospodarowicz and Ill, 1980) and Sertoli cells (Hadley et. al., 1985).

Our results also raise the possibility that extracellular matrix components, such as laminin, are involved in the inductive interactions that give rise to the retina and RPE during development. The results of experimental embryological manipulations indicate that the inductive signals are widespread in the embryo; contact with any mesenchymal cells is necessary for appropriate RPE development (Holtfreter, 1939), whereas contact with many other epithelial tissues such as the ear vesicle, nasal placode or peritoneum can induce the developing RPE to become retina (Dragomirow, 1933a; Dragomirow, 1933b; Ike, 1937). That changes in the composition of the extracellular matrix act as inductive signals in development is an emerging concept in the morphogenesis of many tissues in the embryo, and the results of the present study suggest this may be true for neuronal tissue as well.
Figure 1. Photomicrographs of the stages of transdifferentiation of *Rana catesbeinna* RPE cells when plated on laminin. 

a, Primary culture of RPE cells, just before they were subcultured. 
b, Network-like aggregate of RPE cells, two days after subculture on laminin (100mcg/ml). 
c, Neuronal cluster, six weeks after subculture on laminin (100mcg/ml). 
Note the fine, neuritic processes emanating from the cluster of small phase-bright cells.
d, Similar neuronal cluster, now stained with the neuron-specific monoclonal antibody, 2D3. 
Scale bar, in a, b, and c 100 microns. 
e, Hoffman interference optics image and 
f, corresponding fluorescent micrograph, showing neurofilament staining of neuronal cluster eight weeks after subculture on EHS basement membrane. 
Scale bar, e and f, 100 microns.
Figure 2. Immunoblot of tadpole basement membranes probed with anti-laminin antisera. The blot shows the relative amounts of laminin immunoreactivity in sclera (lane 1), RPE (lanes 2 and 6), vascular membrane (lane 3), brain (lanes 4 and 7), lens (lane 5), and heart (lane 8). Equal amounts of protein were loaded in each lane using a 26 gauge needle adapted to a Gilson P 20 pipetman. Arrowheads, positions of migration of the A and B chains of laminin from EHS-sarcoma; the A chain typically does not blot as well as the B chain. Similar blots with laminin antisera using two other extraction procedures gave comparable results.
<table>
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<th>Substrate</th>
<th>Neuronal clusters (% positive wells)</th>
<th>No. of y-crystallin positive cells per well</th>
<th>(^3)H-thymidine incorporation d.p.m. per well</th>
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<td>Plastic</td>
<td>10.5% (19)</td>
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<td>Polylysine</td>
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<td>172±80 (6)</td>
<td>501.1±227 (3)</td>
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<tr>
<td>Fibronectin</td>
<td>0% (15)</td>
<td>-</td>
<td>413±108.8 (3)</td>
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<tr>
<td>Laminin</td>
<td>86% (26)</td>
<td>1 (6)</td>
<td>268±60.5 (6)</td>
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<td>EHS membrane</td>
<td>94% (27)</td>
<td>7±5 (6)</td>
<td>217.5±79.9 (6)</td>
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CHAPTER FIVE

INO Antibody Inhibits Retinal Regeneration In Larval Frogs.
INTRODUCTION

Studies on retinal regeneration in a number of amphibian species have demonstrated that new neurons within the regenerating retina arise, in part from transdifferentiation of retinal pigment epithelial (RPE) cells (Hasagawa, 1958; Keefe, 1977; Stone, 1951a, b; Reyer, 1977). In addition, intraspecific transplantation of pieces of RPE into the posterior chamber of eyes from both urodeles and larval anurans, has further substantiated the RPE as the source of new retinal neuroepithelial cells (Stone, 1950a; Loposhov and Sologub, 1972; Sologub, 1977). Recent studies of Rana retinal regeneration have proposed a role for the extracellular matrix (ECM) in this process (Reh and Nagy, 1987; Reh et al., 1987). During regeneration the RPE cells which are normally adjacent to Bruchs membrane, detach from Bruchs membrane, phagocytose the degenerating retina and associate strongly with the vascular membrane, prior to transdifferentiation (Reh and Nagy, 1987). The composition of these two basement membranes was found to differ most strikingly in the amount of laminin. Densitometry on immunoblots of ECM extracted vascular and Bruchs membranes probed with anti-laminin indicate a 6-fold excess of laminin in the vascular membrane relative to Bruchs membrane (Reh et al., 1987). Moreover, larval Rana RPE cells were shown to undergo neuronal transdifferentiation in vitro, when plated on laminin containing substrates (Reh et al., 1987). To determine whether the high concentration of laminin in the vascular membrane is necessary
for retinal regeneration in vivo, we attempted to block this process with an antibody that specifically inhibits the interaction of cells with laminin-heparin sulfate proteoglycan complex (Matthew and Patterson, 1983; Chiu et al., 1986).

MATERIALS AND METHODS

Midlarval stage (TK/X-XV) tadpole Rana catesbeinna and adult Rana pipiens were purchased from either Nasco Scientific (Fort Atkinson, WI) or Kons Scientific (Janesville, WI). Tadpoles were kept in 5-gal. tanks at 23°C and fed on tetramin. Adult Rana pipiens were stored in a state of semi-hibernation at 4°C until used for breeding. Rana pipiens tadpoles were reared in 5-gal. tanks at 23°C until reaching midlarval stages (TK/X-XV) when they were used for experiments.

Retinal degeneration in both species was induced by total surgical devascularization of the eye following anesthesia on wet ice. Animals were allowed to recover for one day, reanaesthetized, and eyes injected with solution using a 1cc Tuberculin syringe (Becton Dickinson, Rutherford, N.J.) till eyes distended appreciably (approximately 20-42μl). Injected solutions included either INO antibody, Thy1.1 antibody, A2B5 antibody, B50 antibody, Fetal Bovine Serum (FBS) (Gibco, Grand Island, N.Y.) DMEM tissue culture media (Gibco, Grand Island, N.Y.) or frog Ringers’ solution. Antibodies were injected either in the form of hybridoma culture supernatant or as mouse ascites with or without FBS (1:1). All forms of antibody produced similar effects within a particular antibody species. The animals were
allowed to survive for two to four weeks to allow adequate regeneration, sacrificed, and their eyes fixed in 2% paraformaldehyde for two hours. The eyes were subsequently processed for paraffin or cryostat embedding and sectioning. Paraffin sections were stained with hemotoxylin and scored for the presence of regeneration, without knowing the experimental treatment, using a Zeiss III Photomicroscope. Cryostat sections were stained with the appropriate Rhodamine conjugated second antibody to determine the time course of clearance of the injected antibody. Cryostat sections so stained demonstrated persistence of the antibody for at least two weeks.

In assessing regeneration, association of the RPE with the vascular membrane was quantitated in two week regenerating Rana catesbeinna by measuring the percentage of total vascular membrane in contact with RPE cells and/or newly formed germinal neuroepithelium. Microscope images were digitized and the distances measured using an IBAS computer program.

RESULTS

Retinal devascularization results in the complete destruction of the neural retina in R. catesbeinna tadpoles; however, larval R. pipiens retinas are more resistant to this treatment, and in some cases the photoreceptor layer failed to degenerate. When this occurs, no regeneration ensues, and so these cases were not used. In those cases where retinal degeneration was complete, in both species, we used the following criteria to stage the process of retinal regeneration. In the
initial phase of regeneration the retinal pigment epithelial cells associate with the vascular membrane. Next, these cells begin to lose their pigment, and elongate to form a germinal neuroepithelium (Phase 2). This layer of germinal cells then begins to differentiate over the next few weeks (Phase 3) to form a laminated retina. Eyes injected with control solutions (Thy1.1 antibody, A2B5 antibody, B50 antibody, or vehicle alone) for the most part exhibited normal regeneration. All (22/22) R. pipiens and 23/26 R. catesbeinna eyes, injected with the control solutions exhibited either strong RPE association with the vascular membrane (fig.1a) and/or RPE contribution to a new germinal neuroepithelium (fig.1b). When regenerating eyes that had been injected with the INO antibody were examined, both Rana species displayed a considerable reduction in the percentage of regenerating retinas. Two types of aberrant regeneration were observed (fig.1c,d). In one group, the RPE cells were stalled at the initial stages of phagocytosis of the degenerated retina (fig.1d), often forming a mass of cells in the central retina. In other cases (3/11) the RPE failed to associate with the vascular membrane (fig.1c), covering only 65.3% of the surface of this membrane, as compared to 98.4% in the control eyes at two weeks after devascularization. When regeneration is compared between INO antibody injected eyes and control injected eyes regeneration drops to 50% and 64% of control for R. pipiens and R. catesbeinna respectively (fig.2).
DISCUSSION

The INO antibody has previously been used to block axon regeneration within the rat eye (Sandrock and Matthew, 1987). Axonal regeneration to symphectomized irides was significantly reduced by implantation of INO antibody secreting hybridoma cells relative to control hybridoma cell lines. Here we describe a second aspect of neural regeneration in which laminin is involved, where the INO antibody has a similar inhibitory effect. Two lines of evidence from previous work suggest that laminin induces the transdifferentiation to RPE to neurons in regenerating Rana retinas: 1. During regeneration the RPE cells associate with a laminin enriched vascular membrane prior to transdifferentiation and, 2. Laminin can induce transdifferentiation of RPE cells to neurons in vitro. Here we demonstrate that molecules which block laminin-cell interactions will block retinal regeneration in vivo. This block appears to occur at or prior to association of RPE cells with the vascular membrane, which is consistent with the stage at which the ECM would be expected to play a role. Thus it appears that in vivo, laminin plays a necessary role in regulating regeneration of neurons within the central retina. While laminin alone will induce RPE transdifferentiation in vitro, the response is more robust when plated on EHS sarcoma basement membrane extract which contains laminin in complex with heparin sulfate proteoglycan, collagen type 4 and other minor components (Kleinman et. al., 1982) It is possible that heparin sulfate proteoglycan
can enhance the effect by complexing with laminin to leave it in a conformation more conducive to initiating transdifferentiation.

There is a significant body of evidence supporting a requirement for laminin and HSPG in a complex to elicit biological activity. The INO antibody has been used to perturb avian neural crest migration in vivo (Bronner-Fraser and Lallier, 1988). In addition a number of neurite-promoting factors isolated from conditioned media are composed of a laminin-HSPG complex (Calof and Reichardt, 1984; Davies et. al., 1985; Lander, et. al., 1983). While antilaminin antibodies will block neurite outgrowth on a laminin substrate, they fail to block neurite outgrowth on these conditioned media derived neurite-promoting factors (Edgar et. al., 1984; Lander et. al., 1983b; Manthorpe et. al., 1984). Further, those antilaminin antibodies which do block neurite extension on laminin are directed against the major HSPG binding fragment of laminin (Edgar et. al., 1984). Finally, laminin alone fails to influence adrenergic neuron development from cultured neural crest while intact EHS sarcoma basement membrane extract does (Maxwell and Forbes, 1987).

In the normal undamaged eye the RPE are exposed to the extracellular environment present at Bruch's membrane. In this environment the RPE cells are apparently stably differentiated into the pigmented phenotype. However, following devascularization, the RPE cells associate with the vascular membrane. We have found that vascular membrane is highly immunoreactive to laminin, as well as the INO antibody, while
Bruch's membrane is not. This basement membrane heterogeneity is similar to that observed by Chiu et al (1986); the INO immunoreactivity tended to correspond only to those basement membranes known to promote axonal regeneration in vivo and not to those regions which do not. We propose that in the laminin/HSPG rich environment of the vascular membrane the RPE cells are induced to switch from their typical RPE phenotype to that of retinal neurons. These results exemplify the important role of the ECM in influencing cellular phenotype both in vitro and in vivo.
Figure 1. Two week regenerating retinas displaying normal and blocked regeneration. A, Regenerating retina exhibiting strong association of RPE cells (rpe) with the vascular membrane (vm) (144X). B, A more advanced regenerating retina displaying a germinal retina (gr), originating from transdifferentiated RPE cells, continuous with differentiated retina (dr) (100X). C, INO injected eye exhibiting decreased RPE cell association with the vascular membrane (144X). D, INO injected eye with RPE cells stalled during phagocytosis of the degenerated retina (120X).
Figure 2. Comparison of retinal regeneration in both *Rana catesbeinna* and *Rana pipiens* between all eyes injected with control solutions and eyes injected with the INO monoclonal antibody. Statistical significance was assessed using a single variable independent chi squared test comparing cases of regenerating versus nonregenerating retinas.
Rana Retinal Regeneration

% REGENERATING RETINAS

- catesbienna
- pipiens

All Controls

INO

n=22
n=26
n=26
n=21
n=12

*p<.02
*p<.001
CONCLUSIONS

The data presented in this thesis demonstrate that retinal regeneration in larval Anurans occurs in a manner similar to that previously described in Urodeles, with new neurons within the Anuran retina arising from both increased proliferation at the ora serrata and transdifferentiation of RPE cells. Further, the data suggest that the mechanisms involved in regulating this process may be similar to those observed during development of the retina. The results also indicate that one of these factors may be the extracellular matrix. During retinal regeneration RPE cells detach from Bruch's membrane, migrate across the retina and form a strong association with the vascular membrane prior to transdifferentiation. We have shown that the vascular membrane is extremely rich in laminin immunoreactivity. Further, we have demonstrated that laminin-rich substrates in vitro will induce RPE to neuron transdifferentiation. Finally, we have shown that injection of the INO monoclonal antibody into devascularized eyes will inhibit retinal regeneration in vivo. These results strongly suggest that laminin, present in the vascular membrane, is responsible for inducing the transdifferentiation of RPE cells into neurons during retinal regeneration. Such a role for the extracellular matrix is also possible during normal development of the eye. The extracellular matrix has been shown to influence the differentiation of developing cell types including glomerular epithelia (Ekblom et al., 1980; 1981; Ekblom, 1883), muscle (Menko and Boettiger, 1987) and neural crest (Maxwell and Forbes,
Further, embryonic transplantation studies suggest that the decision to differentiate into RPE rather than neural retina may be a function of the extracellular matrix with which the cells contact. Contact with mesenchymal tissue promotes differentiation of the cells to RPE while contact with many epithelial tissues induces differentiation to neural retina (Dragomirow, 1933a; 1933b; Ikeda, 1937).

Growth factors have been also been shown to play roles in fundamental developmental processes. Factors similar to Fibroblast Growth Factor and Transforming Growth Factor-Beta have been implicated in *Xenopus* mesoderm induction (Kimmelman and Kirschner, 1987; Slack et. al., 1987; Smith, 1987; Weeks and Melton, 1987; Rosa, et. al., 1988). Further, lens regeneration from newt iris is stimulated by retina-derived growth factors in vitro (Cuny et. al., 1986). Since all culture experiments described within this thesis were done in the presence of serum, a role for serum associated growth factors in transdifferentiation cannot be excluded. It is possible that the extracellular matrix could act in conjunction with a growth factor to induce transdifferentiation. Growth factors have been reported to modulate secretion of extracellular matrix components in a number of cell types (Gordon et. al., 1985; Ignitz and Massague, 1986; Mescher and Munaim, 1986; Varga and Jimenez, 1986; Thorne et. al., 1987; Savage et. al., 1987). Further, the extracellular matrix can influence the response of a cell to growth factors (Tomomura et. al., 1987; Madri et. al., 1988).
Therefore, it is possible that normal regenerative or developmental processes may be a consequence of the presence of both the proper growth factors as well as the proper extracellular matrix. Future studies directed towards examining this possibility should provide powerful insights into the role of both the extracellular matrix and growth factors in influencing cellular phenotype during normal developmental or regenerative processes.
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