THE ROLE OF β_1 INTEGRINS IN THE EARLY DIFFERENTIATION OF THE RPE

AND NEURAL RETINA IN THE CHICKEN EMBRYO

by

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ABSTRACT

The early differentiation of the retina and retinal pigment epithelium (RPE) of the chicken embryo involves a complex series of morphological changes and cell migrations. The interactions of extracellular matrix (ECM) molecules, cell adhesion molecules such as β_1 integrins, and soluble growth factors are believed to mediate this process.

The temporal and spatial distribution of ECM molecules laminin and fibronectin and the integrin subunits β_1, α_3, α_4, α_5, α_6, and α_v was determined by immunohistochemistry in the chicken embryo between stage 8 (E2) and stage 24 (E4). Laminin and fibronectin were present in basement membranes throughout the stages examined, and intracellular fibronectin was observed in stages 8 through 24. Fibronectin staining in ganglion cells at stages 22-23 is reported for the first time.

A pericellular staining pattern was observed for β_1 integrin subunit at all stages examined. Alpha 3, α_4, α_5, and α_6 integrins showed a polarized distribution in the RPE post-optic cup formation. Alpha v integrin displayed a diffuse, vesicular staining pattern throughout the stages examined except for a distinctive zipper-like staining pattern at the RPE-NR interface. This pattern is possibly an indication of cell-cell interactions between the neuroblasts of the outer NR and the RPE. Alpha 3 integrin appeared to be heavily expressed in the differentiating RPE, and in the early migrating neuroblasts. The distribution of α_4 integrin is mainly in ectodermal structures before optic cup formation, but became more widespread within the NR and especially in the RPE basement membrane. The α_5 integrin distribution indicated a role in early differentiation of lens fibres and ganglion cells. Alpha 6 was only expressed in the post-optic

cup retina. This distribution suggests a role in later developmental events such as neurite extension and maintenance of differentiated phenotype.

The distribution of β_1 and α integrin subunits with respect to morphological changes observed during retina development point to a significant role of integrin-ECM interactions.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
E	embryonic day
ECM	extracellular matrix
FGF	fibroblast growth factor
ILM	inner limiting membrane
NCAM	neural cell adhesion molecule
NE	neuroepithlium
NR	neural retina
PBS	phosphate buffered saline
RGD	peptide sequence, Arg-Gly-Asp
RGES	peptide sequence, Arg-Gly-Glu-Ser
RPE	retinal pigment epithelium
IKVAV	peptide sequence, Ser-Ile- -Val-Ala-Val
SPARC	secreted protein, acidic, rich in cystine
w/w	weight per weight
w/v	weight per volume
YIGSR	peptide sequence, Tyr-Ile-Gly-Ser-Arg

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INTRODUCTION

The retina is derived from an evagination of the diencephalon at an early embryonic stage. Through a complex and rapid series of morphological changes the ectodermally-derived neuroepithelium differentiates to form the neural retina (NR) and retinal pigment epithelium (RPE). In the chicken (Gallus *domesticus*) embryo these changes occur early in development between 26 hours (stage 8; Hamburger and Hamilton, 1951) and 50 hours (stage 15) of incubation. The neuroepithelium (NE) of the neural tube evaginates laterally to form the optic vesicles. When the neuroepithelium contacts the head ectoderm it then invaginates to form the optic cup, at which point the retina has two distinct layers which are joined at the margins of the optic cup. From this stage the outer layer will develop into a simple cuboidal epithelium (RPE) which will form part of the pigmented, light-scattering blood-retina barrier. The RPE basal membrane is markedly infolded and contains fibrils anchoring into the basement membrane. There are junctional complexes on the apical-lateral membranes which form a permeability barrier characterized by fusion of adjacent cell membranes. As mentioned above, the RPE forms part of the blood-retina barrier, and it regulates the flow of nutrients to the NR and scavenges waste from the NR. The apical processes of the RPE interdigitate with photoreceptor outer segments, providing protection, and phagocytose cellular debris from the photoreceptor outer segments. The RPE cells also synthesize and transport glycosaminoglycans and vitamin A metabolites between the NR and the choroid. The inner neuronal layer further differentiates into a multi-layered complex of rods, cones, and other neurons forming the sensory component of the retina. These morphological changes involve cell shape change, migration, proliferation,

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and differentiation, activities which are mediated by molecules of the extracellular matrix (ECM) and cell surface receptors.

Previous studies on normal retinal development and regeneration have shown that fibronectin, laminin, and basic fibroblast growth factor (FGF-2) play key roles in these processes (reviewed by Reh and Nagy, 1987; Park and Hollenberg, 1991; Pittack et al., 1997). There is also evidence that integrins may mediate interactions between the ECM and cytoskeleton in the developing eye (Reichardt and Tomaselli, 1991). Most of the current knowledge of the temporal and spatial distribution of these molecules is based on studies of later stage embryos (day 7-15) when the optic cup formation is complete and differentiation of the RPE and NR has occurred (Rizzolo, 1990; Rizzolo and Heiges,1991; Rizzolo et al., 1994; Cann et al., 1996; Martinez-Morales et al., 1996; Yip et al., 1998). This point in retinal development coincides with the loss of the capacity of the retina to spontaneously regenerate following damage to the NR. By investigating the earlier stages of retinal development it may be possible to correlate changes in expression of ECM components, cell surface receptors, and cell markers to morphological changes seen during differentiation of the NR and RPE.

Eye Development in the Chicken Embryo

Events of Eye Development

The development of the eye begins with an evagination of the NE of the rostral neural tube at stage 8 to form the primary optic vesicle at stages 9 to 11 (Fig 1a). At stage 11 the optic vesicles begin to constrict medially to form a sealed optic vesicle at stage 12. The NE at this

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point completely surrounds the optic vesicle except for a small optic stalk through which the optic nerve will extend to the brain. At stage 13, when the optic vesicle has extended laterally to closely appose the surface ectoderm (Fig 1b), the optic vesicle begins to invaginate to form the optic cup by stage 14 (Fig. 1c) The surface ectoderm at stage 13 thickens in the area immediately adjacent to the lateral outermost extremity of the optic vesicle to form the lens placode. The remainder of the surface ectoderm adjacent to the lens placode will develop into the cornea (Fig. 1c). During the optic cup formation the outer, medial NE becomes the presumptive RPE and the invaginating lateral NE becomes the NR (Fig. 1d).

Differentiation of the Neural Retina and Retinal Pigment Epithelium

The presumptive NR and presumptive RPE at stages 14 to 16 consist of multiple layers of undifferentiated NE cells. The presumptive RPE is still mitotically active at this stage and the presumptive NR cells are rapidly dividing. Beginning at stage 16, the presumptive RPE produces pre-melanosomes with tyrosinase activity as determined by cytochemistry and transmission electron microscopy (Ide, 1972). Although not a definitive marker for RPE phenotype, pre-melanosome production is an indication that presumptive RPE has begun to differentiate to RPE. Tyrosinase can be detected at stage 16 (Ide, 1972) and increases between stages 17 to 22 (Zimmerman, 1975). From stage 24 on embryonic day 4 (E4) to E5 there is a rapid increase in tyrosinase activity as the DNA synthesis in the RPE decreases to 5-6% of stage 16 levels (Zimmerman, 1975; Stroeva and Mitashov, 1983). The level of DNA synthesis and mitosis declines rapidly from stage 16 through 22 and during this time the RPE becomes a single layer of tall cuboidal cells. From stages 22 to 24 RPE cells become moderately pigmented and

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Figure 1.   Illustation of early eye and retina development in chicken.

(A) Stage 11 embryo head in cross section showing the evaginating optic vesicle moving laterally toward the head ectoderm. (B) Early stage 13 embryo showing the medial constriction of the optic vesicle prior to optic cup formation. The beginnings of the lens placode can be seen at the surface ectoderm. (C) Stage 14 the lateral optic vesicle neuroepithelium (presumptive NR) begins to invaginate toward the medial neuroepithelium (presumptive RPE). The cornea and lens begin to develop from the surface ectoderm. (D) At stage 16 the invaginating NR closely apposes the RPE and he optic cup has formed.

A — Ventricle, Optic vesicle, Head ectoderm

B — Optic vesicle, Lens placode

C — Presumptive RPE, Presumptive NR, Invaginating lens, Presumptive cornea

D — RPE, NR, Lens, Cornea
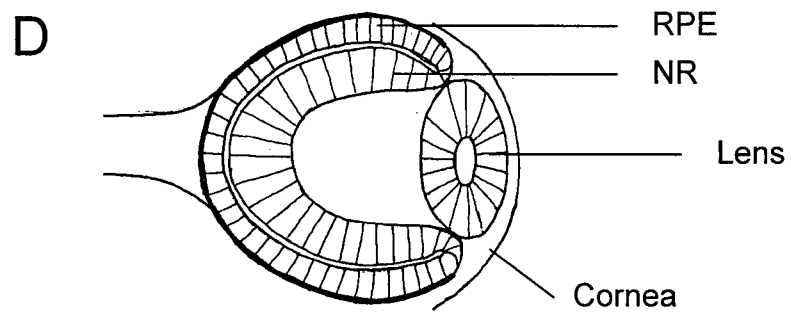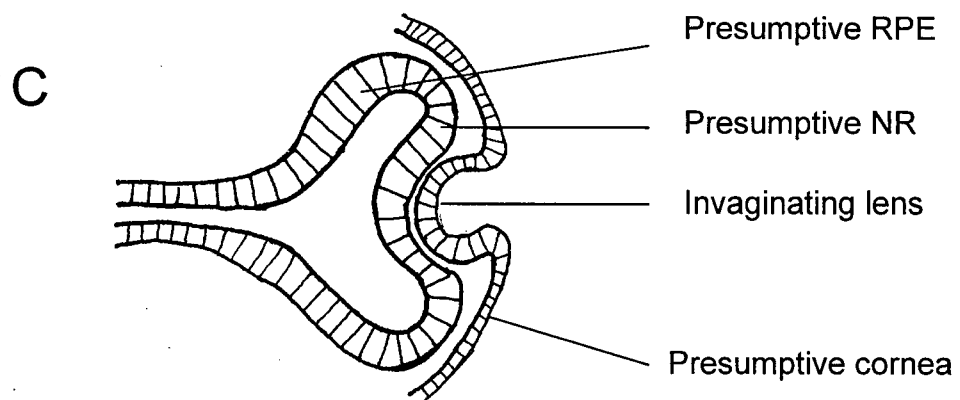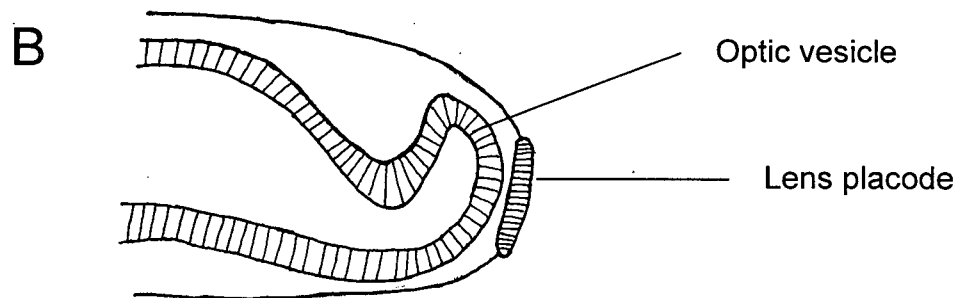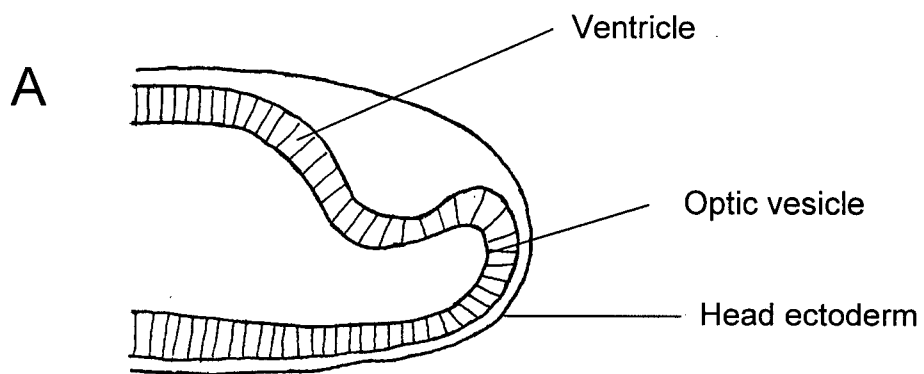
assume a cuboidal shape. Markers other than melanin have been used to identify RPE, however, these markers were used to identify RPE cells in culture where there is a limited number of cell types that could express the embryonic markers used (Neill and Barnstable, 1990; Chu and Grunwald, 1991; Hergott et al., 1993; Zhou and Opas, 1994).

The presumptive neural retina begins to thicken after formation of the optic cup the first NR cells to differentiate are the ganglion cells. The ganglion cell progenitors begin to migrate to the inner NR and extend axons by stage 16, then differentiate at stage 24 (E4) to E6 (Halfter et al., 1985; Cohen et al., 1987). The ganglion cells continue to develop over days 5-9 to form three rows of cells. The ganglion cells then extend their axons along the ILM via laminin until E6 at which point $\alpha_6\beta_1$ laminin receptors are no longer expressed and neurite extension along laminin ceases (Cohen et al., 1989). From E5 to E9 there is neurite extension along vitronectin mediated by $\alpha_v\beta_3$ integrin (Martinez-Morales et al., 1996). By day 12 the ganglion cells have rearranged into a single layer. The photoreceptor precursors and Müller cells appear on days 5-8, at which point the bases of the Müller cells have formed the inner limiting membrane immediately adjacent to the vitreous space. The inner and outer plexiform layers appear between days 8-15; the amacrine, horizontal, and bipolar cells, respectively, also appear at this time. The photoreceptor outer segments develop between days 15-19 and continue to develop throughout the NR until two days after hatching (Coulombre, 1955; Romanoff, 1960; Layer and Wilbold, 1993; Rizzolo et al., 1994).

Transdifferentiation of the Retinal Pigment Epithelium

Prior to the formation of the optic cup, the cells of the neuroepithelium retain the

potential to differentiate to either RPE or NR (Pittack et al., 1997). Experiments in amphibians (newts) in which the future RPE cells were transplanted to the area adjacent to the surface ectoderm resulted in the formation of a fully developed NR (Detwiler and van Dyke, 1953;1954). Other experiments involving transplantation have resulted in both sides of the optic vesicle differentiating to RPE (Lopashov and Stroeva, 1964). While amphibian species (newts, frogs) retain the ability to regenerate NR cells from differentiated RPE (Lopashov and Sologub, 1972; Reh and Nagy, 1987; Reh et al., 1987), mammals and chickens do not retain this ability beyond formation of the optic cup (Lopashov and Stroeva, 1964; Coulombre and Coulombre, 1965). Coulombre and Coulombre (1965) also reported that chicken RPE up to stage 26 can transdifferentiate to NR following surgical removal of the existing NR if a fragment of the excised retina is placed in the vitreous space. Park and Hollenberg (1989) subsequently demonstrated that FGF-2 administered via slow release polymer implants into the vitreous space *in vivo* following surgical removal of stage 24 chicken embryo NR resulted in transdifferentiation of RPE to NR. These findings have since been reported by others using *in vitro* experiments (Pittack et al., 1991;Reh et al., 1991; Guillemot and Cepko, 1992; Opas and Dziak, 1994). Other growth factors have been tested under similar conditions (TGF-β, NGF, EGF), but none have resulted in transdifferentiation of RPE (Park and Hollenberg, 1991; Pittack et al., 1991; Guillemot and Cepko, 1992).

Further *in vitro* experiments (Pittack et al., 1997) using chicken stage 9-10 optic vesicles and cultured RPE demonstrated that a double retina lacking RPE resulted from the vesicles cultured with FGF-2. Furthermore, in the absence of FGF-2 the vesicles developed into a normal RPE and NR, while the same system with added anti-FGF resulted in an RPE phenotype

in both the presence and absence of FGF-2. The RPE cultured with FGF-2 transdifferentiated to NR while the RPE cultured in the presence of FGF-2 and anti-FGF-2 retained the RPE phenotype.

The importance of cell-ECM and cell-cell contact as a requirement for transdifferentiation has also been demonstrated (Reh et al, 1987; Reh and Radke, 1988; Zhou and Opas 1994; Opas and Dziak, 1994). RPE cells cultured separately or spread out on a flat surface did not transdifferentiate, but rather retained an epithelial form. *In vitro* studies (Reh et al., 1987) in which frog RPE cells were cultured on laminin, fibronectin, plastic, EHS sarcoma basement membrane, polylysine, or collagen I demonstrated that trandifferentiation only occurred in cells cultured on laminin and EHS basement membrane (contains laminin, fibronectin, and other ECM components). Similar experiments (Pittack et al., 1991) comparing frog and chicken RPE revealed that chicken RPE could only transdifferentiate in the presence of FGF-2, and only if the cell aggregates were shaken to prevent adhesion and spreading on the culture plate.

Role of Extracellular Matrix and Integrins in Retinal Cell Differentiation In Vitro and In Vivo

Extracellular Matrix Molecules in the Developing Eye

The extracellular matrix (ECM) consists of insoluble structural protein molecules which form a skeleton upon which cells can migrate, differentiate or proliferate (Paulsson, 1992; Yurchenco and O'Rear, 1994). The ECM is a reservoir for soluble factors, some of which are bound to the insoluble proteins in an inactive form. During development ECM molecules are

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produced by epithelial and mesenchymal cells. The basement membrane, the foundation upon which an epithelium resides, consists of three layers: the *lamina lucida* and *lamina densa* which are produced by the epithelial cells, and the *lamina fibroreticularis,* produced by mesenchymal cells underlying the epithelium. Cells can interact with the ECM through integrin receptors. Integrin binding with ECM ligands results in initiation of intracellular signal transduction through which cells can undergo significant changes in expression or function. Integrins expressed along cell-cell interfaces can extend the effects of ECM ligand binding throughout a tissue by effecting signal sequences from cell to cell (Hynes,1992; Lin and Bissell, 1993; Juliano and Haskill, 1994; Haas and Plow, 1994; Schwartz and Ingber, 1994; Ruoslahti, 1996; Sjaastad and Nelson, 1997). Ligands in the ECM include a vast array of molecules, notably fibronectin and laminin, which may play important roles in ocular development.

The extracellular matrix of the developing embryo provides a network of support molecules which are essential for development as well as maintenance of homeostasis of cells and tissues. The major components of the matrix are collagen IV, laminin, perlecan, nidogen, SPARC (secreted protein acidic and rich in cysteine), and fibulin-1, all of which are components of basal laminae (Yurchenco and O'Rear, 1994). Fibronectin is also associated with basement membranes and is present throughout developing mesenchyme as a fine web of fibrillar material.

With the exception of the sclera, extracellular matrix in the eye is less dense and collagenous than in other areas of the body. In the retina, the only collagenous area is Bruch's membrane which supports the RPE. This basement membrane is a fused membrane consisting of basal laminae of the choriocapillaris on the outer extremity and the RPE on the inner side. In between there is a central elastin-rich area flanked on either side by a collagenous layer

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containing collagen I, collagen III and collagen IV. Within this layered network there are considerable amounts of laminin as well as fibronectin (Turksen et al., 1985; Hilfer and Randolph, 1993). In the developing retina, fibronectin is expressed in the underlying membrane of the optic vesicle neuroepithelium, the later developing Bruch's membrane, and in the inner limiting membrane but is not seen elsewhere in presumptive RPE or presumptive NR. In stage 13-22 embryos laminin becomes increasingly prominent in developing membranes while fibronectin is said to retain the same distribution but change in intensity or increase slightly in staining intensity in association with Bruch's membrane (Turksen et al., 1987). Collagen IV and perlecan also increase in expression during development, but to a lesser extent and later than both fibronectin and laminin. SPARC and fibulin-1 have not been described in ocular tissues to date.

Fibronectin is a glycoprotein multimer containing a variety of protein modules. Each monomer is composed of three distinct modules (F1, F2, F3) which are known to exist in proteins other than fibronectin (Potts and Campbell, 1996). The module of particular interest in ocular development is the F3 fragment, which contains the Arg-Gly-Asp (RGD) amino acid sequence (Kleinman et al., 1993; Potts and Campbell, 1996; Ruoslahti, 1996). This sequence is the ligand for a number of integrins found in the developing eye. *In vivo* experiments (Svennevik and Linser, 1993) have shown that RGD peptides injected into the interstitium between the surface ectoderm and NE of chicken optic vesicles at stage 9 can disrupt normal lens development. In addition to fibronectin, RGD is present in other ECM proteins (laminin, vitronectin, collagen, NCAM; Ruoslahti, 1996, Yip et al., 1998).

Laminin is a glycoprotein constructed of an α, β, and γ chain. There are eight genetically

distinct subtypes of the chains ($\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, $\beta2$, $\beta3$, $\gamma1$, $\gamma2$) yielding seven different isoforms

of laminin designated laminin-1 to laminin-7 (Timpl and Brown, 1994). The role of laminin in

development has been reviewed (Kleinman et al, 1993; Ekblom, 1993; Timpl and Brown, 1994).

Laminin has a number of adhesion recognition sequences which have been identified, among

which are RGD (cryptic), Tyr-Ile-Gly-Ser-Arg (YIGSR, on the $\beta1$ chain), and Ile-Lys-Val-Ala-

Val (IKVAV, on the α chain). The YIGSR pentapeptide has been reported to inhibit metastasis

of melanoma and carcinoma, and has also been implicated in cell adhesion and migration (Graf

et al., 1987; Iwamoto et al., 1987; Kawasaki et al., 1991, Kleinman et al., 1993). In addition,

YIGSR has been reported to block angiogenesis (Sakamoto et al., 1991). IKVAV, which has

also been shown to promote adhesion and migration of cells during development, has also been

demonstrated to promote metastasis in melanoma. While none of the peptide sequences

identified in laminin have been used on developing ocular tissues, their close association with

laminin-cell interactions has potential implications in eye development. Antibodies against

laminin have been used successfully in blocking studies. Nagy and Reh (1994) demonstrated

that injecting antibodies *in vivo* against a laminin-heparan sulfate complex, which binds FGF-2

and containins the $\alpha_3\beta_1$ integrin binding site in the E3 domain of laminin, could block

transdifferentiation of RPE to NR in the *Rana* tadpole, which retains the capacity to regenerate

NR up to metamorphosis.

Integrins in the Developing Eye

Integrins are non-covalently linked glycoprotein heterodimer receptors constructed of α

and β transmembrane subunits. Ligands for integrins are diverse, comprising a number of ECM

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insoluble proteins such as fibronectin, laminin, collagen IV, and vitronectin, soluble blood components (fibrinogen, coagulation factors), viral protein, and other cell adhesion molecules or integrins (Hynes, 1992; Schwartz and Ingber, 1994; Haas and Plow, 1994; Hillis and Macleod 1996). Ligand specificity is conferred by the particular pairing of any number of combinations of the fifteen α and eight β subunits. The cytoplasmic domain of the β subunit is linked to the cytoskeleton via α-actinin, talin and vinculin. Once bound by an ECM ligand or a receptor of an adjacent cell, mechanical stress can be transmitted through the cytoskeleton, thereby triggering the appropriate response (migration, spreading, gene expression, etc.).

Different ligands can be recognized by a single integrin; conversely, different integrins can be bound by a given ligand. The integrins known to recognize laminin include: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_7\beta_1$. Integrins which bind fibronectin include: $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_4\beta_7$ and $\alpha_v\beta_7$, some of which also react with vitronectin (Haas and Plow, 1996; Hillis and Macleod, 1996).

The distribution of integrins in the eye is not as well described as in other tissues. Investigations have assessed integrins in normal human eye (Brem et al., 1994), human eyes with proliferative retinal membrane disease (Robbins et al., 1994), and a review has been published (Elner and Elner, 1996) describing integrins found in both post-natal and embryonic eyes of various species. Alpha 6 integrin has been described from the optic cup stage onward (Bronner-Fraser et al., 1992). Most of the integrins described are not located in the retina proper, but rather the cornea, lens, sclera and choroid (Elner and Elner, 1996).

There is very little information about the distribution of integrins during the early developmental stages in the chicken embryo (stages 8-15), when the optic vesicles invaginate

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to form the optic cup and differentiation of retinal cells begins to occur. Rizzolo and co-workers (1994) assessed integrin distribution in the RPE by immunohistochemistry, revealing the temporal and spatial distribution of β_1 integrins from day 7 to day 15 of chicken development. These investigators also probed for α subunits and found that α_3, α_6 and α_v were present on both day 7 and day 15. The distribution of α_3 subunits was confined to the basal membrane on day 7 and day 15. Alpha v also retained expression in the same locations on both day 7 and day 14, but its distribution was on apical, basal, and lateral membranes. There was also intracellular staining of α_v at day 7 which diminished by day 14. The distribution of α_6, however, changed; on day 7 the apical staining was more intense than the basal, while on day 14 the pattern became reversed. The presence of α_6 subunits in the NR from the optic cup stage throughout ganglion cell development and into adulthood was reported by Bronner-Fraser et al. (1992), indicating interactions with laminin in the later stages of retina development The presence of α_3 and α_v subunits would seem to indicate that fibronectin binding was occurring at the stages where they were expressed. Although fibronectin was not reported in the apical regions where integrins were located, perhaps the effect of the fibronectin binding was transmitted via cell-cell contacts. Alpha 4 and α_5 subunits also bind fibronectin, but have not been identified in either the RPE or NR at early stages of development thus far.

Blocking experiments involving anti-β_1 integrin antibodies have been done on developing ocular tissues. In addition to the experiment using RGD peptides to block normal eye morphogenesis, Svennevik and Linser (1993) also used anti-β_1 integrin antibodies to achieve the same result. *In vitro* studies by Hergott and co-workers (1993) involved the inhibition of RPE cell migration to denuded Bruch's membrane following wounding in cultured

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RPE. Under normal circumstances RPE cells in culture migrate into the wound site to repair the damage. However, in the presence of antibodies directed against β_1 integrin migration was markedly inhibited. Another interesting finding was that RPE cells which had spread over a laminin substratum detached upon exposure to anti-β_1 integrin antibodies. Chu and Grunwald (1991) also investigated RPE-substrate adhesion *in vitro* by treating cultures of fibroblasts and RPE on laminin with the anti-β_1 integrin antibodies, 2A10 and CSAT. The result was that adhesion and migration on laminin were impaired. However, in cultures on fibronectin, only the 2A10 antibody prevented adhesion and migration while the CSAT antibody had no effect. If the cells were first allowed to adhere and spread on laminin and fibronectin, then followed by exposure to 2A10 and CSAT, both antibodies caused the cells to detach on laminin, but neither antibody had any effect on the cells cultured on fibronectin.

Rationale

From the information presented it is clear that the early differentiation of the optic vesicle neuroepithelium into RPE and NR is influenced by growth factors, the ECM, and integrins which interact with the ECM. FGF-2 has been implicated in the normal development of retina *in vitro* and transdifferentiation of the RPE to neural retina *in vitro* and *in vivo*. ECM molecules have also been demonstrated to play a role in controlling the decision to become RPE or NR during both normal development and RPE transdifferentiation. Fibronectin and laminin are the earliest ECM components to be detected in the developing membrane underlying the neuroepithelium of the developing eye. During eye development in the chicken embryo, there is early and widespread expression of fibronectin, while laminin, which is present in small

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amounts at stage 8 becomes heavily expressed later in development. Interaction of the presumptive RPE and NR with ECM components is believed to be mediated through a number of integrin receptors. The integrin subunits that have been described in chicken RPE and NR are β_1, α_3, α_6, and α_v. However the spatial and temporal distribution of these subunits has not been established at the early stages of eye development. Beta-1 integrins have been shown to be necessary for ocular morphogenesis but their precise role in RPE and NR differentiation has not been investigated. Svennevik and Linser (1993) injected their blocking agents into the space between the neuroepithelium and ectoderm in an attempt to block morphogenesis of the lens. Their study did not mention any effects on retinal development nor was there any mention of changes in either RPE or NR morphology. It is not clear which β_1 integrins are involved in early eye development because the distribution of α subunits and their co-expression with the β_1 subunit in the early embryonic retina has not been established. Further, the role of β_1 integrins in retinal development between the optic vesicle stage and the optic cup stage has not been studied.

The aim of the present study was to define roles for the above molecules during the differentiation of the early NE into NR and RPE using the chicken embryo as a model. The chicken embryo model offers several advantages. In contrast to commonly used mammalian models, such as the mouse, in which the staging system is based on time post-coitum, staging of the chicken embryo is event- and feature-dependent and therefore more precise. In addition, this model is inexpensive, fast and simple and allows for rapid analysis of many specimens. Unlike the mouse embryo, the chicken embryo is easily accessible and amenable to surgical manipulation.

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Indirect immunofluorescence was used to localize ECM components (fibronectin, laminin) and integrin subunits (α_3, α_4, α_5, α_6, α_v, and β_1). Epifluorescence microscopy was used to visualize these molecules in the developing eye. Once the patterns of expression were established, the second part of the study was carried out in order to investigate the role of pertinent molecules. One approach was to block key receptor-ligand interactions at specific points in time. Svennevik and Linser (1993) used this approach but restricted the study to stage 9 prior to complete optic vesicle formation. The optic vesicles at this point in development are still continuous with the neuroepithelium of the developing brain, and therefore not sealed off on the medial side. The consequence of injecting at this stage is that blocking agents may diffuse into the surrounding space, thereby becoming diluted or affecting cell types other than presumptive RPE and NR. Furthermore, the previous authors' interest was in lens development and consequently they did not address NR and RPE differentiation.

In the present study an antibody to β_1 integrin subunit (CSAT) and peptides containing integrin recognition site RGD were administered via microinjection into the optic vesicle at stage 11 to block receptor-ligand interactions preceding key cellular events during early eye development. Injecting blocking agents at the optic vesicle stage offers the potential to restrict the blocking agents to a smaller space containing a specific population of cell precursors. The optic vesicles at stage 11 are constricted medially and could perhaps retain more of the administered agent than a fully open stage 9 optic vesicle. Moreover, the cell precursors present in the optic vesicles at this stage retain their labile, multipotential character, thereby offering the potential for altering the phenotype or marker expression of the affected cells. The aim of using these agents was to attempt to disrupt normal interactions of the respective molecules and their

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ligands in order to gain insight into their relationship during RPE and NR development *in vivo*.

Hypothesis

The interactions of optic vesicle neuroepithelium with fibronectin and/or laminin, mediated by integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$ and/or $\alpha_v\beta_1$, govern the choice of differentiation pathway to either NR or RPE.

Objectives

The objectives of this study were to:

i) determine the spatial and temporal distribution of β_1, α_3, α_4, α_5, α_6, and α_v integrin subnits and the integrin ligands laminin and fibronectin, during early differentiation of the RPE and NR (stages 8-24) in the chicken embryo.

ii) develop and assess a method for administering agents into the eye of the chicken embryo *in ovo* at the optic vesicle stage (stage 11).

iii) determine the effects of injection of agents (antibodies, peptides) to disrupt β_1 integrin-ECM interactions on RPE and NR differentiation in the chicken embryo *in ovo*.

MATERIALS AND METHODS

Animal model

Fertilized White Leghorn chicken (*Gallus domesticus*) eggs were incubated in a humidified atmosphere (56%) at 38°C. Experimental manipulation of embryos was carried out *in ovo* under aseptic conditions. The eggs were candled over a 150 W bulb to locate the position of the embryo and the position was circled on the shell with a pencil. The pencilled area and blunt end of the egg were then wiped with 70% ethanol. Approximately 2 ml of albumin was removed from the blunt end of the egg using a sterile needle (1½ inches, 20 G) and syringe. Using alcohol-cleaned scissors, a circular hole ("window") was cut in the shell over the embryo prior to manipulation. All openings were then covered with clear adhesive tape. Non-toxic, sterile-filtered India ink (Pelikan; Hannover, Germany) suspended in Tyrode's saline (Stern, 1993) was injected beneath the developing embryo using a sterile needle (⅝ in, 25 G) and syringe to provide contrast for visualization *in ovo,* thereby facilitating staging and surgical manipulation. Developing embryos were staged according to the morphological criteria outlined by Hamburger and Hamilton (1951). In some experiments, blocking agents (antibodies, peptides) were administered by microinjection (see below) into the optic vesicle.

Administration of Blocking Agents

Blocking agents were administered by beads or microinjection. An incision was made in the vitelline membrane, amnion, and medial region of the right optic vesicle of stage 8-11 embryos with a tungsten needle. Heparin-coated acrylic beads (75-100 μm diameter, Sigma

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Chemical Co., St. Louis, MO) were inserted with a watchmaker's forceps. The beads had been previously stored in sterile Tyrode's saline. The "window" in the shell was sealed with transparent tape and embryos were returned to the incubator to continue development to stage 24.

For the microinjection technique, micropipettes were pulled from borosilicate glass capillary tubing (100 mm in length, 1 mm outer diameter, 0.75 mm internal diameter; TW 100F-4, World Precision Instruments, Sarasota, FL) containing a filament along the inner wall. The pulling was done with a vertical pipette puller (Model 700a; David Kopf Instruments, Tujunga, CA). The micropipettes were subsequently bevelled with a K.T. Brown Type micropipette beveller (Model BV-10; Sutter Instruments, Movato, CA) and sterilized by exposure to UV irradiation for 24 h.

The antibodies used were: anti-β_1 (CSAT antibody, gift of Clayton Buck, Wistar Institute, Philadelphia, PA) and rabbit anti-mouse IgG (Prod #R1008, Sigma Chemical Co., St. Louis, MO). The following peptides were used: RGD (Arg-Gly-Asp; Sigma) and RGES (Arg-Gly-Glu-Ser; Sigma). Lyophilized peptides were diluted with sterile Tyrode's saline to 10 mg/ml and 100 mg/ml. All handling of the solutions from this point was carried out under aseptic conditions using sterilized pipette tips and sterile containers. Dilutions were made using sterile filtered Tyrode's saline.

Embryos that had reached the 13 somite stage (stage 11 according to Hamburger and Hamilton, 1951) were used for the experiments with blocking agents. The tape covering the "window" in the shell was cut away and the opening was enlarged to allow access to the embryo. Blocking agents (antibodies and peptides) were administered by micropipettes attached

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to the end of plastic tubing. The reagent was drawn into the pipette by applying negative pressure to the other end of the tubing by mouth. Approximately 50 nl was drawn from a droplet (2 µl) of the appropriate reagent on a sterile weigh boat placed in a humidified chamber. The bevelled micropipette was used to pierce the right optic vesicle and overlying vitelline membrane and deliver the blocking agent. The doses of antibodies and peptides applied to the embryos were based on amounts and concentrations used by Svennevik and Linser (1993). A piece of transparent adhesive tape was applied to re-seal the opening in the shell, and the embryos were replaced in the incubator and allowed to develop to stage 24.

Fixation and Embedding

For studies of normal eye development, embryos were removed at appropriate stages. For the experiments with blocking agents, injected embryos and controls were dissected 24h following treatment.

The extraembryonic membrane was cut in a 1 cm circle around the embryo, then the cut disc was grasped with watchmaker's forceps and removed from the egg. The embryo was placed in a plastic Petri dish (35 mm by 10 mm) containing phosphate-buffered saline (PBS). The vitelline membrane was removed and excess yolk was washed from the ventral side of the disk with PBS using a disposable pipette. The embryo was then spread flat in the dish with forceps and PBS was withdrawn using a pipette. Bouin's fixative (Kiernan, 1990) was added carefully to the dish by pipette so as not to disturb the disk. For immunohistochemical experiments, embryos were fixed at room temperature for 15 min. The embryos were then rinsed in PBS, and all extraembryonic tissues were dissected away. Embryos were embedded in gelatin using a

modification of the method of Stern (1993). The embryos were transferred by grasping the caudal region with forceps and placing them in glass vial containing 5% (w/v) sucrose in PBS. The embryos remained in the 5% sucrose/PBS until they sank to the bottom of the vial. The 5% sucrose/PBS was then replaced by 20% (w/v) sucrose in PBS and the embryos were infiltrated overnight at 4°C. The 20% sucrose /PBS was replaced with a 37°C solution containing 7.5% (w/v) gelatin in 10% (w/v) sucrose/PBS for 5 h at 37°C with occasional swirling. The embryos were transferred to disposable Tissue-Tek intermediate cryomold (Miles Inc., Elkhart, IN) and the cryomolds were filled with gelatin infiltration solution. The cryomolds and embryos were allowed to solidify at room temperature. The solidified embryos in gelatin were then removed from the molds using flat forceps and a block of gelatin containing the embryo was cut out. The remaining gelatin was discarded and the embryo block was appropriately oriented and re-embedded in the same cryomold containing Tissue-Tek O.C.T. compound (Miles Inc.) . The O.C.T.- embedded embryos were then rapidly frozen in liquid nitrogen-cooled isopentane. Serial cryosections were cut at 5 μm on a Reichert-Jung Cryocut cryostat at -26°C and placed on Superfrost/Plus slides (Fisher Scientific, Nepean, ON), allowed to air dry for 30 min and stored at -20°C.

Immunohistochemistry

The immunohistochemical techniques involved the use of appropriate primary antibodies subsequently detected with a corresponding fluorescently labelled secondary antibody. Rinses consisted of 0.1 M phosphate-buffered saline at pH 7.4 containing 150 mM NaCl (PBS). The primary antibodies were commercially available immunoglobulins against embryonic chicken

NCAM (clone 5e; Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA), chicken β_1 integrin subunit (clone W1B10; Sigma Chemical Co., St. Louis, MO), human α_v integrin subunit (rabbit polyclonal; Chemicon, Temecula, CA), human α_3 integrin subunit (rabbit polyclonal; Chemicon), human α_4 integrin subunit (rabbit polyclonal; Chemicon), chicken α_5 integrin subunit (clone A21F7; DSHB), and chicken α_6 integrin subunit (clone P2C62C4; DSHB). Fibronectin and laminin were detected with antibodies against chicken fibronectin (rabbit polyclonal; Chemicon) and human laminin (rabbit polyclonal; Monosan, Uden, the Netherlands). Antibodies were diluted with 0.1 M PBS buffer pH 7.4 containing 1% (w/v) bovine serum albumin (BSA), 1% (v/v) heat-inactivated goat serum, 0.1 % (w/v) sodium azide, 0.01% (v/v) Triton-X100. The appropriate dilutions of antibodies were determined by staining a series of sections with primary antibody dilutions of 1:100, 1:300, 1:500, and 1:1000, followed by detection with a fluorescent secondary antibody in a 1:250 dilution. Optimal dilutions were selected by determining the combination of primary antibody dilution and secondary antibody dilution which resulted in maximal staining of positive control tissue sites while simultaneously achieving minimal non-specific background staining. The final primary antibody dilutions were 1:200 for all of the purified preparations of antibodies raised against alpha integrin subunits, and 1:300 for the β_1 integrin subunit, fibronectin, laminin and NCAM. The monoclonal antibodies A21F7 used to detect α_5 integrin subunit and P2C62C4 for α_6 integrin subunit were purchased as supernatants and were applied neat.

Slides stored at -20°C were removed from the freezer and allowed to air dry for 15 min. Rings of 1 cm in diameter were etched around the sections using a diamond pencil. Slides were then placed in PBS warmed to 37°C for 15 min to remove gelatin (Stern, 1993) and rinsed in

PBS. The slides were de-lipidized by placing them in methanol at -20°C for 6 min followed by acetone at -20°C for 3 min. The slides were rinsed in PBS, and 5% heat-inactivated goat serum in PBS was applied for 15 minutes. The goat serum was poured off the slides and the primary antibodies were applied at approximately 50 µl per section on the slide. The primary antibodies were applied to sections for 16 h at room temperature in a humidified chamber. The secondary antibodies (goat polyclonal anti-rabbit IgG or goat anti-mouse IgG heavy and light chain tagged with either Oregon Green 514 or Texas Red-X; Molecular Probes, Inc., Eugene, OR) were applied in a 1:250 dilution in PBS for 2 h at room temperature. Following incubation, the slides were washed in three changes of PBS. Negative controls consisted of the primary antibodies being replaced by blocking, non-immune goat serum in primary antibody diluent. After the final PBS rinse, the slides were mounted using Gelvatol (Stern, 1993) and coverslipped.

Photomicrography

The stained sections were viewed on a Zeiss Axiophot photomicroscope equipped with epifluorescence components. Fluorescence and phase contrast photomicrography was carried out with Ektachrome P1600 (2 pushes on development) colour reversal film for 35 mm slides (Kodak, Rochester, NY). A Nikon LS-1000 slide scanner (Nikon, Tokyo, Japan) with Nikon Control Version 2.0 software was used to scan the slide images. Scanned images were converted to grayscale and saved as TIF files using Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA). The slide were scanned at 2700 pixels/inch to provide maximum resolution of the images. The scanned images were subsequently resized to 600 pixels/inch to reduce the file size to facilitate computer manipulations. The photographic plates were assembled in Adobe Photoshop 4.0

(Adobe Systems, Inc) and dye sublimation-printed at 300 dpi.

RESULTS

Immunohistochemical Localization of Cell Markers, ECM Components and Integrin Subunits

During Eye Development

The objective of this study was to determine the spatial and temporal expression of fibronectin, laminin, β_1 integrin (subunits α_3, α_4, α_5, α_6, and α_v) prior to and during RPE and NR differentiation in the chicken embryo. In addition to the ligands mentioned above, the distribution of the neuronal marker, NCAM, was determined. Embryos were collected at stages 8 through 24 (n=3 per stage). The embryos were cryoprotected, embedded in gelatin, and cryosectioned at 5 μm. Indirect immunohistochemistry to detect the aforementioned ligands was carried out as described in the preceding section. Prior to immunostaining, sections were treated with a sequence of absolute methanol followed by acetone each carried out at -20°C to de-lipidize intact cell membranes.

Positive tissue controls consisted of extraocular and ocular embryonic chick tissues taken at stages ranging from stage 8 to stage 24. Fibronectin, laminin, NCAM and the integrin subunits under investigation are known to exist at various stages in these tissues. Negative controls consisted of serial sections of the same tissues treated with non-immune serum in place of the primary antibody followed by application of the appropriate fluorescently-labelled secondary antibodies. All negative controls for all ligands studied showed no observable fluorescence in any tissues (Fig. 2 a-d).

The gelatin embedding allowed for excellent orientation, cryoprotection, and reliable serial sectioning at 5 μm, resulting in good preservation of morphology. The brief fixation in

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Figure 2.   Assessment of non-specific binding of fluorescent-labelled secondary antibodies.

Fluorescently-labelled goat anti-mouse secondary antibody was applied following incubation with (A) non-immune goat serum (B) mouse anti-chicken NCAM. Fluorescently-labelled goat anti-mouse secondary antibody was applied following incubation with (C) non-immune goat serum and (D) mouse anti-α_5 integrin antibody. Immunofluorescence microscopy. 240X
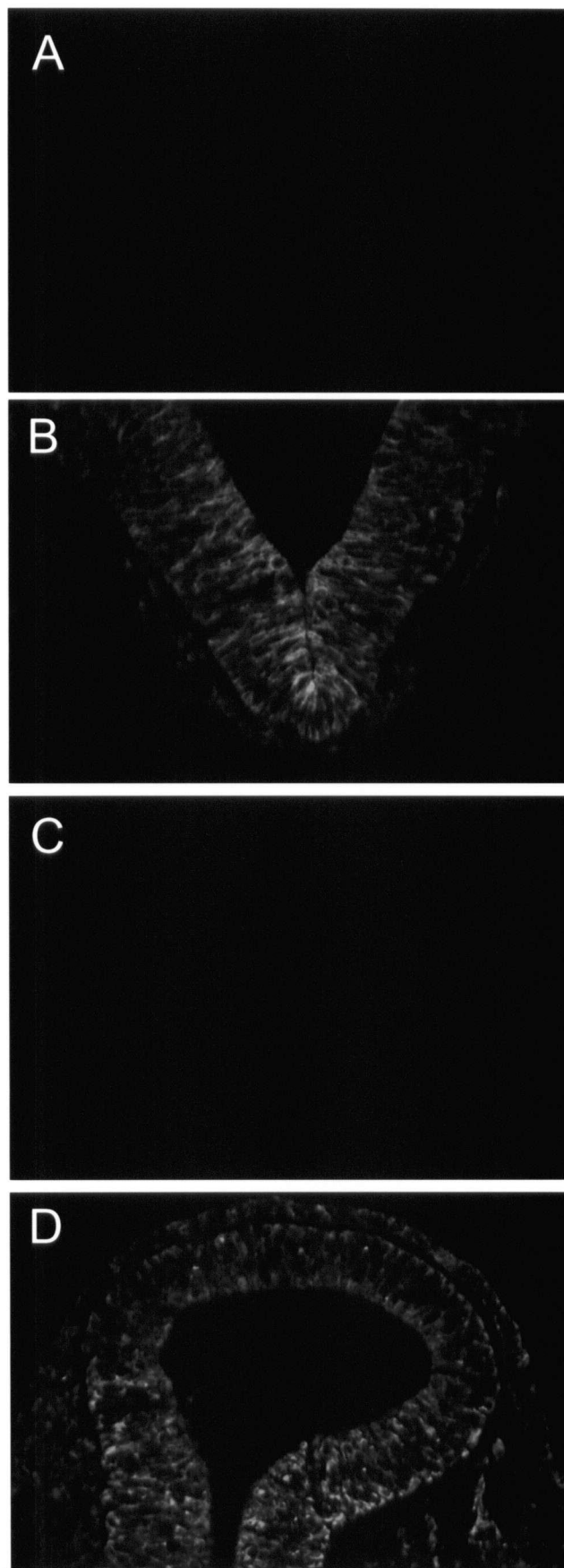
Bouin's fixative resulted in good immunoreactivity and preservation of cellular detail. In a preliminary experiment, immunohistochemical staining for fibronectin, laminin, and β_1 integrin (n=3) resulted in specific fluorescent staining of the ligand with minimal or no background immunoreactivity. De-lipidizing prior to staining for integrins improved the specificity and intensity of the fluorescent signal.

Antibodies to several molecules were run at the same time, thereby minimizing the number of tissue sections sacrificed for negative controls. For example, serial sections from four or five different stages of embryos were mounted on successive slides, and each slide was stained for a separate molecule. In this manner one negative control sufficed for a series of stains. Stages were also overlapped from run to run to ensure a valid comparison basis from one run to the next.

ECM Components

Laminin was present at stage 8 in the outer NE surface (Fig. 3a). Scattered cells expressing laminin were also observed in the NE and developing mesoderm adjacent to the neuroepithelium. At the stages examined, staining of ocular tissues was largely restricted to the developing Bruch's membrane, cornea and lens basement membrane, and the inner limiting membrane of the NR (Figs. 3b-d). The intensity of laminin immunoreactivity in the basal laminae appeared to increase steadily through stage 24. There was a trace amount of staining in the lateral epithelium of the optic vesicle at stages 11 to 13 (Fig. 3b) which disappeared at later stages. There was persistent staining within the cells of the cornea at least up to stage 24 (Figs. 3c, 3d). Staining within the cells of the cornea persisted at least up to stage 24.

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Figure 3.   Immunolocalization of laminin during eye development in the chicken embryo.

(A) Stage 10 embryo, right optic vesicle facing right. Intense immunostaining at basement membrane of NE, and scattered cells (arrows) 120X (B) Stage 11 embryo, right optic vesicle facing top. Intense immunostaining in basement membranes of NE and surface ectoderm. Note cellular staining on inner surface of NE (arrows). 240X (C) Stage 17 embryo, right optic vesicle facing top. Intense immunostaining of basement membranes of RPE, NR, lens and cornea basement membranes. Note the absence of NR (large asterisk) or RPE (small asterisk) staining. 120X. (D) Stage 24 embryo. Immunofluorescence of basement membranes of NR, RPE, lens, and cornea are stained. Immunofluorescence microscopy. 60X.
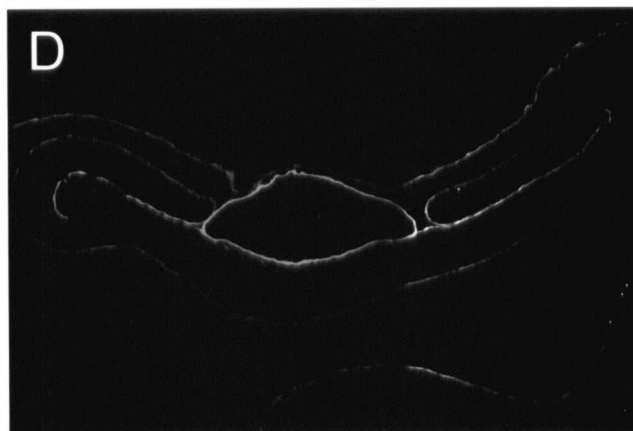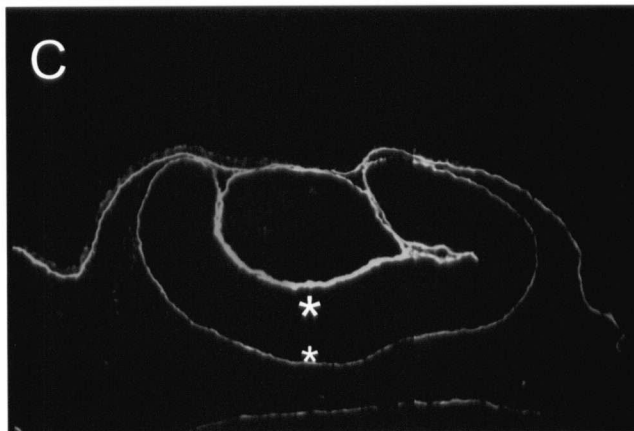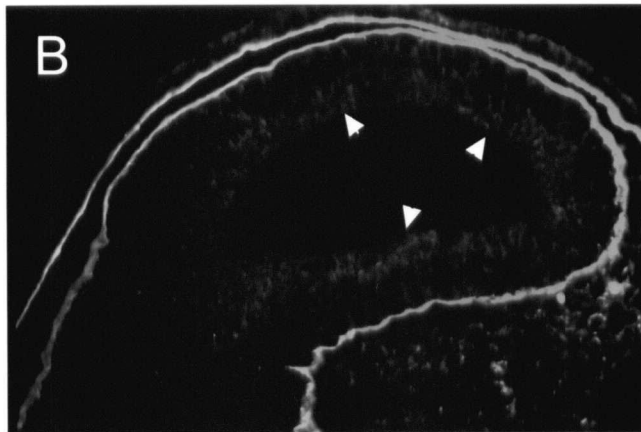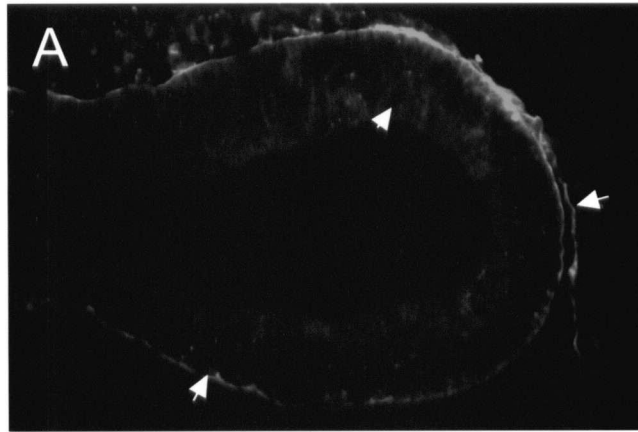
Fibronectin immunoreactivity was observed in the developing basement membranes of the NE, ectoderm, and mesoderm at stages 8 to 11 (Fig. 4a). Fibronectin surrounds the optic vesicle epithelium and was continuous around the optic cup epithelium, lens epithelium, and cornea. There were also trace amounts of expression within the presumptive RPE at stages 15 through 17 (Fig. 5a) which persisted through stage 24. The corneal cells and basement membrane showed staining at stages 14 through 24. The NR showed fibronectin staining from the inner surface to the outer surface with increased amounts at either extremity. At stage 22 there was intense staining in the differentiating ganglion cells which diminished by stage 24. The fibronectin staining at stage 24 increased from stage 22. There was also expression in the apices of the RPE and intense staining of Bruch's membrane at stage 24 (Fig. 5b). Strong immunoreactivity for fibronectin was observed in the mesenchyme throughout all of the stages studied.

NCAM

Pericellular NCAM expression was present throughout the NE at stage 8. The fluorescence was most intense at the apices of the inner NE cells but was present surrounding cells elsewhere in the NE (Fig. 6a). The signal intensified and spread throughout the NE of the optic vesicle at stage 13, particularly on the inner and outer surfaces (Fig. 6b). The staining in the apical, basal, and lateral margins of the corneal epithelial cells was intense and remained so through to stage 24. After formation of the optic cup at stage 14 the staining of the presumptive NR and RPE was markedly diminished while the corneal epithelium and lens epithelium were intensely positive. At stage 15 the presumptive NR showed moderate staining

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Figure 4.   Immunolocalization of fibronectin during eye development in the chicken embryo.

(A) Stage 10 embryo, right eye facing right. Intense immunostaining of NE basement membrane, ectoderm basement membrane, mesenchyme, and scattered cells. (B) Stage 13 embryo, right eye facing top. Intense immunostaining of NE basement membrane, ectoderm basement membrane, and mesenchyme. Note moderate intracellular staining of NE (asterisk). Immunofluorescence microscopy. 220X.
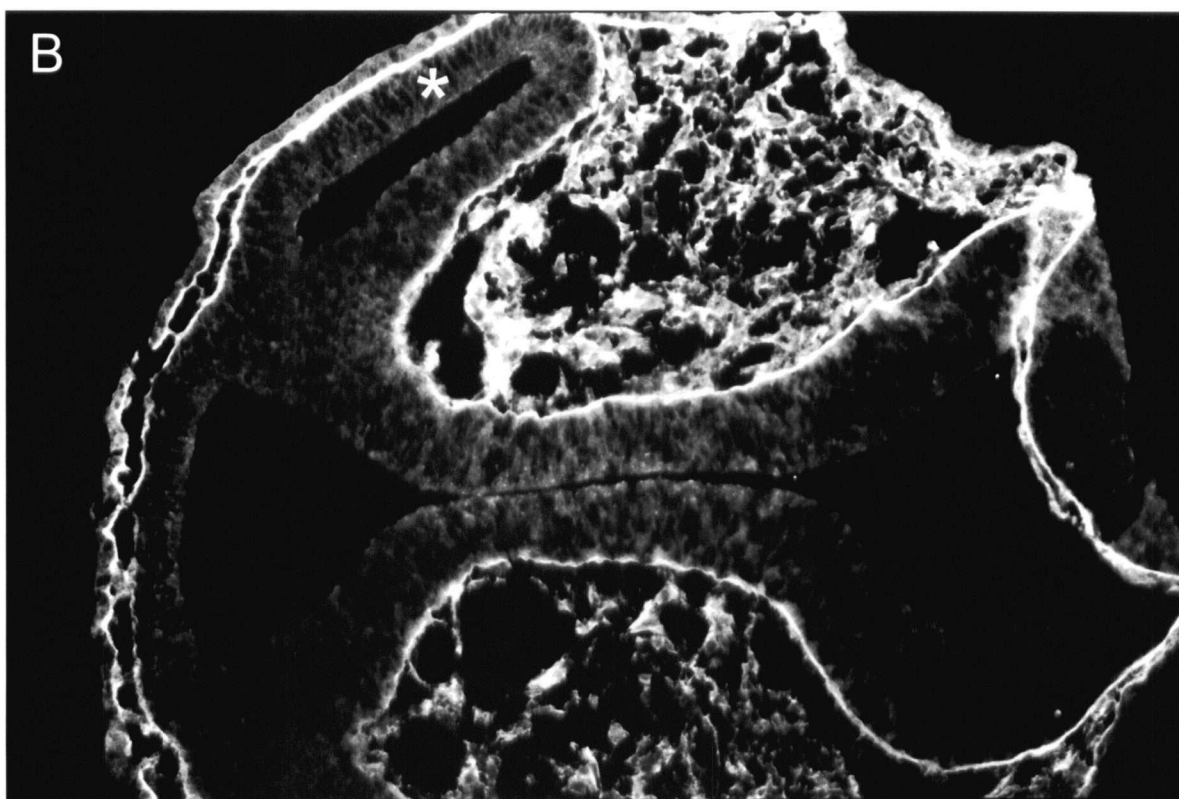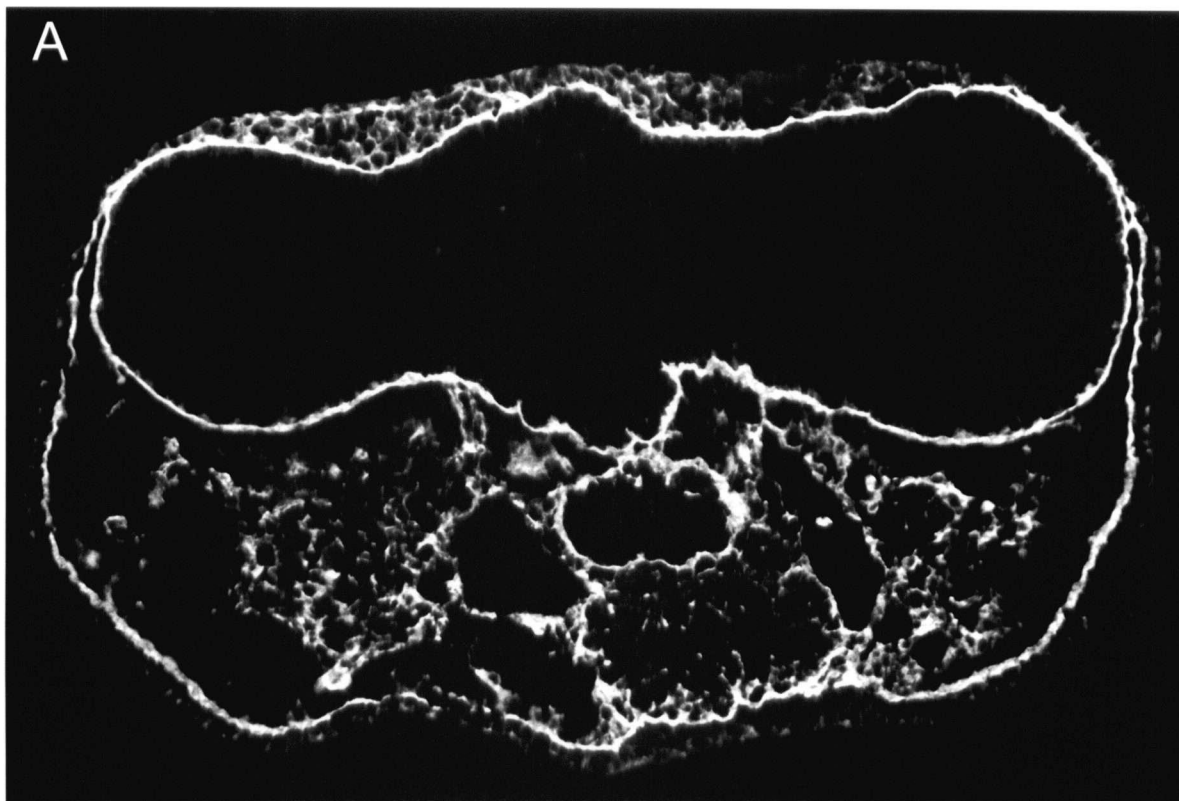
Figure 5   Immunolocalization of fibronectin during eye development in the chicken embryo.

(A) Stage 22 embryo, cornea facing top. Immunostaining of ILM (small arrows), RPE basement membrane, cornea basement membrane. Note intense staining of ganglion cells in NR (large arrow), lens fibres (large asterisk), and moderate staining of apical RPE (small asterisk). (B) Stage 24 embryo, cornea facing top. Intense membrane staining, increased staining of NR (large asterisk), moderate staining in RPE (small asterisk). Immunofluorescence microscopy. 220X
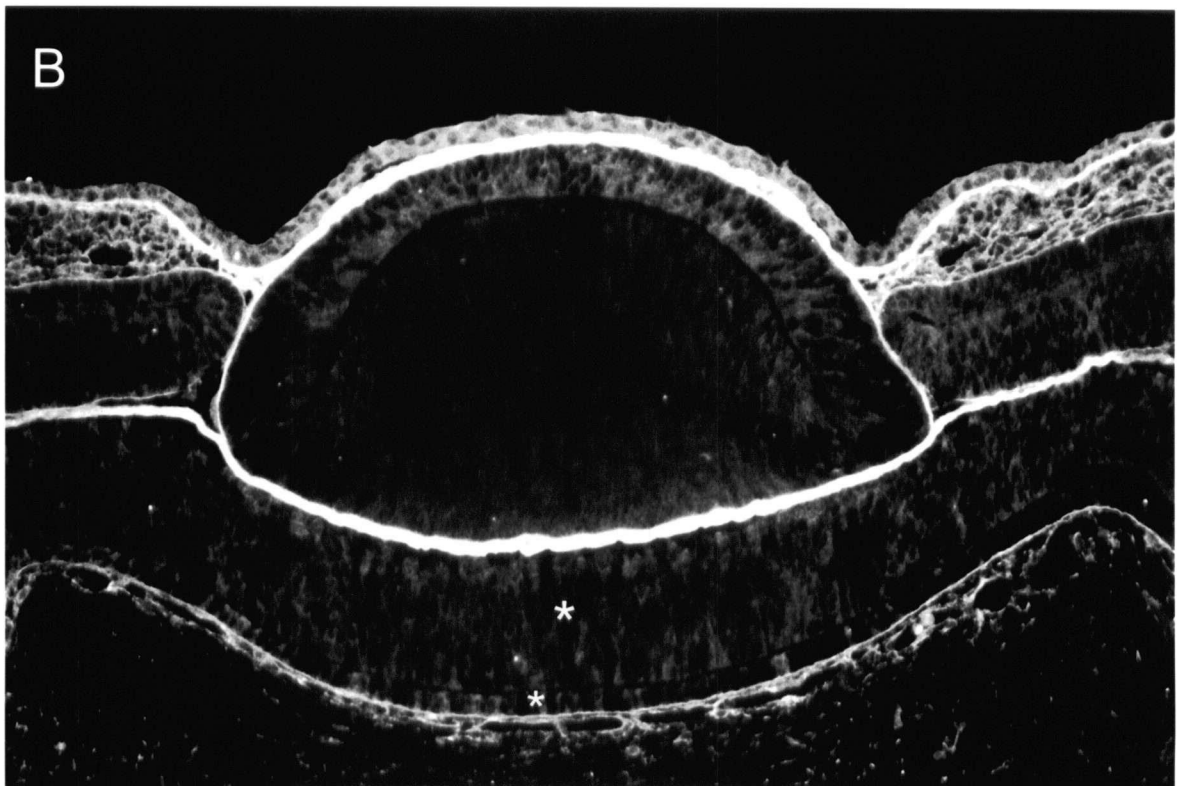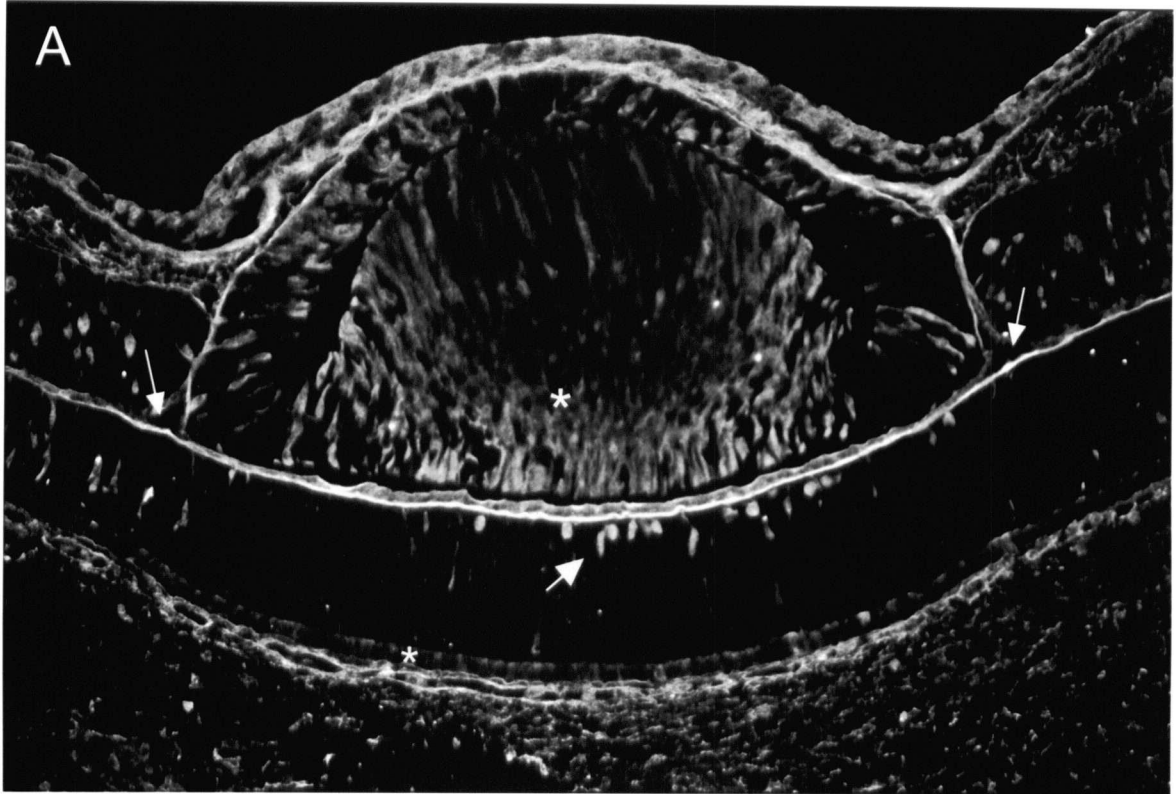
Figure 6.   Immunolocalization of NCAM during eye development in the chicken embryo.

(A) Stage 8 embryo neural tube facing top. Immunostaining surrounding NE cells. (B) Stage 13 embryo, right optic vesicle facing top. NCAM staining is intense in the ectoderm, pericellular staining in the NE. (C) Stage 15 embryo, right optic cup facing top. Decreased staining in RPE (small asterisk) increased staining in central NR (large asterisk). Note intense staining of cornea and lens. (D) Stage 24 embryo. Complete loss of RPE staining (asterisk), intense staining in NR. Immunofluorescence microscopy. 240X.
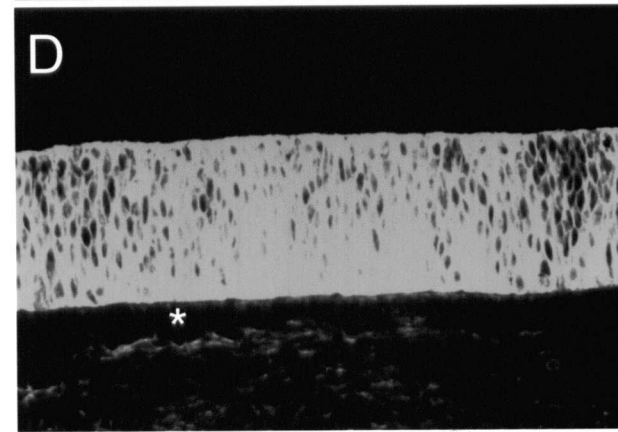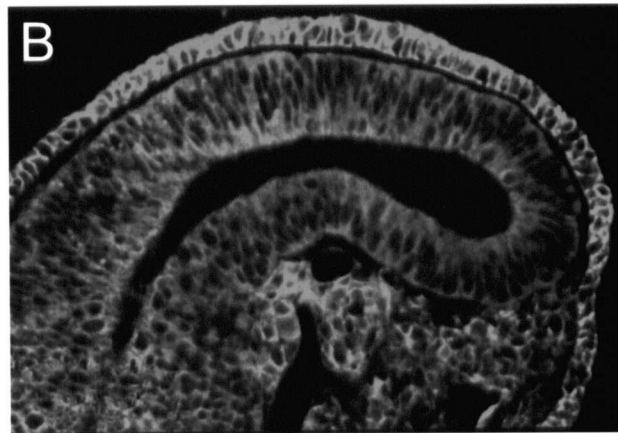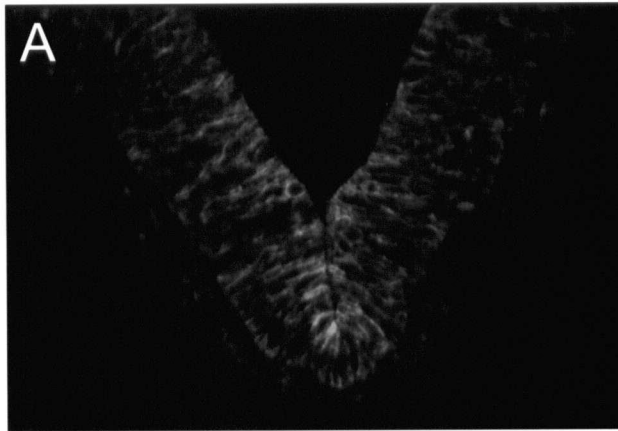
in the central portion but was negative at the margins (Fig. 6c). At this stage, the RPE was

unstained and remained so through subsequent stages (Figs. 6c-d). In contrast, the NCAM

staining increased throughout the NR up to stage 24 when it was intensely positive and had

extended to the margins (Fig. 6d).

Beta 1 Integrin Subunit

The distribution of β_1 integrin expression was ubiquitous throughout the developing

eye from stages 8 through 24. At stage 8 the cells of the NE are surrounded by moderately

stained membranes throughout most of the epithelium (Fig. 7a). There was increased β_1

staining in and around the inner NE surface which persisted through stage 13 when there was

strong staining surrounding the cells of the inner regions of the optic vesicle (Fig. 7b). The

apical, basal and lateral margins of the corneal epithelial cells at stage 13 were strongly

immunoreactive and remained so through stage 24. By stage 15 the lens epithelium, corneal

epithelium and developing retina showed strong staining. Where the outer region of the NR

closely apposes the apical RPE the staining of the NR and the RPE was most intense (Fig. 8a).

The staining was equally intense at the inner surface of the NR and gave the appearance of

clusters. The staining intensity continued to increase in all ocular tissues through stage 24

when intense staining was seen surrounding the RPE cells. At this stage, the cell margins of

the NR displayed strong β_1 immunoreactivity, which appeared as clusters in the vicinity of

the of the inner limiting membrane (Fig. 8b).

Figure 7.   Immunolocalization of β_1 integrin subunit during eye development in the chicken embryo.

(A) Stage 10 embryo, right optic vesicle facing right. Immunostaining around cell margins of NE. (B) Stage 12 embryo, right optic vesicle facing top. Immunostaining has become more prominent on inner surface of NE and surrounding the NE cells. Immunofluorescence microscopy. 440X.
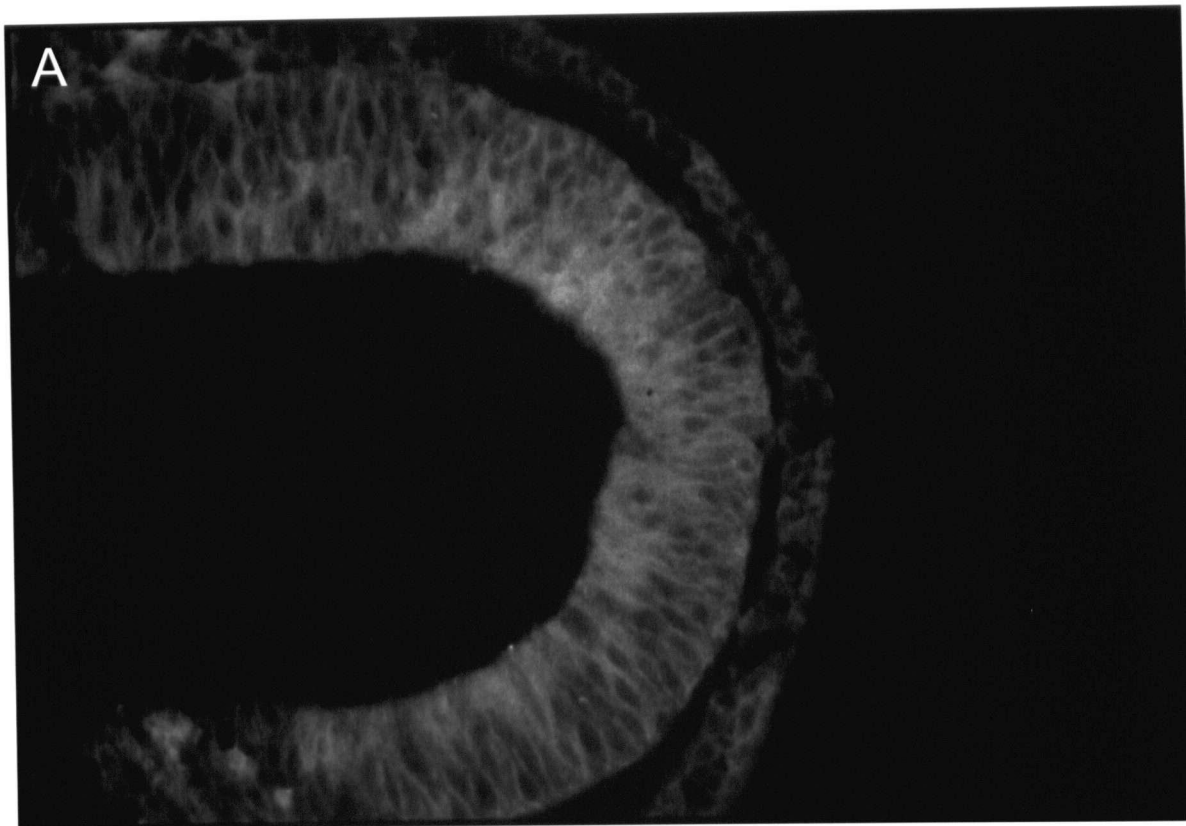
Figure 8.   Immunolocalization of β_1 integrin subunit during eye development in the chicken.

(A) Stage 15 embryo, right eye facing top. Pericellular stainng pattern throughout the entire optic cup.   (B) Stage 24 embryo. Intense staining surrounding the RPE (small asterisk) and throughout the NR (large asterisk). Note increased signal on ILM (arrows).   Immunofluorescence microscopy. 440X
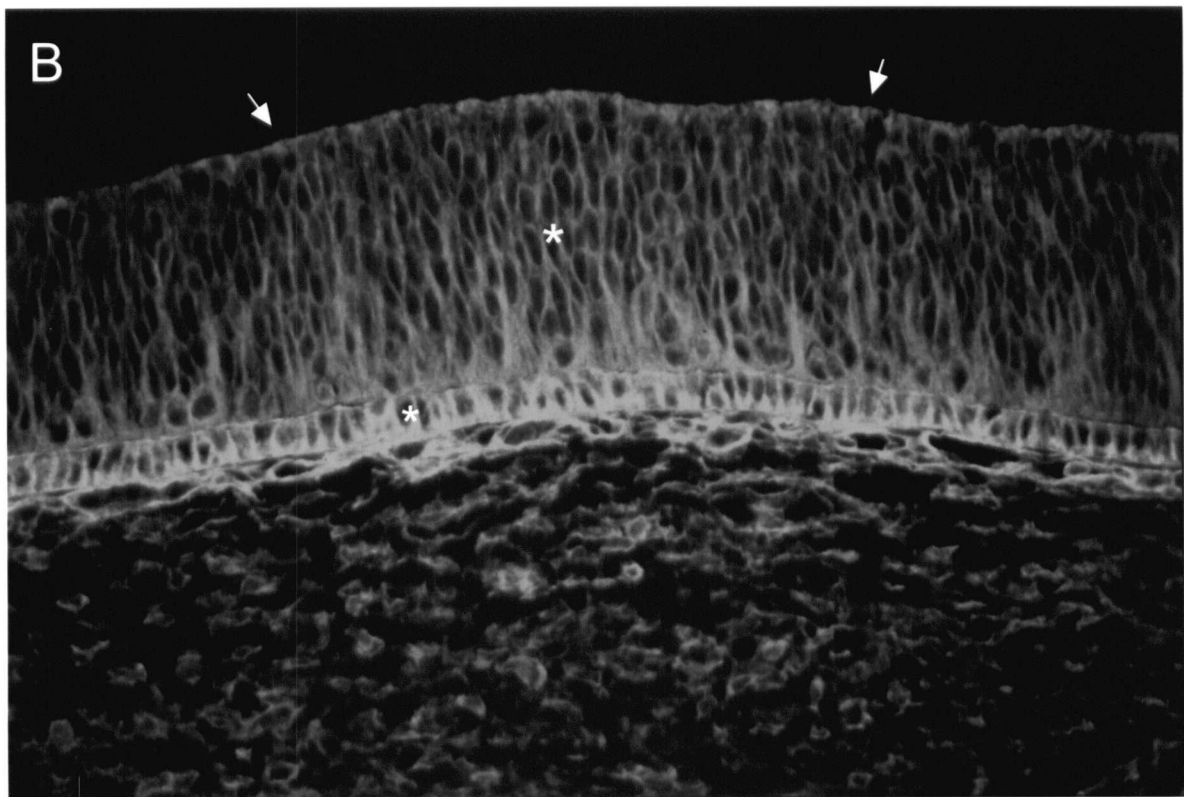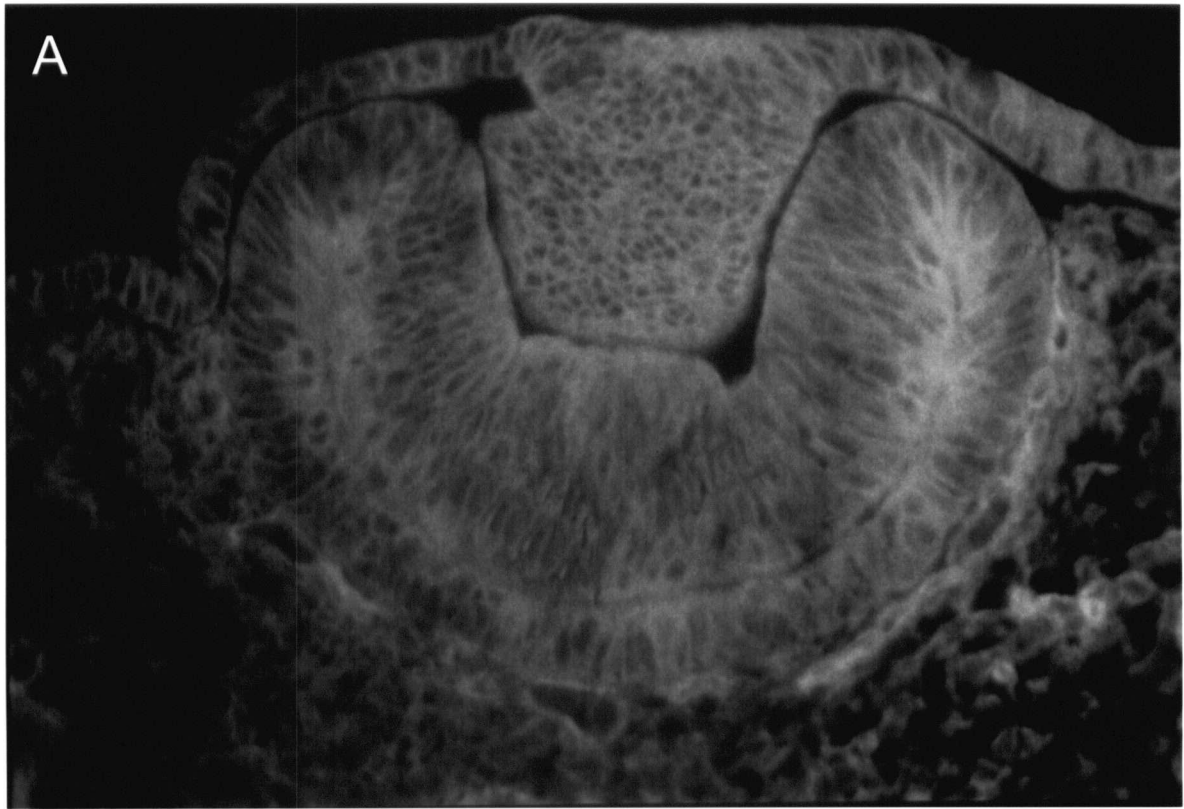
Alpha 3 Integrin Subunit

The staining of α_3 integrin subunit at stage 8 was weak, intracellular, punctate and diffuse with foci of immunoreactivity at the apices of the inner NE and bases of the outer NE (Fig. 9a). This pattern persisted through stage 13 with increasing intensity at each stage. At stage 14 the expression was markedly increased to strong staining in the apical, basal, and lateral surfaces of the corneal epithelium and continued to increase to intense staining by stage 16. The staining intensity did not increase in the cornea from stages 17 to 24. Similarly, the staining of the lens epithelium increased to a maximum at stage 16 then appeared to decrease slightly to moderate staining at stage 17 and remained constant through stage 24. Cells in the central portion of the presumptive NR and in the apices of the RPE showed increased pericellular staining at stage 14 (Fig. 9b). At the margins of the optic cup, staining was less pronounced showing weak staining in both the NR and RPE. By stage 24, the inner and outer regions of the NR showed strong staining. The distribution of α_3 in the NR from stages 17 through 24 appeared pericellular (Fig. 10a). The pattern in the RPE progressed from moderate staining at the apices and weak at the bases at stage 14 to strong staining on the basal, lateral, and apical surfaces of the RPE by stage 18. The intense immunoreactivity of the apical, basal, and lateral aspects of the RPE was mainly restricted to the central area of the RPE at stage 17. By stage 22 the entire RPE was equally stained throughout to the margins (Fig. 10b). There was no change in the pattern of α_3 integrin subunit staining thereafter to stage 24.

Figure 9.   Immunolocalization of α_3 integrin subunit during eye development in the chicken embryo.

(A) Stage 8 embryo, neural tube facing top. Punctate vesicular staining in NE cells (arrows). (B) Stage 14 embryo, right eye facing top. Pericellular and diffuse intracellular staining in NR and RPE. Note moderately intense staining in cornea.   Immunofluorescence microscopy. 440X.

Figure 10   Immunolocalization of α_3 integrin subunit during eye development in the chicken.

(A) Stage 19 embryo. RPE cells intensely stained (asterisk). The NR is moderately stained, and the early migrating ganglion cells (arrows) are more intensely stained than the remainder of the NR is seen. (B) Stage 24 embryo. Intense staining of the cornea, RPE has become monolayer, and shows moderate pericellular staining (small asterisk). Note decrease in NR staining (large asterisk).
Immunofluorescence microscopy. 440X.

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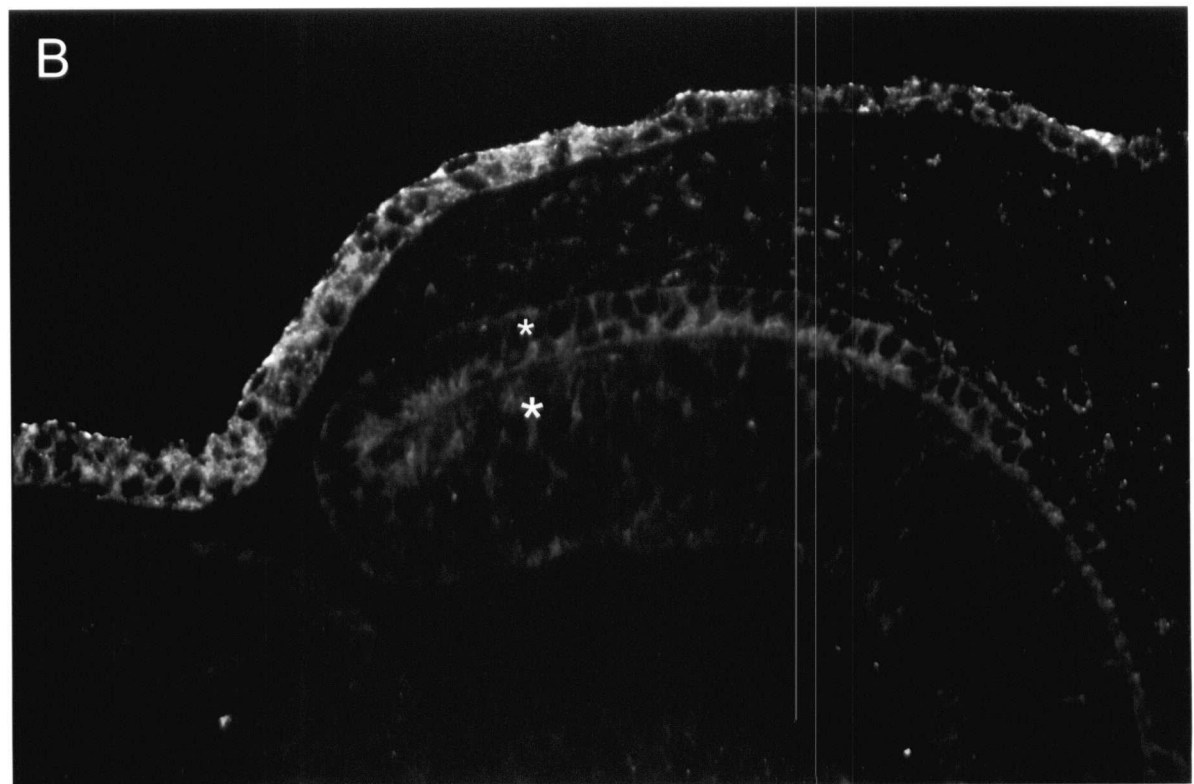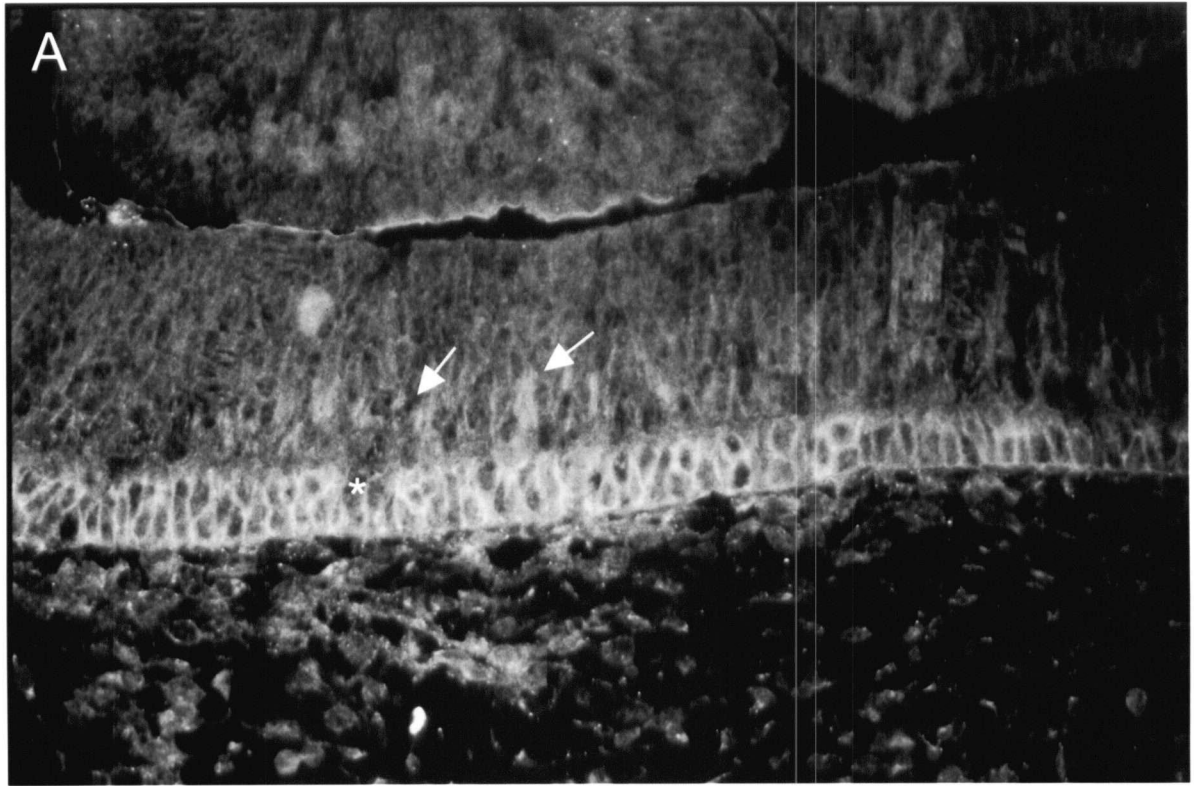
Alpha 4 Integrin Subunit

Staining for the α_4 integrin subunit at stage 8 displayed a distinct, intracellular punctate pattern in the apices of the cells of the inner NE and in the bases of the cells of the outer NE (Fig. 11a). There were more intense clusters of immunoreactivity along the ectodermal surface of the embryo which increased by stage 12 when the presumptive cornea is forming. The early lens placode and cornea stained with equal intensity. Staining of the lens cells diminished and showed consistent moderate staining from stage 15 through 24. The weak, diffuse staining pattern remained in the NR and RPE through to stage 17. At stage 17 (Fig. 11b) the staining intensified at the inner NR region and was largely intracellular; the outer NR remained moderately stained. The distribution of signal did not change up to stage 24 but there was an increase in intensity of staining in the outer regions of the NR (Fig. 12b). The inner NR fluorescence was intense at stage 22 and appeared more diffuse at stage 24 but was not significantly changed in intensity (Figs. 12a, 12b). In the RPE, staining was observed at the apical surfaces at stage 17 and increased in the apical and basal areas by stages 22 and 24. At stage 22 (Fig. 12a) there was strong, clustered, discontinuous staining at the Bruch's membrane surface of the RPE and in developing blood vessels of the choroid. By stage 24 the basal staining was continuous and intense and apical expression of intracellular α_4 integrin was increased (Fig. 12b).

Alpha 5 Integrin Subunit

At stages 8 through 10 the α_5 integrin subunit was mainly localized to the developing basal lamina of the NE: strong discontinuous staining was observed at the basal extremity of
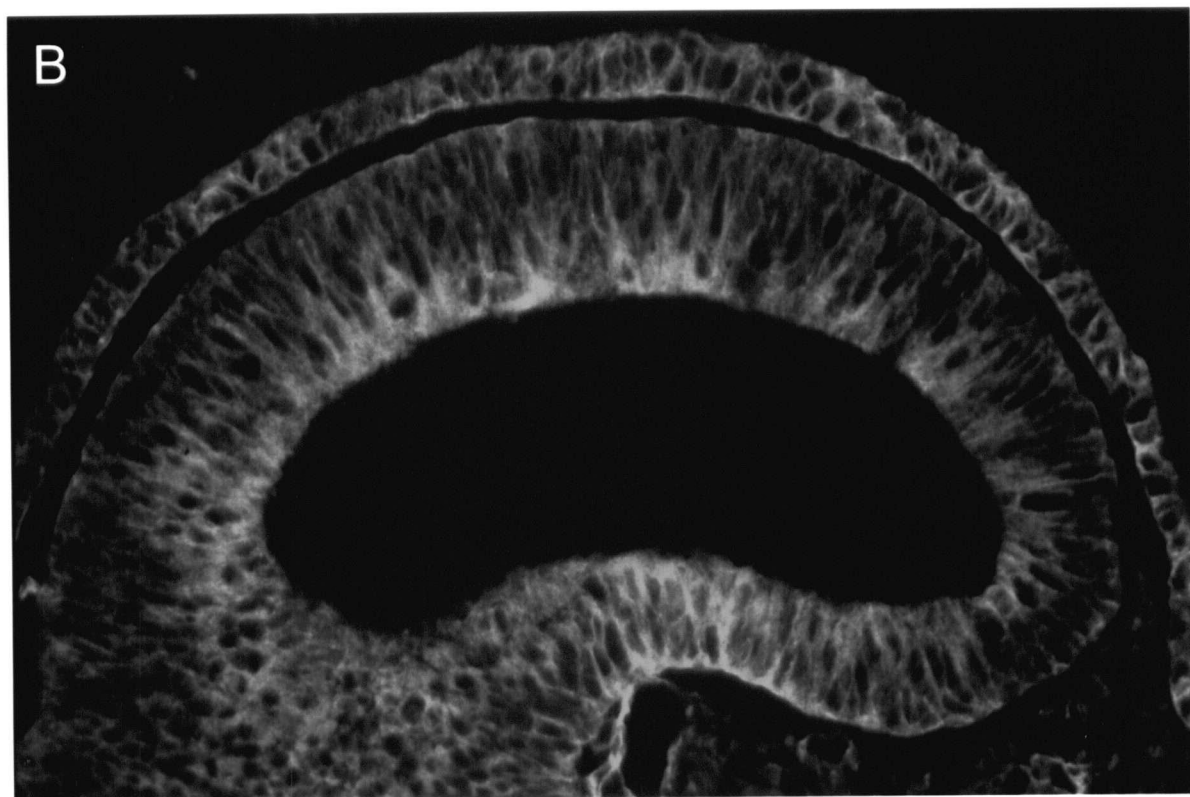
Figure 11.   Immunolocalization of α_4 integrin subunit during eye development in the chicken embryo.

(A) Stage 8 embryo, neural tube facing top. Intense punctate staining on inner and outer NE, punctate vesicular staining within the NE. (B) Stage 17 embryo, right eye facing top. Increased staining in lens, NR, RPE, and cornea. Note diffuse staining pattern in NR (large asterisk) and RPE (small asterisk). Immunofluorescence microscopy. 440X.

Figure 12.   Immunolocalization of α_4 integrin subunit during eye development in the developing chicken.

A Stage 22 embryo. There is intense, clustered staining along the ILM (arrows), increased staining in NR, and intense discontinuous staining of RPE basdement membrane and choriocapillaris (arrowheads). (B) Stage 24 embryo. Increased NR staining and continuous staining along RPE basement membrane (arrowheads).   Immunofluorescence microscopy.   440X.
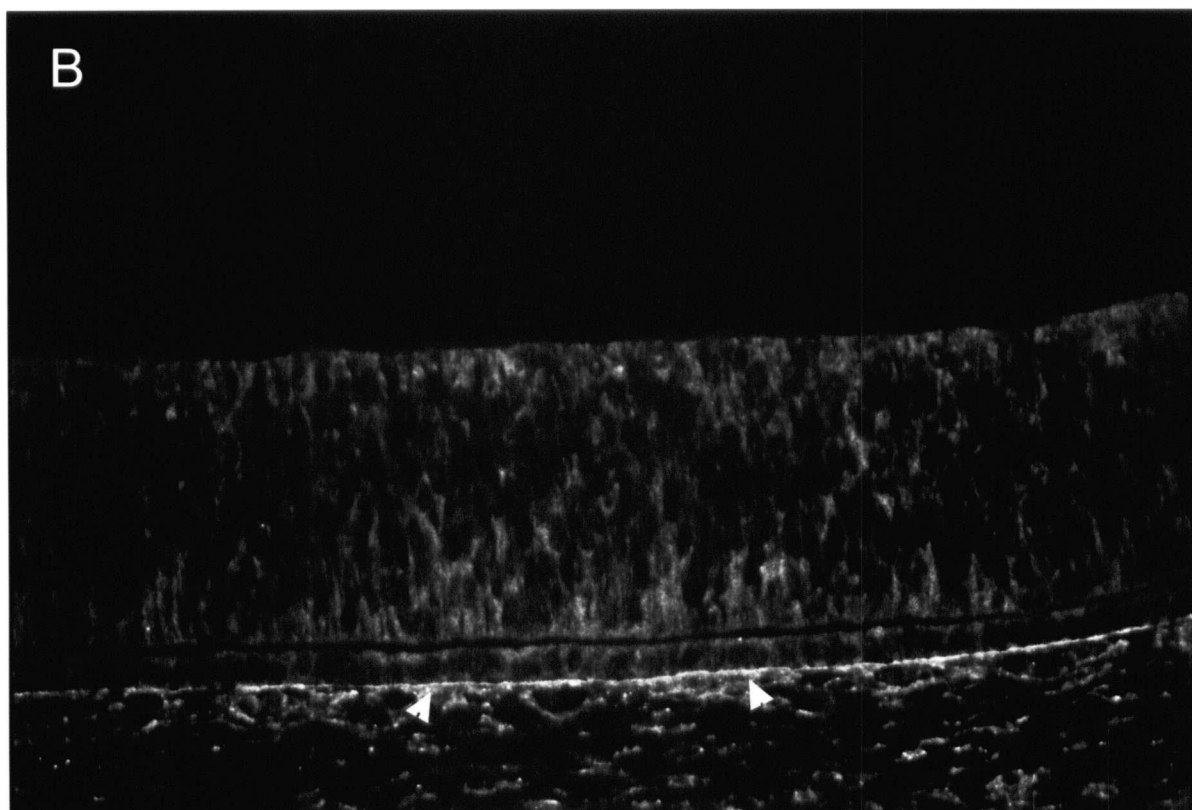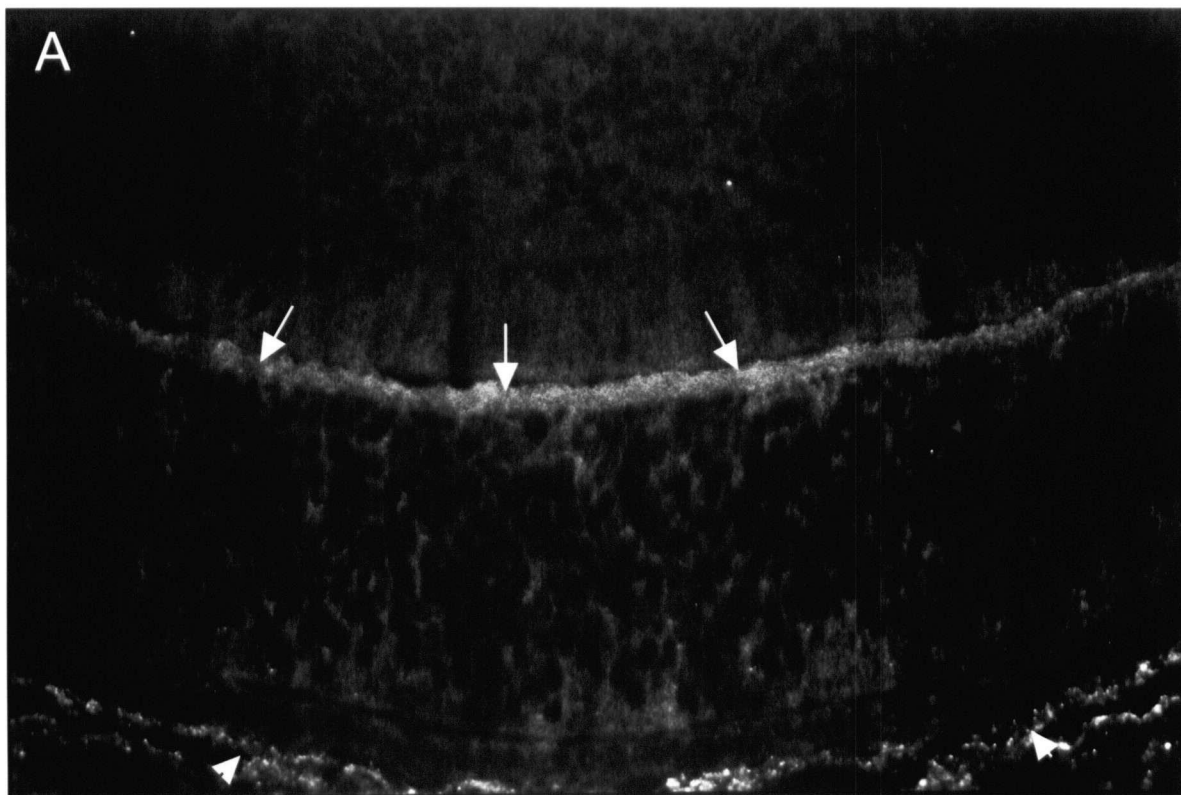
the outer NE (Fig. 13a). There was also more diffuse basal staining in the cells of the outer NE. The distribution of α_5 integrin subunit changed markedly from stage 10 to 11. At stage 11 the pattern changed to that of clumps of diffuse moderate to strong fluorescence in both the NE and corneal epithelium. The α_5 subunit was localized mainly in the apices of the inner (medial surface) and bases of the outer (lateral surface) of the NE of the optic vesicle. There were also cells throughout the epithelium showing strong immunoreactivity (Fig. 13b). The apical and basal corneal epithelium were moderately stained and this pattern persisted from stage 11 through 24. There was an enormous increase in staining intensity of the lens fibres by stage 18; this strong fluorescence persisted through stage 24 (Fig. 14a, 14b). By stage 18 the NR and RPE showed similar moderate staining intensities (Fig. 14a). Staining was observed within cells throughout the central portion of the NR from the inner to the outer regions. In the RPE, clusters of moderate to strong fluorescence were seen in the basal and apical intracellular areas. At stage 22 the staining pattern was not remarkably altered, but there was strong staining in cells which were likely developing ganglion cells on the basis of their morphology and location (Fig. 14b). The distribution of α_5 integrin subunit did not change appreciably thereafter to stage 24.

Alpha v Integrin Subunit

Weak, diffuse intracellular staining for α_v subunit was seen in scattered cells throughout the NE at stage 8 (Fig. 15a). This same distribution remained apparent, albeit in an increasing number of cells, through stage 11. By stage 13 the staining intensity increased throughout the optic vesicle, presumptive cornea and lens epithelium showing an ubiquitous
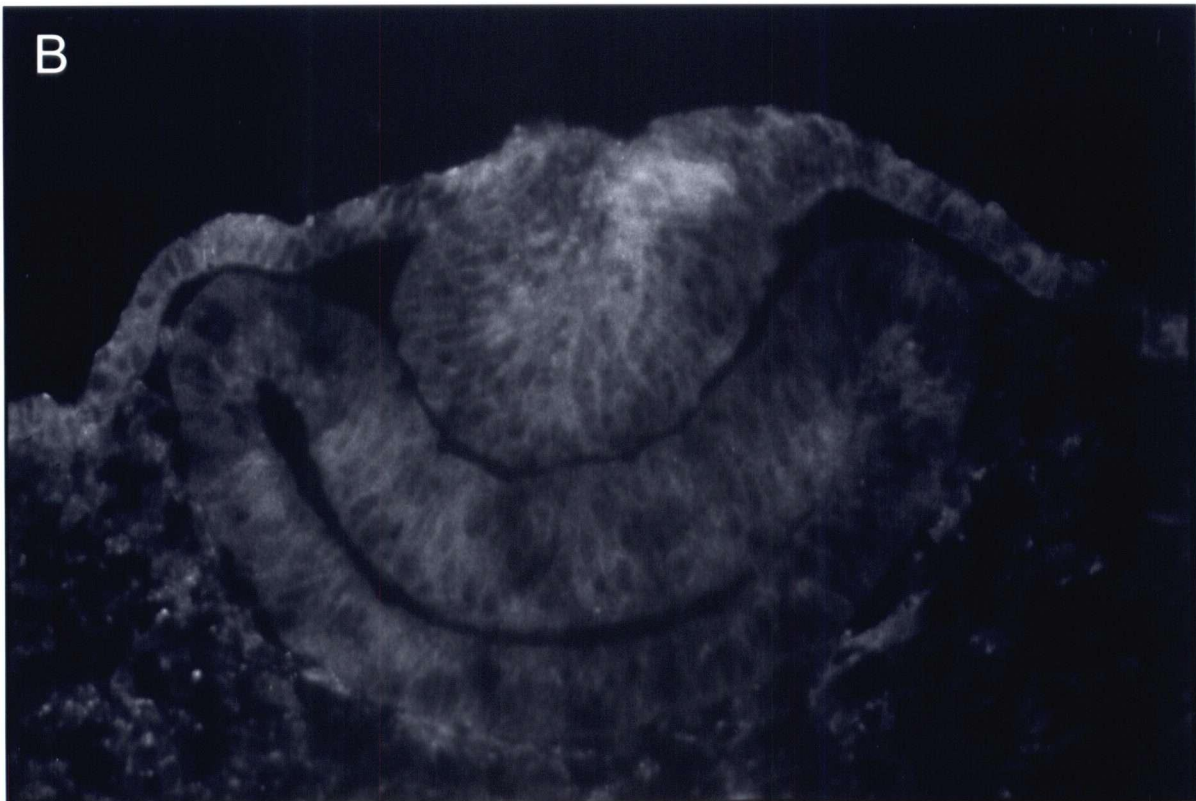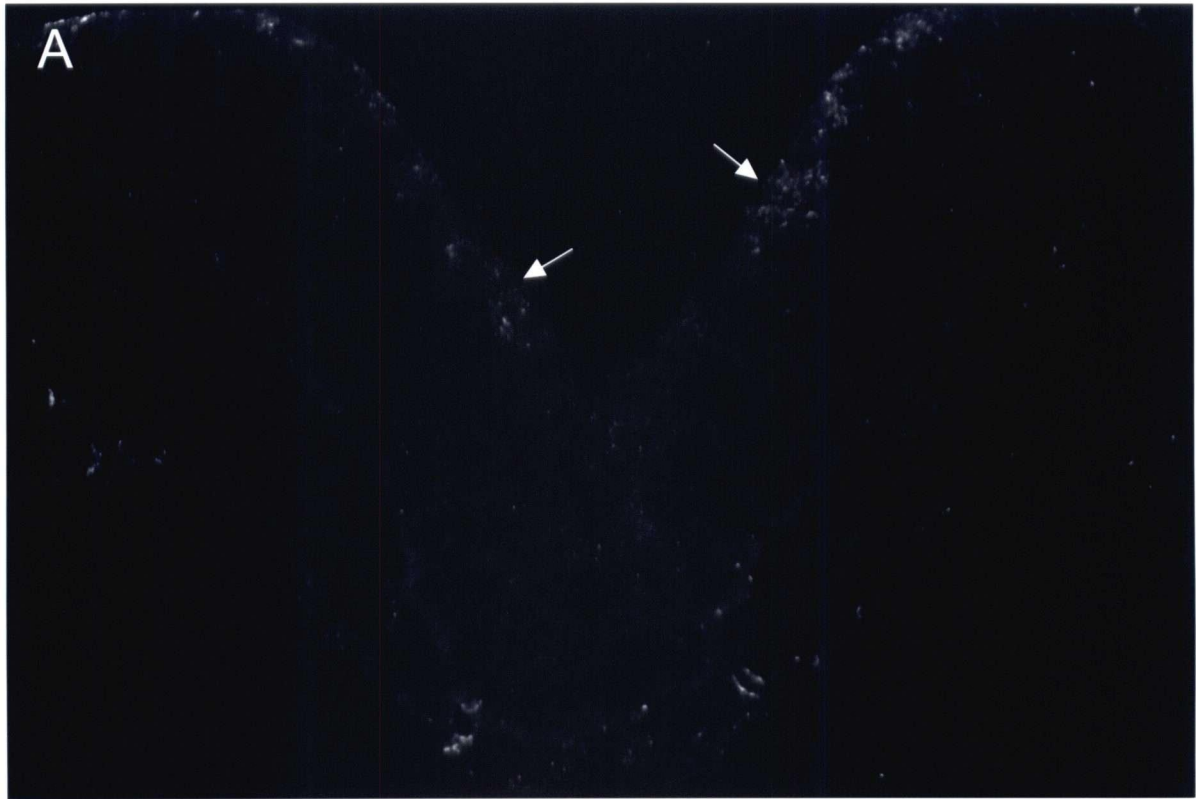
Figure 13.   Immunolocalization of α_5 integrin subunit during eye development in the chicken embryo.

(A) Stage 9 embryo, right eye facing right. Intense staining along the basement membrane and in the mesenchyme. Note the punctate vesicular staining within the NE (arrowheads). (B) Stage 11 embryo, right optic vesicle facing top. Focal staining of basement membrane at stage 9 has been replaced by a clumped staining pattern at outer NE surface. Intracellular staining has increased, and pericellular staining can be seen. Immunofluorescence microscopy. 440X.
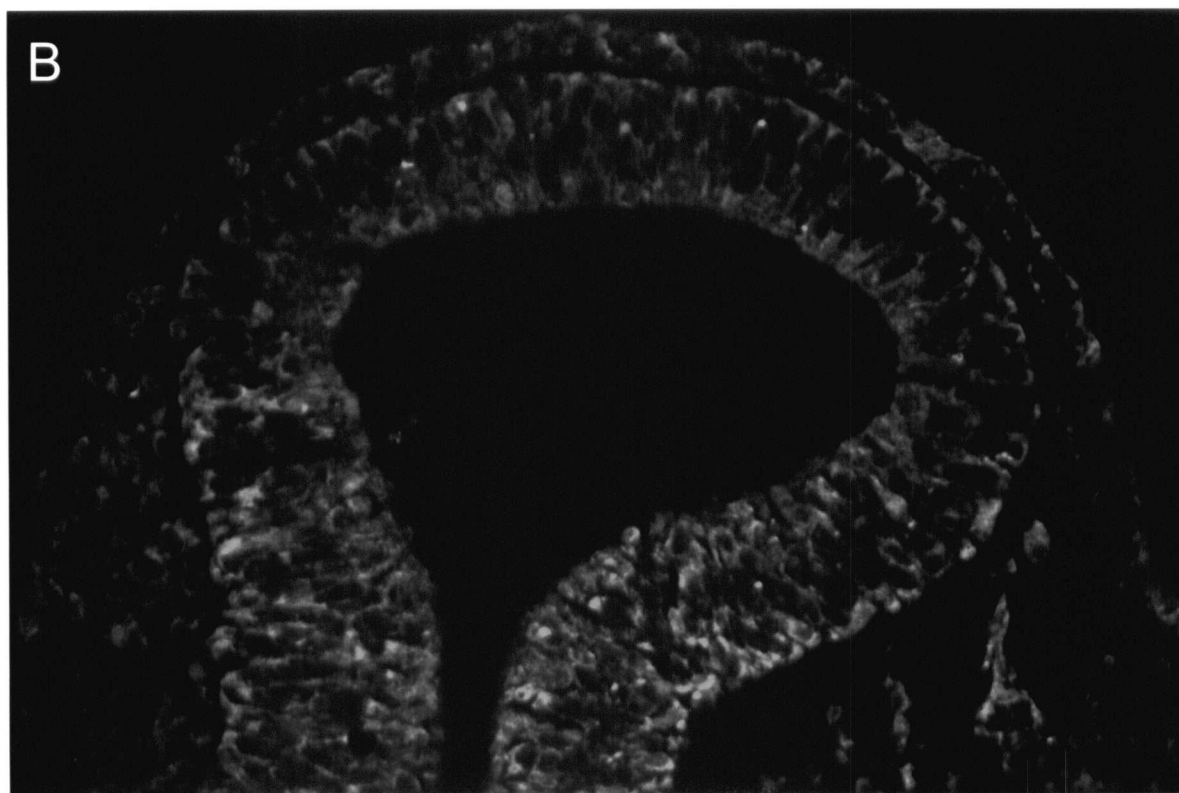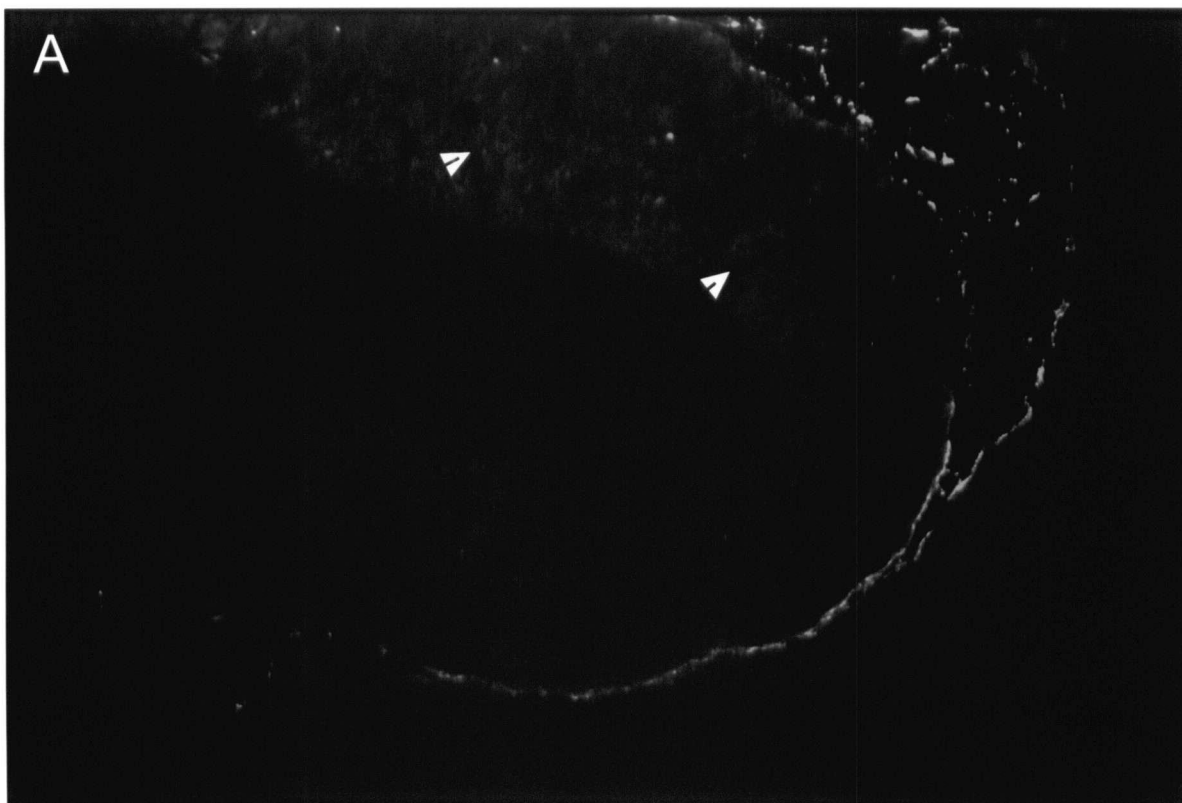
Figure 14.   Immunolocalization of α_5 integrin subunit during eye development in the chicken embryo.

(A) Stage 19 embryo, right eye facing top. Intense staining of lens fibres , some polarized apical and basal staining in RPE (asterisk).   (B) Stage 22 embryo, right eye facing top.   Intense staining of lens fibres and ganglion cells (arrows). Ganglion cell staining diminishes by stage 24.   Immunofluorescence microscopy. 440X.
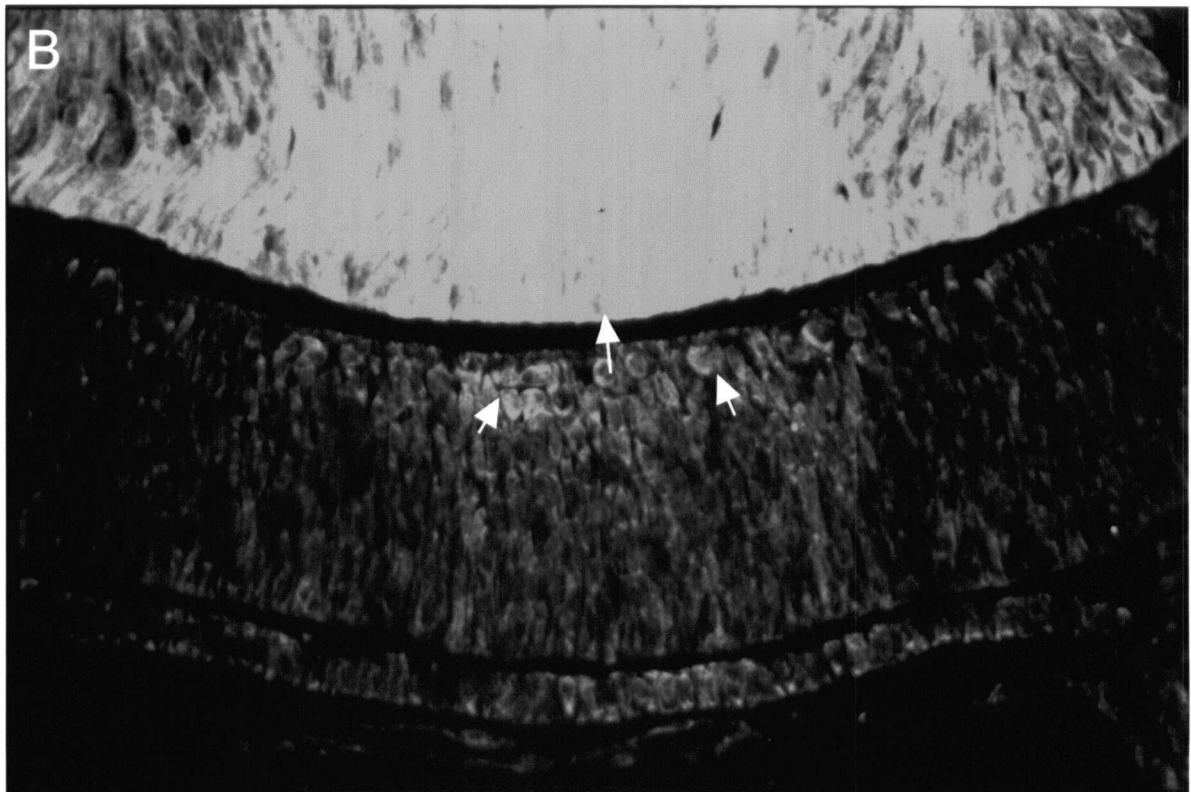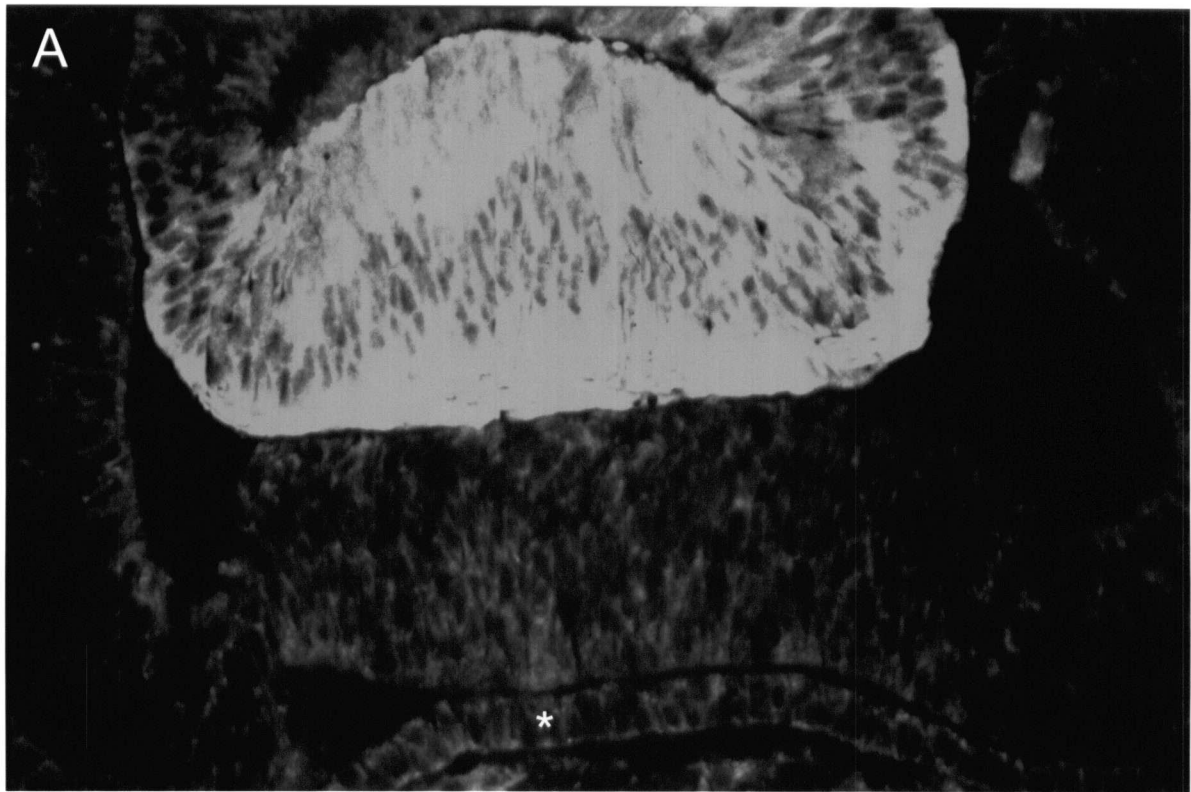
Figure 15.   Immunolocalization of α_v integrin subunit during eye development in the chicken embryo.

(A) Stage 8 embryo, neural tube facing top.   Scattered diffusely stained cells in NE. (B) Stage 14 embryo, right eye facing top. Large increase in staining intensity throughout all tissues. Note the more intense staining at outer NR and apical RPE surfaces (arrows). Immunofluorescence microscopy. 440X.
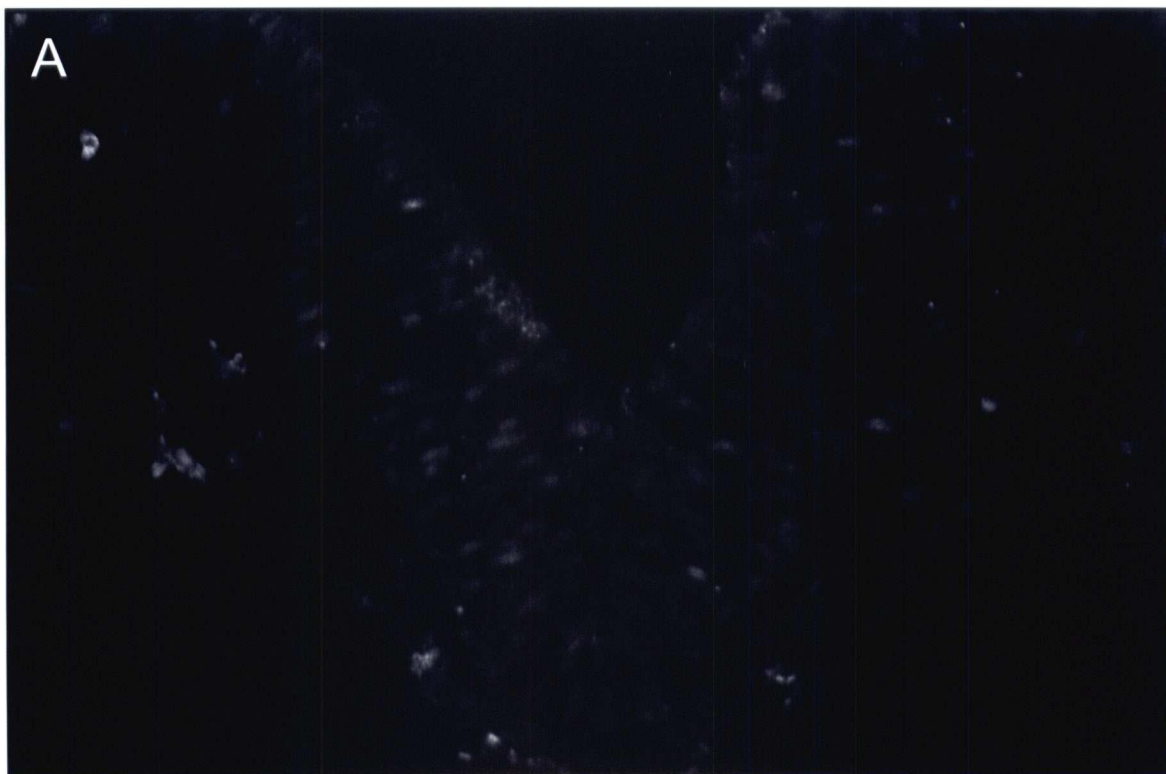
distribution. By stage 14 the corneal epithelium and lens epithelium were moderately stained with an area of strong fluorescence on the inner surface of the lens. The presumptive NR and RPE displayed moderate staining for α_v throughout at stage 14 with strong fluorescence on the outer NR surface and the apices of the RPE (Fig. 15b). By stage 16 the outer region of the NR and apical region of the RPE were strongly stained and by stage 19 a distinctive zipper-like distribution of α_v was seen at the interface between NR and RPE (Fig. 16a). The staining throughout the remainder of the NR and RPE remained diffuse and moderate. By stage 19 developing blood cells of the choroid stained intensely and the fluorescence persists through stage 24. From stage 20 onward the zipper-like pattern of α_v disappeared and the staining became more diffuse throughout the NR and RPE (Fig. 16b).

Alpha 6 Integrin Subunit

The distribution of the α_6 integrin subunit differed markedly from that of the other α subunits examined at stages 8 through 13. There was barely detectable expression of α_6 integrin until stage 14 when a trace to weak staining was seen throughout the presumptive NR except for the inner regions which stained moderately (Fig. 17a). In the lens epithelium and corneal epithelium staining remained weak to moderate from stages 8 through 24 but the distribution of fluorescence in the lens changed from ubiquitous to localized in the surface facing the NR. Between stages 14 to 16 there was a significant increase in α_6 immunoreactivity in the region of the developing inner limiting membrane which appeared as clumps (Fig. 17b). There was also increased staining in the apices of the RPE cells by stage 16 which persisted and intensified slightly through stage 19 (Figs. 18a, 18b). At stage 22
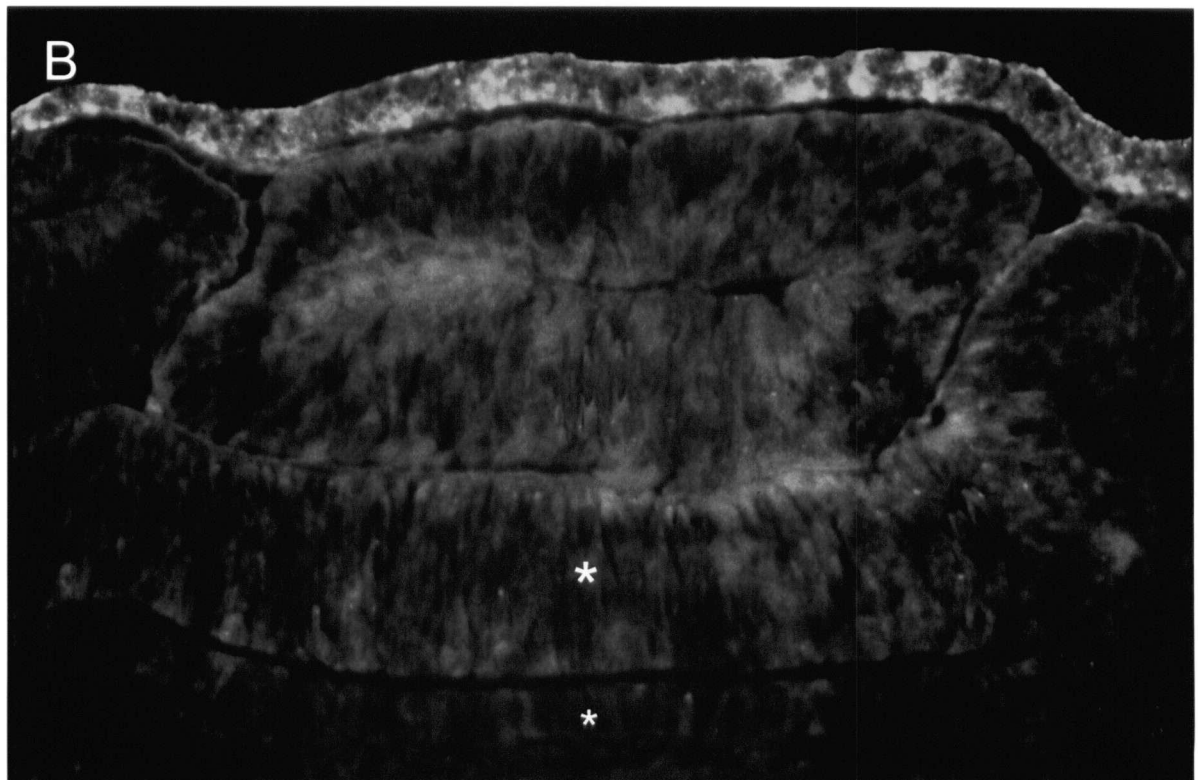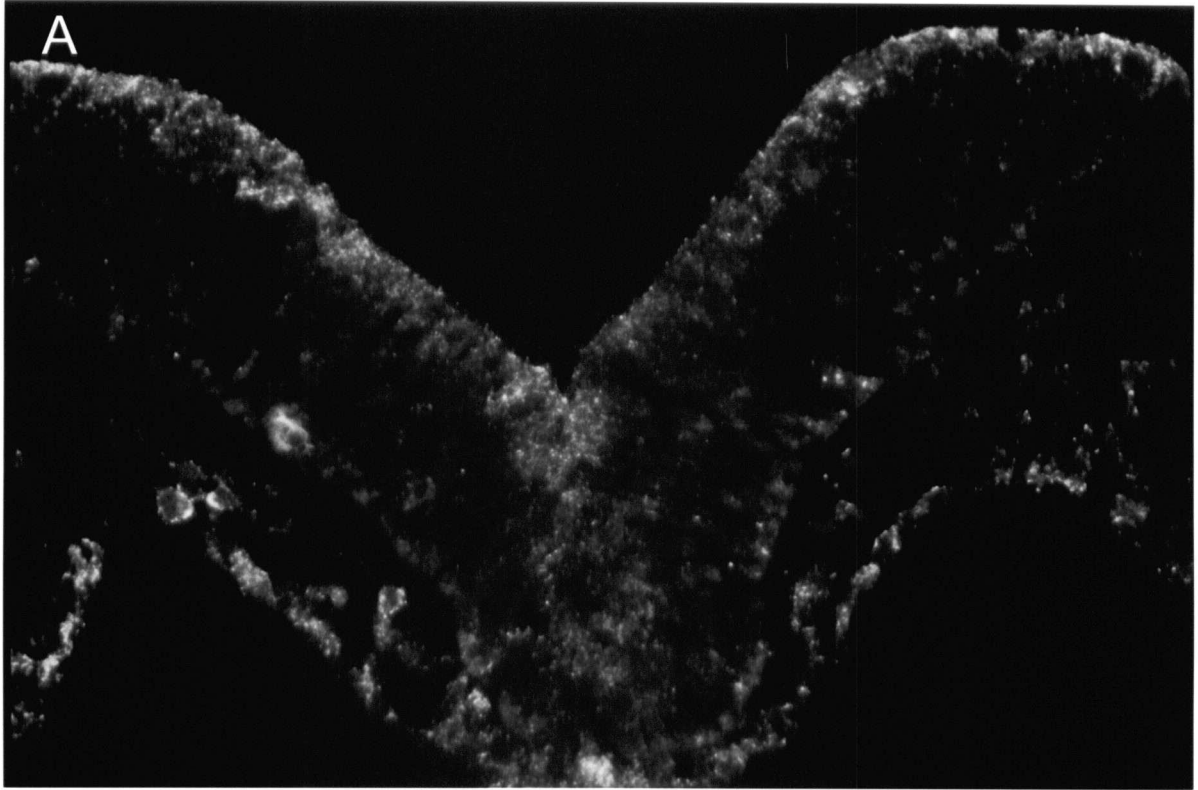
Figure 16.   Immunolocalization of α_v integrin subunit during eye development in the chicken embryo.

(A) Stage 19 embryo. Zipper-like intense staining at interface of NR and RPE. (B) Stage 24 embryo. Zipper-like staining pattern has been lost and replaced by diffuse, punctate staining in NR and RPE. Note intensely staining developing blood cells in the choriocapillaris. Immunofluorescence microscopy. 440X.
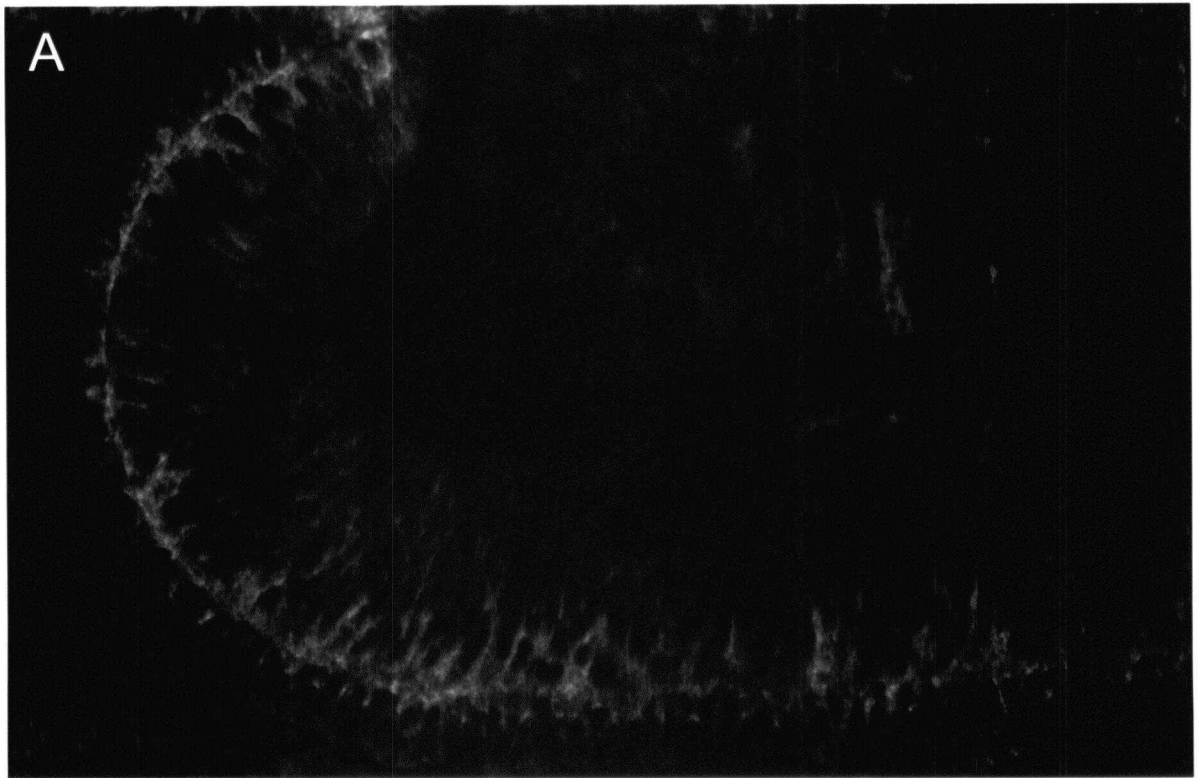
Figure 17.   Immunolocalization of α_6 integrin subunit during eye development in the chicken embryo.

(A) Stage 8 embryo, neural tube facing top. There was no visible staining prior to optic cup formation. (B) Stage 17 embryo, right eye facing top. Staining has increased particularly at the ILM (arrowheads) and surrounding the lens. The RPE has apical and basal staining (asterisk). Immunofluorescence microscopy. 440X.
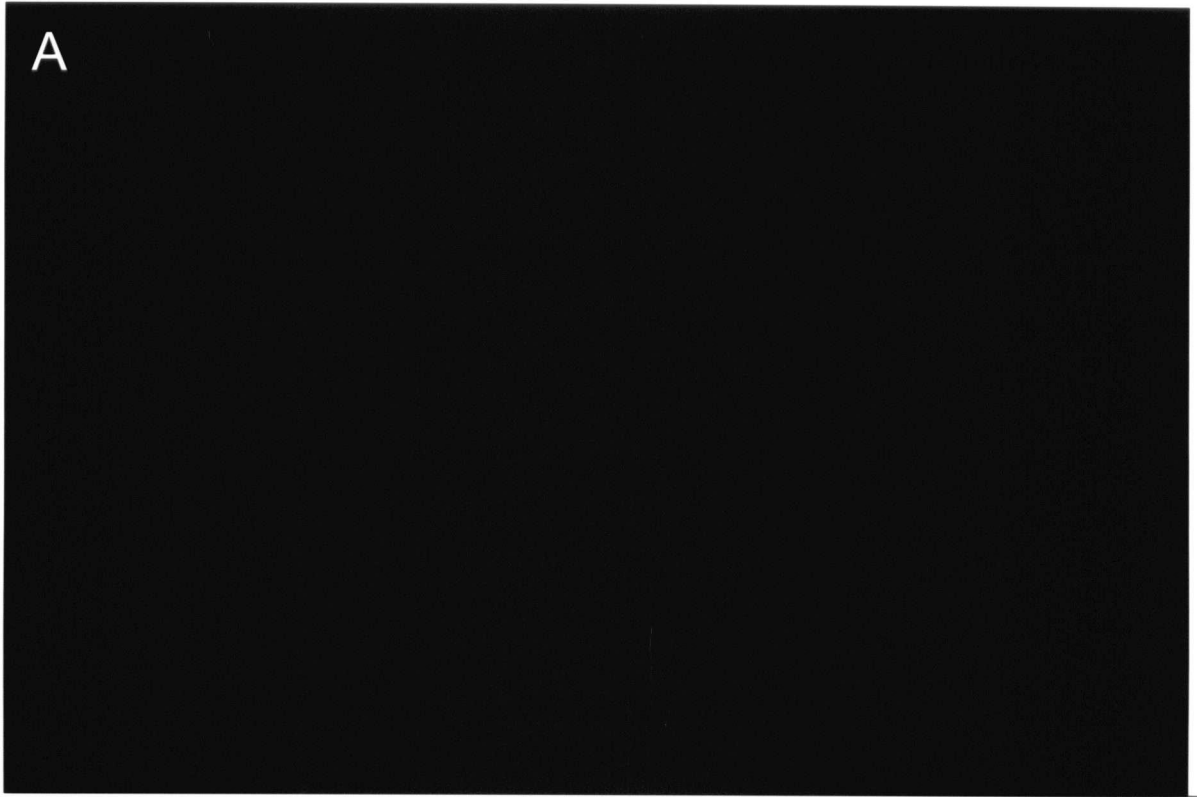
Figure 18.   Immunolocalization of α_6 integrin subunit during eye development in the chicken embryo.

(A) Stage 22 embryo, right eye facing top. Polarized apical and basal staining in RPE (asterisk). Ganglion cells in the inner NR are staining (arrows). (B) Stage 24 embryo, right eye facing top. Increased ganglion cell staining is observed, more intense staining is seen surrounding migrating neuroblasts. Immunofluorescence microscopy. 440X.

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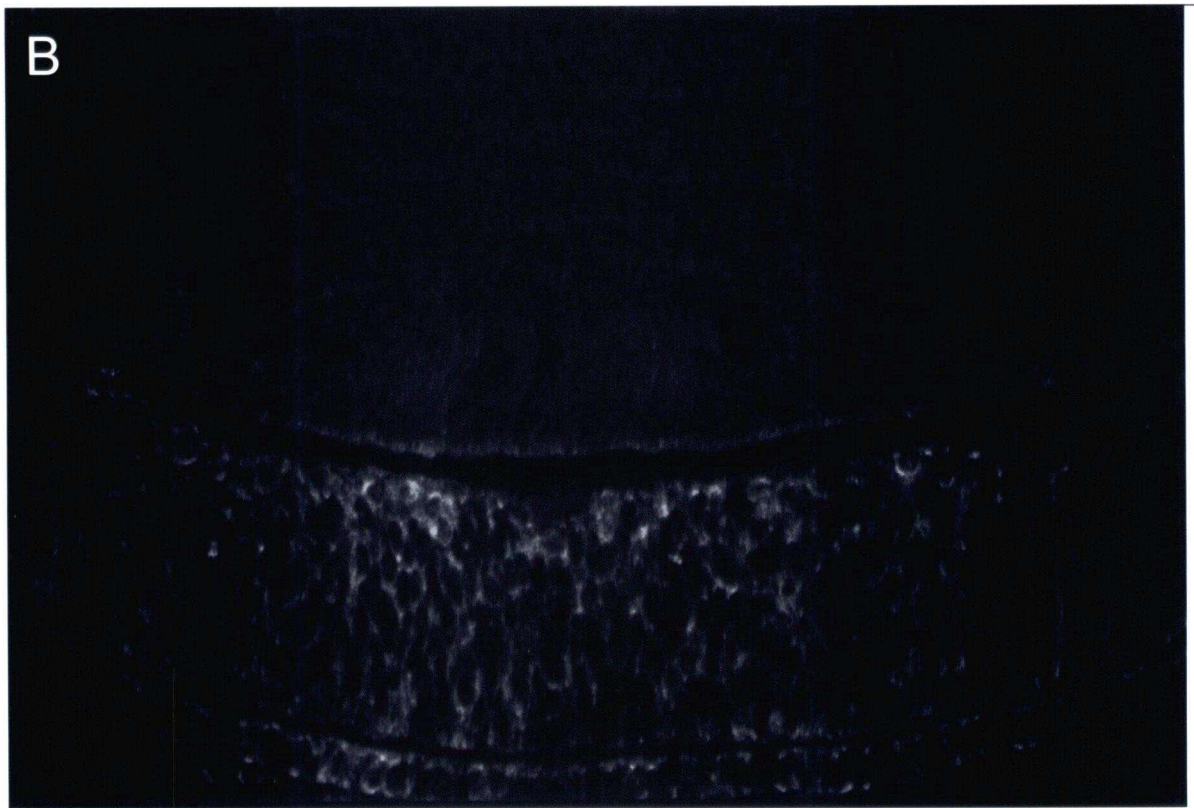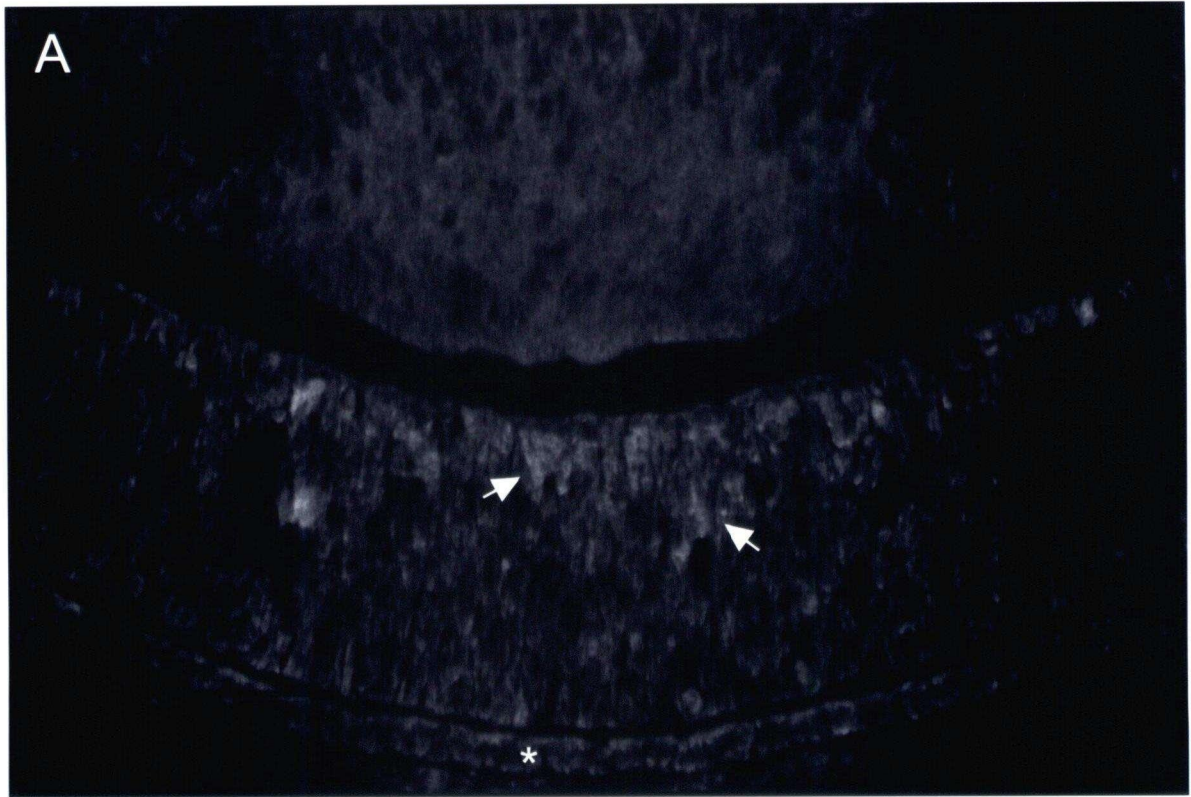
there are cells in the inner region of the NR, likely ganglion cells based on their morphology and distribution, which showed an increased expression which persisted to stage 24. The RPE at stage 19 showed a distinct apical and basal distribution of staining which intensified through stage 24.

Experimental Manipulation of Embryos *In Ovo*

The technique of injecting India ink diluted in Tyrode's saline through the vitelline membrane beneath embryos was employed to provide a black background upon which the translucent embryos could be observed and manipulated. Two types of non-toxic ink were tested. Koh-I-noor 9150-D ink (Germany) (diluted 300 μl in 10 ml of Tyrode's) resulted in poor survival (7/19, 37%) at 5 days incubation, probably due to preservatives such as sodium azide. A second ink solution (Pelikan; Hannover, Germany;India Drawing Ink) was used with the same concentration and diluent resulting in much better survival at 5 days (58/67, 87%).

Two techniques for administration of blocking agents into the optic vesicle *in ovo* were developed and assessed. The first of these employed heparin-coated acrylic beads of 75-100 μm in diameter which were implanted in early stage chicken embryos (Dr. Joy Richman, personal communication). The beads were implanted in the rostral neural tube at stage 8 and optic vesicles at stages 9, 10, and 11. The embryos were then allowed to develop to stage 24 whereupon they were dissected, visually assessed, and processed for sectioning in glycol methacrylate. The embryos were subsequently sectioned, stained with Toluidine blue O and evaluated by light microscopy. Surviving embryos showed no abnormal histology. Although the embryo survival was good (62/71, 87%), the beads tended to dislodge from the implanted

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site during subsequent development. Beads implanted in the neural tube became trapped in the developing head mesenchyme; those implanted in optic vesicles were extruded back into the developing brain ventricles as the vesicles constricted further during optic cup formation and the bead position was inconsistent. It was therefore necessary to investigate an alternate technique for delivery of blocking agents.

Microinjection had previously been used by Svennevik and Linser (1993) as a successful mode of delivery for peptides and antibodies in early chicken embryos. In order to determine if the injection procedure would be damaging to the embryos and to determine the degree of difficulty of the technique in terms of handling and targeting the injection site, a preliminary study was conducted. Approximately 50 nl Tyrode's saline was injected into the left optic vesicle of four stage 11 embryos; the right side optic vesicles were left untouched and served as a control. All injected embryos survived and developed normally to stage 24. Subsequent processing, sectioning, staining and observation by light microscopy showed normal histology in both eyes. Eight embryos were pierced through the left optic vesicle but not injected in order to determine if the injection procedure itself caused damage (sham injection). All 8 embryos survived and developed normally as assessed by light microscopic observation. Due to the apparent ease of performance compared to bead implantation, good survival rates and lack of perturbation of development, microinjection was adopted as the mode of delivery of peptides and antibodies in the present study.

Administration of blocking agents

Efficacy of microinjection technique

The initial series of injections were carried out by first breaking off the sealed tip of the micropipette against a sterile surface, then allowing the pipette to fill by capillary action. To estimate the volume per injection, 1 μl of Tyrode's saline was repeatedly drawn from, and the total number of injection aliquots per microlitre was counted. Out of 6 different pipettes tested, the average volume was worked out to be 20-35 nl. This estimate was based on filling the solutions to 1/3 of the distance from the tip to the sloped shoulder of the pipette. The volume was difficult to standardize due to the difference in tip break size and gauging the exact level of fluid in the micropipette. Furthermore, the micropipettes are not exactly identical probably due to physical variation in the glass structure. Since the broken tips could not penetrate the vitelline membrane, chorion, and amnion, it was necessary to pierce and cut the amnion before injecting into the embryo. The previously injected ink sometimes obscured the injection site, leading to uncertainty as to the efficacy of the injections. Early results of anti-β_1 and RGD injections were inconsistent, either resulting in normal development or death of the embryo. Survival rates were low ($<40\%$) perhaps due to the injected blocking agents in some cases, or damage incurred during surgery. In order to standardize the tip size and allow penetration into the optic vesicles through the unopened amnion, micropipette tips were bevelled. Injection through the amnion resulted in a markedly easier targeting of the optic vesicle since the optic vesicle remained more or less steady during the injection.

In order to evaluate the efficacy of the microinjection procedure and localize the

injected material, a series of embryos were injected with mouse anti-β_1 integrin subunit (CSAT) and the embryos were collected at 3 different times: immediately following injection (0 hours; n=17), 1 hour post injection (n=6), and 2 hours post injection (n=6). These times permitted evaluation of the diffusion rate of injected material following injection. The embryos were processed for cryomicrotomy as described above, then stained with Oregon Green 514-labelled goat anti-mouse IgG or Texas Red-X-tagged goat anti-mouse IgG. Embryos collected immediately following injection showed fluorescent staining in the injected optic vesicle in 47% (8/17) of specimens (Fig. 19a). In 18% (3/17) of embryos, fluorescence was observed immediately outside the vesicle between the ectoderm and NE with only small amounts within the vesicles (Fig. 19c). There was some leakage of injected material into the developing brain and the contralateral eye of all of the successfully targeted embryos. In embryos examined at 1 hour and 2 hour post-injection, the injected material diffused rapidly out of the injection site and was barely discernible.

Injection of Anti-β_1 Integrin Subunit

In the preceding parts of this investigation, the molecules associated with key morphological events were identified and a delivery technique was developed and assessed. The objective of the second part was to disrupt events mediated by β_1 integrins and determine the effect this had on NR and RPE differentiation. An antibody to β_1 integrin (CSAT) was administered by microinjection as described above into the optic vesicles of developing embryos at stage 11, prior to formation of the optic cup. Control embryos received injections of the following: a) the blocking antibody was omitted and replaced with monoclonal mouse

Figure 19.   Diffusion of injected antibodies in the chicken embryo.

Injected antibodies were detected with fluorescent-labelled goat anti-mouse antibody: 0 h after injection of (A) CSAT and fluorescently-labelled goat anti-mouse IgG showing fluorescence in the injected eye (right) with leakage to the ventricle and contralateral eye. (B) phase contrast photo of (A). (C) Zero hours after microinjection of CSAT and fluorescently-labelled goat anti-mouse IgG showing leakage outside the optic vesicles (arrow).   (D) phase contrast photograph of (C).
A, C.   Immunofluorescence microscopy. 240X.
B, D.   Phase contrast microscopy. 240X

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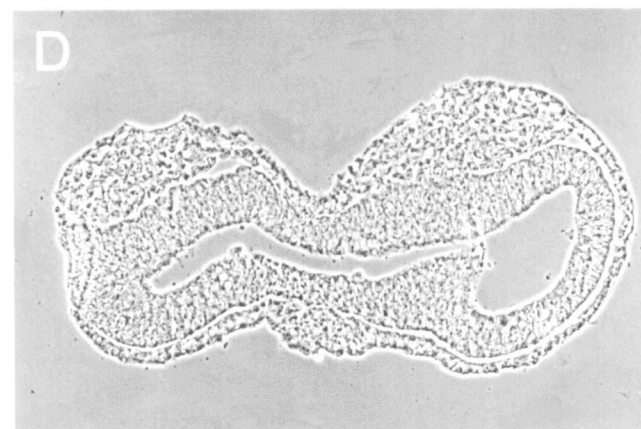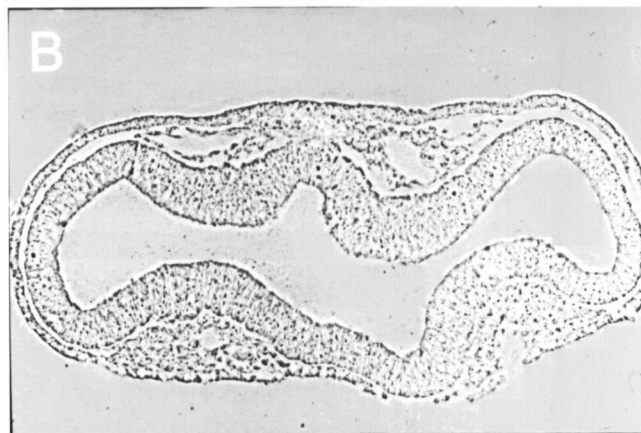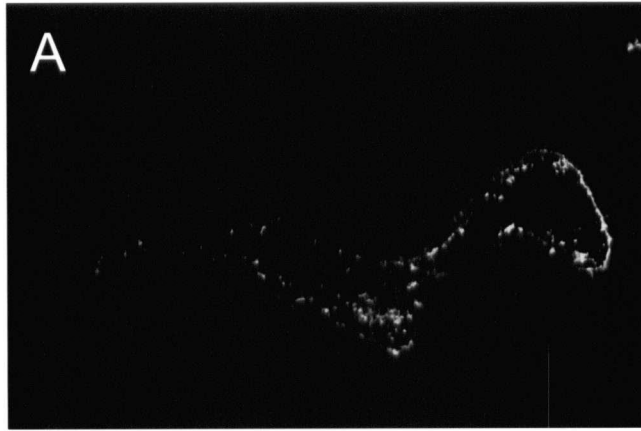
anti-rabbit IgG, an irrelevant antibody; b) the blocking antibody was omitted and replaced with the same volume of Tyrode's saline. The doses were 0.5 ng/nl and 5 ng/nl for both antibodies. Embryos were allowed to continue development and were collected at stage 24, by which stage the NR and RPE have differentiated and can be distinguished. Embryos were processed for light microscopy as described above to assess the morphological changes that occurred as opposed to control embryos.

The 5 ng/nl injections resulted in a survival rate of 33% (2/6) for β_1 integrin and 100% (2/2) for IgG. The 0.5 ng/nl injections resulted in a survival rate of 80% (4/5) for β_1 integrin and 100% (2/2) for IgG. Injections consisting of Tyrode's saline alone resulted in a survival rate of 60% (6/10). The surviving embryos, harvested at stage 24 were sectioned and stained with Toluidine blue O and found to be grossly and histologically normal.

Injection of Peptides

The purpose of this experiment was to examine the effects of blocking integrin interactions with ECM components by using RGD peptide, a recognition sequence for fibronectin and laminin, on NR and RPE differentiation *in ovo*. RGD and RGES peptides were dissolved in sterile Tyrode's saline to the appropriate concentrations, then microinjected into the optic vesicles at stage 11. RGES peptide was used as a negative control since it is a small, similar peptide to RGD in terms of size and charge, and has not been shown to be involved with interactions of laminin and fibronectin. The doses of peptides were 10 ng/nl, and 100 ng/nl. Following injection, embryos allowed to develop to stage 24 and were processed for light microscopy as described above.

Injections of RGD at 100 ng/nl and 10 ng/nl were either fatal or resulted in morphologically normal embryos. In embryos that did not survive beyond stage 13 or 14 there was also no development of blood vessels surrounding the embryo as assessed by visual inspection *in ovo*. Preliminary light microscopic evaluation of normal-appearing embryos revealed normal histology in the developing eye of the embryos which survived to stage 24. The embryos that died did so consistently at stages 12-14 and showed significant disintegration due to autoloysis when collected; they were unsuitable for light microscopic examination. Of the embryos injected with RGD at 100 ng/nl 33% (11/33) survived and were grossly normal. At doses of 10 ng/nl of RGD a similar pattern was observed of either normal development or death with 47% (27/58) injected embryos surviving. The RGES control injections at 100 ng/nl and 10 ng/nl resulted in survival rates of 50% (10/20) and 57% (17/30), respectively. In sham injections where Tyrode's saline was injected, a survival rate of 60% (12/20) was seen.

DISCUSSION

Technical Considerations

Manipulation of Embryos

The technique of injecting India ink *in ovo* through the vitelline membrane into the space underlying the embryo was employed to facilitate viewing the embryos during manipulation. The embryos are translucent and therefore difficult to see and virtually impossible to stage against the yellow colour of the yolk. The contrast provided by the ink allowed for accurate staging, better localization of injections into the optic vesicles, and easier viewing of the earliest stage embryos when harvesting. The ink subsequently diffused away from the embryo as development ensued. The India ink chosen was tested in experiments in our laboratory and in the laboratory of Dr. Joy Richman to determine its toxicity to the embryos. In a study in our laboratory the Pelikan India Drawing ink chosen was compared to Koh-I-noor 9150-D non-toxic India ink (the commercial replacement of the Pelikan ink). Stage 8 embryos were exposed to diluted ink injections surrounding the embryo and incubated to 5 days. Since the experiments were done *in ovo* with the intention of causing as little trauma as possible to the embryos it was necessary to use an isotonic, physiological diluent. An initial study using Hank's solution as a diluent for Pelikan ink resulted in a survival rate of 34% (20/59). The same concentration of the Pelikan ink diluted with Tyrode's saline showed a survival rate of 87% (58/67). The Koh-I-noor ink was subsequently tested diluted in Tyrode's and resulted in a survival rate of 37% (7/19). Despite the fact that both inks were marketed as non-toxic, there was a significant difference in embryo survival upon exposure

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from stages 8 through 25. The results point to the need to test toxicity *in ovo* of both ink and diluent prior to selecting injectable inks since the manufacturer's toxicity data may not necessarily apply to all animal models.

Fixation and Processing

The choice of Bouin's fixative was based on the nature of its reactivity with tissue macromolecules associated with immunoreactivity. The fixative ingredients in Bouin's are picric acid, acetic acid, and formaldehyde. Buffered formaldehyde was not recommended as a fixative by the manufacturers of the antibodies used in the subsequent study as it was said to destroy immunoreactivity. Bouin's, on the other hand, is known to give good to excellent immunoreactivity with numerous antibodies (Elias, 1990).

Immunoreactivity depends on a series of molecular interactions between the antibody and its epitope. The successful binding of antibody and epitope requires close association followed by attractive forces which contribute to the precise "lock and key" binding pocket. Antibodies in an isotonic solution of appropriate ionic strength can gain intimate contact with their epitopes but require specific chemical configurations to bind. The first long distance attractive force is electrostatic force between oppositely-charged amino acid side chains on both the antibody and epitope (Jefferis and Deverill, 1991). The ionic attraction brings the antibody in closer contact with the epitope where other attractive forces such as hydrogen bonding, hydrophobic interactions, and van der Waals forces can take effect once water is extruded from the binding site. The success of binding, therefore, is directly related to the number of ionic charges available on the epitope. The amino groups found on lysyl and

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guanidyl residues, and amino terminals on proteins are the sources of positive charges in tissue (Kiernan, 1990). These same groups are covalently crosslinked by aldehyde fixatives and are therefore no longer available for detection with ionic dyes or antibodies which bear negatively charged groups in the areas associated with the binding pocket. Presumably, the failure of some antibodies to react with aldehyde-fixed tissues is associated with the amino group blockade resulting from this mode of fixation.

Bouin's fixative contains picric acid which rapidly binds ionically in a salt linkage to the amino groups in tissue. The formaldehyde in Bouin's fixative cannot bind to amino groups which are already occupied by picric acid, nor can it bind covalently to protonated amino groups induced by the low pH of the fixative. The amino group blockade by picric acid is reversible; the ionic linkage between picric acid and tissue is easily disrupted by raising the pH of environment of the tissue to approximately neutrality where the picric acid is replaced by hydroxyl ions in an ion exchange phenomenon. Furthermore, the short fixation time employed with Bouin's in this study (15 minutes) was not sufficient to cause extensive damage to the epitopes by the acidic nature of the fixative. Presumably, the short exposure was sufficient enough to destroy endogenous hydrolytic enzyme activity within the tissues, thus preventing autolysis and diffusion of cellular components. Since the amino group blockade was reversible, there was no significant chemical alteration of the reactive groups in the epitopes. As a result, the preservation of immunoreactivity was achieved for all antibodies employed.

The choice of gelatin processing of the embryos as described above followed by cryosectioning was necessary due to the virtually shapeless, mucoid nature of the embryos as they were collected from the egg. The texture as well as the extremely small size of the

embryos made the crucial task of orienting and serially sectioning through the eyes very difficult by traditional cryoprocessing with OCT. The advantage of the gelatin processing was that the gelatin provided some firmness to the embryos so that they could be manipulated more easily. The infiltration of the gelatin also made the embryos lie flat so that they could easily be subsequently re-embedded before freezing so that the head was in the proper orientation for serial eye sections to be taken. The gelatin processing also provided remarkably good morphology compared to embryos frozen immediately in OCT. The OCT-frozen embryos showed poor morphology, with badly damaged cells, numerous cracks in epithelial structures, and distortion of hollow structures. The gelatin processed embryos, conversely, showed morphology approximating that of paraffin-processed tissue. The cryoprotection afforded by adding sucrose to both the infiltration buffer and gelatin resulted in the ability to re-freeze the sections without causing freeze-thaw artefact often seen in tissues that have been re-frozen for storage.

Controls for Immunohistochemistry

The small size of the embryos made it possible to mount up to 5 different embryos at different stages on a single slide. A series of sections were then cut and consecutive stages were placed on each slide which was useful as a "within run control" since the morphology and variety of structures at different stages provided useful information about specificity and positive control sites. The step of de-lipidizing the tissue by treatment with absolute methanol and acetone, each at -20°C, was added to the staining protocol to eliminate background staining due to hydrophobic interactions between the antibodies and intact cell membranes.

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The treatment also facilitated entry of the antibodies through intact membranes to cytoplasmic target sites. The methanol-acetone treatment was augmented by the addition of Triton X-100 to staining reagents and buffer rinses. These extra steps eliminated spurious staining in negative control sections and enhanced the immunoreactivity of integrin subunits.

The controls employed were designed to provide positive and negative staining information, and provide within run, and run-to-run control. The technique of omitting primary antibody and replacing it with non-immune serum is a standard negative control in immunochemistry. The secondary fluorescent antibody is subsequently applied, thereby providing a control which can provide information on autofluorescence of the tissue, non-specific binding of the secondary antibody, and a measure of specificity of the primary antibody which is run on a serial section. The positive control tissue and within run control was achieved by having multiple embryos at different stages mounted on the same slide. As the morphology of the embryos changes during development and new structures arise the variety of positive tissue sites change from stage to stage. This provides a good positive control since the distribution of positive sites changes in a fashion that becomes predictable from one staining run to the next. This was crucial to the study since it would have been impossible to stain all sections of all embryos at the same time. The run-to-run control was reinforced by overlapping stages from run to run so that a measure of consistency could be maintained. Lastly, the staining runs consisted of several different antibodies applied to serial slides. This enabled information about specificity to be obtained since the changes in staining patterns from different antibodies on serial sections was obvious.

Spatial and Temporal Distribution of NCAM During Eye Development

NCAM is a neuronal marker which is expressed at the neural plate stage of embryonic development. It has been used in previous studies as a marker for neuronal precursors and NR (Pittack et al 1997; Opas and Dziak,1994; Rizzolo et al, 1994). The intense immunoreactivity seen in the non-neural developing cornea and lens at stages 13-17 is consistent with previous studies that have reported NCAM expression in non-neural tissues (Tosney et al, 1986). The intensity of NCAM staining increased to stage 13 prior to the invagination of the optic vesicles to form the optic cup. The NCAM expression then decreased in both the presumptive NR and presumptive RPE at stage 14 after the optic cup has formed. This is an interesting finding since it occurs immediately preceding events marking the differentiation of the RPE. Within 8 hours of optic cup formation the presumptive RPE begins to produce premelanosomes and undergoes a marked decrease in DNA synthesis and mitotic activity (Ide, 1972; Zimmerman, 1975; Stroeva and Mitashov, 1983). These events correspond to the disappearance of NCAM expression in the RPE. Conversely, the presumptive NR undergoes a marked increase in NCAM expression, particularly in the central areas, from stage 15 onward. Presumably this marks the beginning of NR differentiation although the first neurons to differentiate, the ganglion cells, will do so 24 hours later. While the lens epithelium continued to stain intensely through stage 24 the lens fibres and corneal epithelium diminished to weak staining by stage 21. As in the case of the RPE, this appears to coincide with terminal differentiation.

Spatial and Temporal Distribution of ECM Molecules During Eye Development

Laminin

Laminin was present at stage 8 in the developing basement membrane of the NE and in cells producing laminin in the mesoderm and within the NE. The distribution of laminin has been described in the chicken from stage 10 onward (Hilfer and Randolph, 1993) but has thus far not been reported at stage 8. The distribution of laminin remained confined to developing basement membranes and in a few scattered mesenchymal cells throughout stage 24. The laminin membrane upon which the NE resided at stage 8 remained intact through the evagination of the optic vesicles at stages 9 to 12, then extended to completely surround the optic vesicle by stages 13-14 when invagination begins. Throughout the formation of the optic cup the laminin membrane remains intact in the basement membrane of the presumptive RPE and the presumptive inner limiting membrane (ILM) which derives from the membrane of the outer, lateral presumptive NR. At no point during stages 8 through 24 did the laminin membrane surrounding the presumptive retinal components appear discontinuous. Perhaps the flexible laminin sheet beneath developing NE, then later RPE and NR cells can provide the minimal attachment required for cell division and migration without the more rigid structure provided by a full basement membrane. Collagen IV is present at stage 10 in the embryonic chicken eye and is mainly located along developing basement membranes of the NE and surface ectoderm (Hilfer and Randolph, 1993). Following invagination of the optic cup, collagen IV is present in the presumptive ILM and the basement membrane of the presumptive RPE. Presumably the collagen of the basal laminae would have to be digested

to accommodate remodelling associated with the shape changes that must take place to form the optic cup. The laminin sheet may provide a porous medium through which metalloproteases such as collagenase can be secreted to mediate shape changes. Once the optic cup is established the laminin in the basement membranes of the corneal, lens, and RPE appeared to thicken, coinciding with differentiation to the epithelial phenotype. The ILM which serves as the basement membrane for Müller cells and a scaffold for neurite extension from retinal ganglion cells remained strongly positive throughout the stages following optic cup formation.

Fibronectin

Fibronectin was present in developing basement membranes of the NE, surface ectoderm and later corneal epithelium, lens epithelium, RPE, and NR from stage 8 onward to stage 24. In this respect, the results of the present study concur with those of previous studies (Kurkinen et al., 1979; Turksen et al., 1985; Hilfer and Randolph, 1993). As in the case of laminin, fibronectin in chicken embryos has not been reported for stages earlier than stage 10. The investigators in the previous studies where fibronectin distribution was assessed by immunohistochemistry restricted the stages studied in their experiments to stages 10 to 15 (Hilfer and Randolf, 1993), stages 11-16 (Kurkinen et al., 1979), and 4 days (stage 24) to 17 days (Turksen et al., 1985). Consequently, the distribution of fibronectin in embryos at stages 8 - 9, and stages 17 - 23 has also not been previously described. Unlike laminin, fibronectin is expressed within cells of the NE, NR, and RPE in addition to mesenchymal cells. The results

of the present study differ from those published previously in that the earlier studies did not mention intracellular or pericellular staining, only staining of basement membranes. The presence of fibronectin in and surrounding cells raises the possibility that there is interaction between fibronectin and integrin receptors at these sites. The trace amount of fibronectin staining within the NE of stages 8 through 13 appeared to increase to a moderate intensity by the formation of the optic cup at stage 14. The staining within the RPE is polarized to the apices of the RPE cells and along the basal lamina. This finding is consistent with a number of alpha integrin subunits similarly distributed and clusters of Na^+/H^+ antiporter activity reported by others (Ingber et al., 1990; Rizzolo, 1991; Schwartz et al., 1991) at later stages. The presence of intracellular and pericellular fibronectin was consistent in the present study throughout all stages. This finding was reproducible and did not represent non-specific background staining. The negative controls for fibronectin consistently showed no immunoreactivity in all staining runs.

Fibronectin provides a framework upon which migrating cells can move and also contains fragments which can bind α_3, α_4, α_5, and α_v integrin subunits. The binding of integrins can induce signal transduction directly in the cells bound, or can potentiate the cellular response to growth factors or mitogens (Ingber, 1991; Damsky and Werb, 1992; Schwartz et al., 1992; Humphries et al., 1993). The continued presence of fibronectin within and surrounding NE, NR, and RPE cells co-expressed with a variety of integrins throughout stages 8 to 24 would seem to indicate a major role for fibronectin in retinal development. At stages 21-23 there was intense immunoreactivity for fibronectin in cells of the NR which, on the basis of their morphology and location, are probably differentiating ganglion cells. They are distributed at the inner surface of the NR in close proximity to the fibronectin-rich ILM. The accumulation of fibronectin within

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these cells may possibly anticipate secretion of the fibronectin into the extracellular space during the differentiation of these cells. By stage 24 the fibronectin staining within the ganglion cells decreased while the extracellular level of fibronectin appeared to increase. The axons extending from these cells at this point in development may contact the fibronectin and laminin in the ILM which could provide further guidance as the fibres extend to form the optic nerve.

Spatial and Temporal Distribution of Integrin Subunits During Eye Development

Beta 1 Integrin Subunit

At stages 8 to 10, β_1 integrins were expressed in close proximity with fibronectin in the developing basement membrane of the NE, and in numerous sites throughout the mesenchyme. There was also a small amount of fibronectin expressed within the NE where there was abundant expression of β_1 integrin along the cell membranes. At stages 11 to 13 there was a virtually identical distribution of fibronectin and β_1 integrin along the inner surface of the NE of the optic vesicle, which represents the presumptive cleft between the apical RPE and outer NR at later stages. From stage 15 to 24 there was a steady increase in β_1 integrin staining in the NR, RPE and in the lens fibres. At stage 24 the outer NR showed intense foci of staining surrounding neuroblasts in the outer region, while the cells of the adjacent RPE showed intense staining on their basal, lateral and apical margins. The close relationship of the intensely stained adjacent cells may be an indication of cell-cell communication occurring between the differentiating neurons of the NR with the largely differentiated RPE. There was also β_1 integrin staining concentrated along the ILM, suggesting ligand binding by at least one of the possible β_1

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integrins with either laminin or fibronectin. The intense staining within the lens fibres disappears as the fibres elongate, a pattern which was also seen with fibronectin and α_5 integrin.

The findings in the present study have covered the period of ocular development from E2 (stage 8) to E4 (stage 24) which have thus far not been published. Earlier studies reported the distribution of β_1 integrin in chicken eye as of day 7 which was considered an early stage of eye development by other investigators (Philip and Nachmias, 1987; Rizzolo and Heiges, 1991; Rizzolo et al., 1994). Rizzolo and co-workers (1994) described the distribution of β_1 integrin on the apical, basal, and lateral RPE cell margins at E7 and basolateral at E11. A study by Philip and Nachmias (1987) indicated that by E16 the β_1 integrin distribution was confined to the basal regions of the RPE cells and remained so throughout the late embryonic stages and in the mature hatched chick. The decline in the distribution of β_1 integrins in developing retina was also reported by Cann and co-workers (1996) who described an 80% drop in mRNA levels of β_1 integrin from E6 to E12. Thus, β_1 integrin appears to be involved in matrix-cell and, based on its staining distribution, possibly cell -cell interactions from at least stage 8 through to E16, with a maximal expression in developing retina from approximately stage 22 on E3 to E6.

The ubiquitous distribution of β_1 integrins in the developing eye throughout the stages studied is likely to be a reflection of the numerous possible ligands with which these molecules can bind. Several different β_1 integrins are known to bind fibronectin and laminin It has been reported that $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_v\beta_1$ bind fibronectin while $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_7\beta_1$ bind laminin. In addition to the above integrins, fibronectin and laminin are known to be ligands for β_3, β_4, β_6, and β_7 integrins (Elner nd Elner, 1996; Hillis and MacLeod, 1996).

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Alpha Integrin Subunits

The distribution of the α subunits in the developing chicken eye is not well documented. Previous studies report α_6 integrin at stages 14 and beyond (Bronner-Fraser et al., 1992), α_3, α_6, and α_8 at E6 (de Curtis and Reichardt, 1993), α_3 α_5 α_6 and α_v at E7 and E14 (Philip and Menko, 1993; Rizzolo et al., 1994) and α_2, α_4, α_6, and α_8 from E6-E15 (Cann et al., 1996). With the exception of the Bronner-Fraser study (1992) whose focus was on α_6 integrin distribution in the entire chicken embryo, the above mentioned studies investigated NR and RPE during later differentiation when the NR and RPE have acquired different morphology and function. The RPE is differentiated at later stages and is involved in ion transport, support for developing photoreceptors, and has tight junctions which provide a major portion of the blood-retina barrier. The NR at the stages investigated in previous studies consists of a collection of developing neurons organized in distinctive layers as well as glial cells and their associated processes.

There have been no reports to date of the α integrin subunits in stage 8 (E2) to stage 24 (E4) chicken retina with the exception of α_6 at the optic cup stage. The present study examined the NE, and its development through the optic cup formation then through the earliest stages of differentiation prior to the formation of identifiable differentiated cell types. The alpha integrins studied included α_3, α_4, α_5, α_6, and α_v, all of which are known to dimerize with the β_1 integrin subunit.

Alpha 3 Integrin Subunit

The α_3 integrin subunit has only been identified in a heterodimer with the β_1 subunit

(Schwartz and Ingber, 1994; Hillis and MacLeod, 1996; Elner and Elner, 1996). The distribution of α_3 integrin subunit at stages 8 to 10 was diffuse, intracellular, vesicular, and punctate within the NE. This finding contrasts with the pericellular distribution of the β_1 integrin subunit at the same stages. In the absence of any previous reference material it is difficult to interpret upon this discrepancy, but perhaps an explanation may be that the α_3 integrin subunit is being synthesized but not expressed at this time. From stage 11 onward the α_3 integrin distribution matches that of β_1 integrin. The intense pericellular α_3 staining surrounding RPE cells at stages 17 to 24 corresponds well with the pattern of β_1 staining in the same area.

The $\alpha_3\beta_1$ integrin binds the RGD fragment of fibronectin, laminin and collagen I, all of which are present in the developing basement and Bruch's membranes underlying the RPE. Furthermore, the α_3 distribution corresponds to the fine web of fibronectin seen throughout the NR and at the apices of RPE cells at stages 17-24. Since no laminin was detected in the NR at these stages and collagen I is not known to exist in the NR, the most likely ligand in NR is fibronectin. There was a thin dense line of α_3 integrin staining immediately adjacent to the ILM at stages 19-24. In this region the α_3 integrin could be binding laminin or fibronectin, both of which are heavily expressed in the ILM. This finding has not been previously described.

Rizzolo and co-workers (1994) reported the α_3 integrin distribution at both E7 and E14 as being confined to the basal regions of the RPE cells. They postulated that the basal location of the α_3 integrin was an indication of its interaction with the collagen, laminin, and fibronectin of Bruch's membrane, which was thought to be the only location of these ligands near the RPE. Their findings contrast with both the α_3 and fibronectin distributions in the RPE as described in the present study. In the RPE at stages 17-24, the staining for α_3 was pericellular, and

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fibronectin was indeed found to be present within and at the apices of the RPE cells. The progression of α_3 integrin staining from a diffuse intracellular to a definitive pericellular pattern as seen in the corneal epithelium and RPE at these stages could possibly be associated with differentiation to the epithelial phenotype. While there were clearly some areas of the NR and mesenchyme showing weak to moderate staining, the abundant expression surrounding epithelial cells was striking. As differentiation proceeds, the basement membrane thickens as increasing amounts of laminin and collagen are laid down (Timpl and Brown, 1994). Given that the α_3 subunit has binding capability for both these ligands, perhaps the progressive clustering of α_3 integrin toward the basal aspect of RPE cells later in development represents signal integration between the α_3 integrins and ECM maintaining the epithelial phenotype.

Alpha 4 Integrin Subunit

The α_4 integrin subunit binds fibronectin and VCAM-1, and is known to be expressed in the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ dimers (Elner and Elner, 1996; Hillis and MacLeod, 1996). Unlike other fibronectin-binding α subunits such as α_3, α_5, and α_v which bind the RGD fragment of their ligands, α_4 binds the Leu-Asp-Val (LDV) fragment of its ligands (Cox et al., 1994; Elner and Elner, 1996).

The punctate clusters and intracellular vesicular staining seen within cells of the NE at stages 8 and 9 do not fit the pericellular staining pattern shown by β_1 integrin at the same stages. This may be an indication of synthesis and storage of the α_4 subunit or could possibly represent an integrin combination other than with β_1, such as $\alpha_4\beta_7$ or some yet undescribed dimer. The intense clustering of signal at the inner NE and ectodermal staining at stages 8 through 13 did

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not correlate with that of β_1 staining in the same areas. This could again be an indication that an integrin other than $\alpha_4\beta_1$ is present.

Fibronectin, a ligand for both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ is present along the surface ectoderm but not at the inner NE surface at stage 8. From stages 10 through 24 the α_4 distribution approximated that of β_1 integrin in the developing corneal epithelium, lens epithelium, and other ectodermal structures. There was no significant increase in α_4 staining in the presumptive RPE or presumptive NR until stage 17 when diffuse staining was apparent in the apices of the RPE, throughout the NR, particularly near the ILM, and surrounding the lens. The staining at the ILM appeared consistent with ligand binding in that the staining signal was dense and punctate. The intense, clustered staining noted at the base of the RPE and in the developing blood vessels of the choriocapillaris at stages 22 to 24 coincided with that of β_1 integrin in the same area, but was much more intense and concentrated. Again, this raises the possibility that the α_4 expression seen there is due to another dimer combination such as $\alpha_4\beta_7$ in addition to $\alpha_4\beta_1$. The staining also corresponded to the distribution of fibronectin in both the basement membranes of the RPE and choriocapillaris. The distribution of α_4 integrin suggests that it is involved in ectoderm-ECM interactions in the early stages (8-17) of retina development, but becomes associated with NR and RPE development only after RPE differentiation has been initiated.

Alpha 4 integrins have not been previously described at stages 8 to 24 in the chicken. Cann and co-workers (1996) described the distribution of α_4 integrin in E6 to E15 chicken embryos by quantifying its mRNA expression in NR. The α_4 mRNA levels in NR were reported to decrease slightly from E10 to E12, then increased steadily thereon to hatching. Expression of α_4 in the stage 22-24 NR in the present study showed an increase within migrating and

differentiating neuroblasts throughout the depth of the NR. Fibronectin and β_1 integrin were also present in the same region at that time, and the staining clusters of α_4 integrin seen may be an indication of fibronectin binding by the neuroblasts of the NR. When considered in conjunction with the results reported by Cann and co-workers (1996), it seems apparent that there is an overall increase in α_4 levels within the NR through to hatching. The steady increase in NR staining for α_4 throughout the post-optic cup stages of development may be associated with NR differentiation.

Alpha 5 Integrin Subunit

The distribution of α_5 was consistent with that of β_1 with a few notable exceptions. At stage 8 the distribution was vesicular and intracellular in the NE with intense clustering at the basement membrane. The intensity of the basement membrane staining for α_5 exceeded that of β_1 which did not cluster in the same fashion. The vesicular staining of the NE and diffuse intracellular staining for α_5 at stages 8 to 24 could indicate either production and storage of α_5, or the presence of another yet unidentified β subunit dimerized with α_5. By stage 11 the distribution corresponded more closely with that of β_1 at the same stage with clumping of signal on the inner and outer NE and surface ectoderm. The persistent intracellular staining throughout all of the stages studied appeared to be a feature of α_5 integrin. At later stages (14 to 20) the clumped staining pattern was noted in the apical and basal regions of the RPE and throughout the NR which could possibly indicate binding of α_5 integrin to fibronectin since fibronectin was present at those sites.

At stages 17 to 18 there was a large increase in α_5 staining in the lens fibres which

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corresponded exactly to that of β_1 integrin and fibronectin staining at the same site from stages 21-23. The staining intensity for both α_5 and β_1 decrease in the lens fibres as they began to elongate at stages 23 to 24, and the fibronectin staining at that time also diminished. The $\alpha_5\beta_1$ integrin is the only reported dimer containing the α_5 subunit, and fibronectin is the only known ligand for this receptor (Elner and Elner, 1996; Hillis and MacLeod, 1996). There is evidence for the $\alpha_5\beta_1$ dimer in the staining patterns observed, particularly in the NE of stages 10 to 13, RPE at stages 17 to 24, in the lens fibres and epithelium at stages 20 to 24, and in the outer NR at stages 17 to 24. As is the case with α_3 and α_4 integrins, however, there is a possibility that some of the immunostaining reported may be due to α_5 dimerized with another β subunit.

Rizzolo and co-workers (1994) reported that α_5 integrin was not present in the chicken RPE at either E7 or E14. Alpha 5 integrin staining has not been reported in chicken NR, but is present in trace amounts adult human NR in the outer limiting membrane and inner nuclear layer (Brem et al., 1994). The disappearance of α_5 integrin from RPE and lens fibres as differentiation progresses may point to α_5 integrin as a mediator of early developmental events in the eye, in contrast to α_3 and α_4 integrins.

Another interesting finding was the intracellular staining of large, round cells of neuronal appearance located near the inner surface of the NR at stages 22 and 23. These cells are likely differentiating retinal ganglion cells which appear to be producing α_5 integrin. There was concomitant intracellular staining for fibronectin in the same cell population, and α_5 integrin staining along the ILM where it had a clustered appearance. However, the β_1 distribution at stages 22 and 23 did not reveal any intracellular staining. This may be an indication of a β integrin subunit other than β_1, or the production of fibronectin and α within the same cell

population at stage 22 may indicate synthesis for later expression as the retinal ganglion cells extend their axons to the ILM.

Alpha v Integrin Subunit

The distribution of α_v subunit was not typical of the other alpha subunits examined in the present study. Whereas the stage 8 distribution of α_3, α_4, and α_5 subunits was vesicular within the cytoplasm of the NE, α_v revealed a diffuse intracellular pattern in scattered NE cells. In this respect, the α_v distribution does not correspond to that of β_1 integrin which has a pericellular distribution. This could be an indication of either α_v subunit production and storage within the NE cells for subsequent expression or may represent α_v expressed with another beta subunit such as β_3, β_5, β_6, or β_8. The α_v integrin subunit has been reported as a heterodimer in the $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ configurations (Elner and Elner, 1996; Hillis and MacLeod, 1996). The ligands with which $\alpha_v\beta_1$ binds are fibronectin and vitronectin (Elner and Elner, 1996; Hillis and MacLeod, 1996). The staining pattern seems to preclude fibronectin binding since there is no fibronectin distributed in a similar pattern within the NE cells and there did not appear to be any clustering of staining signal which could indicate ligand binding. Vitronectin has thus far not been described in NE at this early stage, but cannot be excluded as a possible ligand.

There was a change in the staining pattern of α_v integrin at stage 14 after formation of the optic cup. The staining intensity in the outer presumptive NR and the apical presumptive RPE was increased with respect to the surrounding tissues. The staining pattern persisted as the outer presumptive NR and apical presumptive RPE become closely apposed, and the staining intensity increased. By stage 17 a "zipper-like" staining pattern was beginning to form between the NR

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and RPE and became progressively more obvious through to stage 19. During this time, the staining intensity elsewhere in the NR and RPE diminished. The staining pattern for α_v integrin at this stage was also consistent with the heavy expression of β_1 integrin in the apices of the RPE cells and outer NR, possibly indicating an $\alpha_v\beta_1$ integrin dimer. Fibronectin is present in the apical RPE and outer NR at stages 17 to 19 and the heavily clustered staining pattern for α_v integrin at the junction of the two apposing tissues would seem to indicate ligand binding.

The continued scattered intracellular and extracellular staining in the NR and RPE for α_v at stages 17 to 19 and beyond in the absence of β could indicate the presence of α in combination with a β subunit other than β_1. The $\alpha_v\beta_3$ integrin has been described at stage 24 in the NR(Martinez-Morales, 1996; Yip et al., 1998) in association with neurite extension on vitronectin. The distribution of vitronectin in chicken embryos earlier than stage 24 has not been determined, and the presence of vitronectin or any of its integrin receptors cannot be ruled out in stage 17 to 24 chicken embryos. From stages 20 to 24 the zipper-like α_v staining pattern is lost and is replaced by a diffuse widespread signal throughout the NR and RPE with occasional vesicular staining in the outer NR. There is also a large increase in staining within the developing blood cells in the choriocapillaris which is not likely due to $\alpha_v\beta_1$ integrin since the β_1 integrin subunit is not associated with blood cells, nor was there any β_1 staining seen in blood cells within the choriocapillaris. The blood cell staining may be due to the $\alpha_v\beta_3$ integrin since it is associated with blood cells and vessels.

Martinez-Morales and co-workers (1996) reported the presence of vitronectin in the extracellular matrix throughout the NR of chicken at E5. Using western blot analysis these investigators showed an increase in vitronectin levels to a maximum at E9 followed by a

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decrease through E13. They also described the α_v and vitronectin distribution in the NR from E5 through E13. The vitronectin staining was seen throughout the NR in a dispersed pattern, on the ILM, and surrounding developing neuroblasts. The staining at E9 was mainly associated with retinal ganglion cells, the fiber layer, and the inner plexiform layer. The α_v distribution was reported from E4, corresponding to stage 24. The pattern of α_v mRNA expression at stage 24 was identical to the immunoreactivity reported for α_v in the present study. At later stages, the α_v distribution was seen in all layers of the differentiated NR, particularly in the ganglion cells and inner plexiform layer. On the basis of their results and the findings of others, particularly Neugebauer and Reichert (1991) who demonstrated neurite extension by retinal neurons cultured on vitronectin, they suggested that vitronectin supported proliferation and differentiation of neuroblasts and provided a substrate for neurite extension mediated by α_v integrin binding. They did not speculate on the nature of the beta subunits associated with α_v. The present study would seem to indicate that $\alpha_v\beta_1$ may be present since the staining patterns of α_v and β_1 show some overlap. The presence of $\alpha_v\beta_3$ can only be speculated, but is possible, particularly at later stages 20 to 24.

Alpha 6 Integrin Subunit

The only report of α_6 integrin distribution in early stage chicken embryos is from Bronner-Fraser et al (1992) who stated that α_6 integrin was present in the optic cup on E2, and in the ganglion cell layer at later stages into adulthood. The early distribution of α_6 integrin contrasts markedly with that of the fibronectin-binding alpha subunits described above. There was no staining above background from stages 8 to 13 prior to formation of the optic cup. At

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stage 14 trace staining was observed in the presumptive NR, presumptive RPE, cornea, and lens. The staining increased progressively in all ocular tissues through stage 17 where clustering was seen at the ILM, around the lens epithelium, and in the apices of the RPE cells.

The only reported ligand for α_6 integrin is the E8 fragment of laminin (Elner and Elner, 1996; Hillis and MacLeod, 1996), and the only integrin configurations reported are $\alpha_6\beta_1$, and $\alpha_6\beta_4$. The $\alpha_6\beta_4$ integrin is laminin-binding and resides in hemidesmosomes where it participates in epithelial cell adhesion to basement membrane (Elner and Elner, 1996). The ILM and lens periphery staining is consistent with laminin binding. Laminin is strongly expressed along the ILM and lens epithelial basement membrane. The apical RPE staining occurs in an area where there is no visible laminin. The polarized staining pattern has been reported at E7 and E14 when RPE differentiation has taken place (Rizzolo et al., 1994). The scattered intracellular staining in the NR from stages 17 to 24 may be associated with neuroblasts. There is staining within cells of the inner NR at stage 22 which are consistent with retinal ganglion cells. The cells may be producing α_6 integrin in anticipation of neurite extension along the laminin of the ILM at later stages. There was also α_6 staining in the neuroblast layer in the outer NR at stage 24. Throughout stage 14 to 24 there was intracellular staining which did not correspond to any detectable laminin distribution. Laminin is expressed later in NR development as neuroblasts extend neurites and differentiate in their respective layers (Cann et al., 1996). The results of the present study suggest that α_6 integrins do not play a significant role in ocular development before optic cup formation, but their increasing presence at stages 16 to 24 appears to indicate involvement in differentiation of both the NR and RPE.

Disruption of Integrin-Ligand Interactions by Injection of Blocking Agents

Injection Technique

The choice of microinjection instead of heparin-coated beads as a mode of delivery for peptides or antibodies was made in an attempt to minimize trauma to the embryos and accurately deliver doses in a consistent and repeatable fashion. The efficacy of the microinjections was determined to be 64% (11/17) by tracing the fate of injected CSAT antibody by detection with a secondary fluorescently labelled antibody. The results of this experiment showed that some of the injected antibody escaped from the optic vesicle immediately and was localized to the interepithelial space between the surface ectoderm and NE. Furthermore, the injected material was found throughout the developing brain and in the contralateral eye. After 1 and 2 hours the injected antibodies diffused out entirely from the optic vesicle.

The survival rate of embryos in the experiment just described approximated the survival rate among the embryos in the peptide and antibody injection experiments. A curious finding was the 60% survival rate among embryos injected with Tyrode's saline alone. During the evaluation of the India ink injections the survival rate among embryos which were exposed to Tyrode's saline and India ink surrounding the embryo was 87%. This discrepancy may be a consequence of intimate exposure of the developing brain and optic vesicles to Tyrode's as opposed to exposing only the surface of an intact embryo to Tyrode's saline. The developing brain and optic vesicles presumably contain soluble mediators of proliferation, growth and survival. It may be possible that an abrupt introduction of injected fluid causes an efflux of these mediators out of the embryo at a critical stage of development.

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The injection volume may also have been a potential cause for the observed low survival rates. In the study of Svennevik and Linser (1993) the equipment available allowed an injection volume of 4 nl to be delivered into the mesenchyme separating the surface ectoderm and NE beneath the presumptive lens placode. The equipment used in the present study did not allow for such exquisite volume control and consequently the total volume and amount of agent in the injections were up to 9 times that of the Svennevik and Linser study. The introduction of 25 to 35 nl of fluid into the optic vesicle in a rapid fashion may also have caused sufficient stretching of the NE to disrupt cell-cell contact or cell-basement membrane contact to cause irreparable damage to the embryo.

In the injections of viscous fluids such as RGD at a concentration of 100 ng/nl and anti-β_1 integrin antibody (CSAT) at 5 ng/nl there was a decreased survival rate (33% for each). In these two cases the viscosity of the fluids may have contributed to the demise of the embryos by binding non-specifically to the lumenal surfaces of the developing brain and optic vesicles, thereby blocking receptors for growth factors and ECM molecules in the vesicular space. Notably, injection of the 100 ng/nl RGES solution which is far less viscous than either RGD 100 ng/nl or CSAT at 5 ng/nl resulted in a survival rate of 50%.

Anti-β_1 Antibody (CSAT)

The small number of embryos subjected to anti-β_1 integrin injections was due to termination of the experiment upon advice from the advisory committee. None of the embryos injected showed any morphological disruption from stage 11 to stage 24. The ability of an antibody to disrupt normal development depends on the epitope against which the antibody is

directed. Chu and Grunwald (1991) found that the anti-β_1 integrin antibodies 2A10 and CSAT did not show equal ability to disrupt RPE cell binding to fibronectin and laminin. The 2A10 disrupted spreading of fibroblasts and RPE cells on fibronectin while the CSAT had no effect. Therefore failure of an antibody to disrupt integrin-ligand binding may not be conclusive.

Peptides Containing Integrin Recognition Sequences

The absence of any disruption of ocular morphogenesis following injection of RGD peptides into the optic vesicle at stage 11 may have been a consequence of the location of the RGD-binding integrins. The widespread distribution of RGD-binding integrins such as $\alpha_3\beta_1$, $\alpha_4\beta1$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$ shown in the immunohistochemical study would seem to indicate an important role of these molecules in development of the eye. The distribution of the integrins at stage 11, however may have precluded any significant contact with the injected RGD. All of the α subunits which bind RGD were present at stage 11 except $\alpha_6\beta_1$, but all were mainly located within the NE cells. The injected material remained in the vesicle for less than one hour and perhaps did not gain access to the integrins, particularly those which reside near the basement membrane. Another possibility could be that the integrins at the inner surface of the optic vesicle may indeed have bound the injected RGD, but the absence of a continuous supply of RGD any daughter cells generated over the next few hours would not have been exposed to ligand. Yet a third possibility is that the charged RGD and RGES fragments in solution may have come into contact with oppositely charged growth factors thereby causing a mutual sequestering by virtue of electrovalent attraction. If this was indeed the case, both the peptide and growth factor concentrations in the optic vesicle would have been diminished thereby

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potentially depriving the embryo of crucial growth factors and diminishing the possibility of morphological disruption by the peptides.

Possible Roles of β_1 Integrins in Early Differentiation of the RPE and Neural Retina

There is evidence for the role of ECM (Reichardt and Tomaselli, 1991; Paulsson, 1992; Lin and Bissell, 1993) and growth factors (Park and Hollenberg, 1991; Pittack et al., 1991; Pittack et al., 1997; Sakaguchi and Reh, 1997) in early events of RPE and NR differentiation, likely mediated by integrins (Rizzolo et al., 1994; Elner and Elner, 1996). Beta 1 integrins, in particular have been studied in both RPE (Chu and Grunwald, 1991; Rizzolo and Heiges, 1991; Hergott et al., 1993; Rizzolo et al., 1994; Menko and Philip, 1995), and NR (Cann et al., 1996; Martinez-Morales, 1996). However, the distribution of β_1 integrins and their ligands during the stages of chicken embryo development investigated in the present study have not been well documented in the literature. Further, their role in cell differentiation in the retina and RPE has not been elucidated.

Two approaches were used in the present investigation: an immunohistochemical study of the identity and distribution of β_1 integrins in stage 8 to 24 chicken retina; blocking the fibronectin-binding alpha subunits coexpressed with β_1 integrin that have been described in RPE and NR development with the exception of α_4 which binds LRV, and α_6 which binds the E8 fragment of laminin (Elner and Elner, 1996; Reichardt and Tomaselli, 1991). In view of the distribution of RGD-binding integrins in the NR and RPE during development, the injection studies were inconclusive. The temporal and spatial information provided by the immunohistochemical study, however, indicates that certain β_1 integrins are present during

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the early development of the RPE and NR and suggests that they may play important roles of NR and RPE differentiation.

In the present study there were no significant differences in distribution of integrin subunits, ECM molecules or NCAM between the presumptive RPE and presumptive NR prior to the formation of the optic cup (Fig. 20a-b). While β_1 integrin appears to be present as dimers with all of the alpha subunits examined, there remain some discrepancies in the distributions of β_1 and α subunits which seem to indicate either as yet undescribed dimers with other β integrin subunits or perhaps expression of β_1 on the cell surface in advance of α subunit expression. The α_3, α_4, α_5 subunits showed a similar intracellular vesicular distribution within NE cells from stages 8 through 10. Only α_5 integrin appeared to be engaging ligand along the NE basement membrane at this time. At these stages, evagination of the NE of the rostral neural tube occurs to form the primary optic vesicle. The intracellular distribution of these alpha subunits at this stage may be an indication of active synthesis of the α_3, α_4, and α_5 subunits in the NE. The staining appearance, however, did not seem to indicate any ligand binding since it is likely that any ligand binding would be seen as focal staining at the cell margins and not in cytoplasmic vesicles. The β_1 integrin staining was mainly localized to cell margins. Humphries and co-workers (1993) suggested that the β_1 subunit can function independently of its α subunit by virtue of its ligand-independent attachment to the cytoskeleton. If this is indeed the case, then perhaps the β_1 integrin distribution surrounding cells at stages where there is no coexpressed α subunits may represent an involvement in cell-cell communication via the cytoskeleton independent of any ECM ligand binding.

After optic cup formation at stage 14 the expression patterns of the α subunits begin to diverge. The expression of α_3 in the RPE increased at the cell margins from stages 15 through 20, coinciding with a number of important changes in the RPE (Fig. 20a). The α_v integrin subunit expression also increaseed during this period, perhaps indicating a correlation to the increase in α_3 and β_1 expression. The commencement of α_6 integrin subunit expression within the presumptive RPE and presumptive NR at this time may indicate an involvement with differentiation events which will take place over the next stages of development. At stage 15 NCAM expression in the RPE was very weak, presumably indicating a loss of the neural phenotype by the RPE. The morphology of the RPE became decidedly cuboidal from stages 16 to 22, and the RPE changed from multilayered to a monolayer. Two striking features of α_3 integrin expression during this developmental period were the heavy intense pericellular distribution around RPE cells at a time when the RPE is very actively differentiating (Fig.20a), and intracellular localization in neuroblasts of the outer NR. The outer NR at stage 16 has ganglion cell precursors which are beginning to migrate and project axons (Halfter et al., 1985; Cohen et al., 1987). Alpha 3 integrin has at least two important ligands: the RGD fragment of fibronectin and the E8 fragment of laminin (Reichardt and Tomaselli, 1991; Elner and Elner, 1996). A single integrin can mediate more than one function, particularly when they are capable of binding more than one ligand (Humphries et al., 1993). The distribution of α_3 integrin between stages 16-22 may indicate two separate functions: the promotion of the epithelial phenotype in RPE; mediating early migration and differentiation in retinal ganglion cells. Both fibronectin and laminin reside in the RPE basement membrane at stage 19, either of which can be bound by α_3 integrin. Fibronectin resides in the NR at stages 19 to 24 and

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Figure 20.   Graphical representation of integrin subunits in the RPE and NR from stages 8 to 24.

(A) RPE at stages 8-24 showing the distribution of integrin subunits during the neural groove stage (I), the optic vesicle stages (II), the optic cup formation (III), and the RPE differentiation stages (IV). (B) NR at stages 8-24 showing the distribution of integrin subunits during the neural groove stage (I), the optic vesicle stages (II), the optic cup formation (III), the ganglion cell migration stages (IV), and the ganglion cell differentiation stages (V). The intensity scale for both graphs is: (0) negative, (1) weak, (2) moderate, (3) strong, (4) intense.

($\blacklozenge$) α_3 integrin subunit, (O) α_4 integrin subunit, (Δ) α_5 integrin subunit,
($\blacksquare$) α_6 integrin subunit, ($\blacktriangle$) α_v integrin subunit, ($\bullet$) β_1 integrin subunit
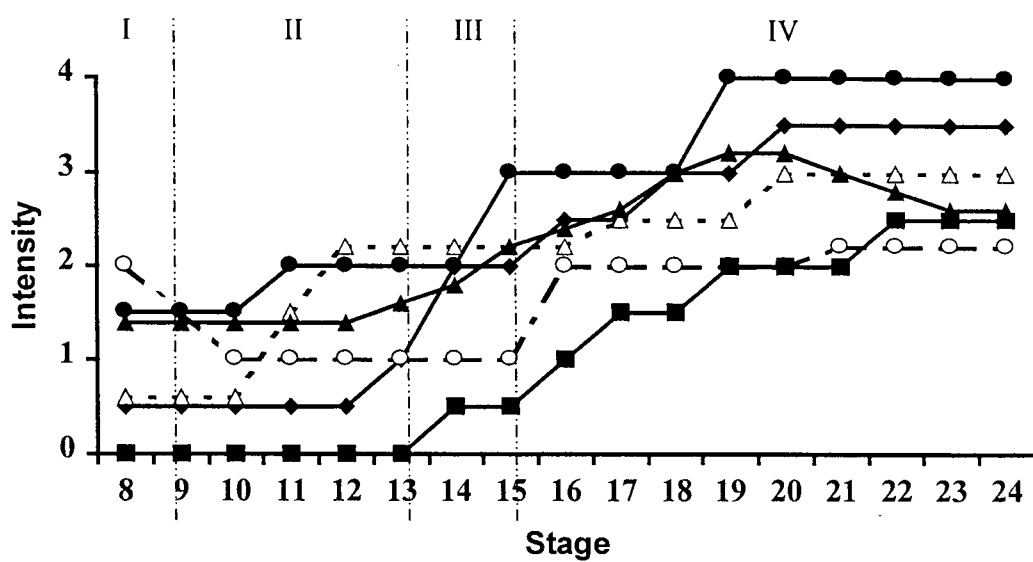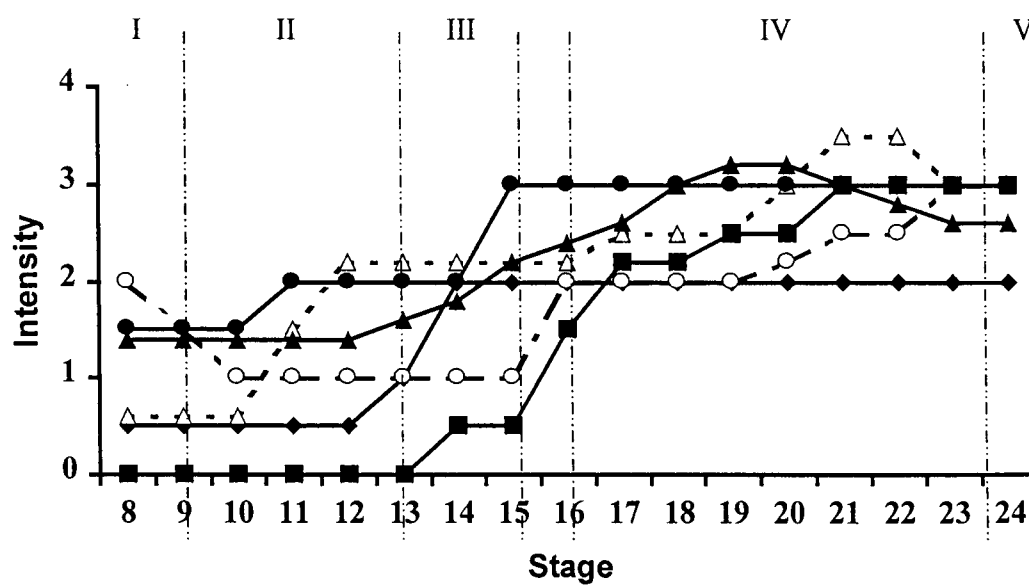
may be providing a support for neuroblast migration as it does elsewhere in the embryonic nervous system (Reichardt and Tomaselli, 1991). Other studies have shown that α_3 subunit disappears from the apical RPE by E7 when it is confined to the basal margins of RPE cells (Rizzolo et al., 1994). At stage 24, α_3 integrin subunit is strongly expressed in the RPE, therefore the subsequent rapid loss of α_3 staining from E4 to E7 may suggest a role in facilitating phenotype change in the RPE and perhaps, through basement membrane matrix binding, maintaining the epithelial phenotype. Studies of RPE cells in culture have shown that RPE cells must maintain contact with their basement membrane to retain the epithelial phenotype (Reh et al., 1987; Reh and Radke, 1988; Zhou and Opas, 1994). These investigators were able to demonstrate transdifferentiation of RPE to neuronal phenotype only in the absence of basement membrane contact. Alpha 3 subunit staining in the NR appears to diminish from stages 22-24 (Fig. 20b). This finding and the fact that α_3 integrin has not been reported in the NR at later stages may indicate a narrow stage range from stage 15 to 22 where α_3 integrin is developmentally active. Alpha 4 integrin does not bind through the RGD fragment but rather the LDV fragment of fibronectin and VCAM-1 (Humphries et al., 1993; Elner and Elner, 1996). The RGD and LDV fragments have been reported to be mutually inhibitory (Mould et al., 1991; Humphries et al., 1993), although the distributions observed for the α_4 integrin and the RGD-binding alpha integrins α_3, α_5, and α_v in this study did not provide any conclusive evidence for mutual antagonism. There was a steady increase in α_4 integrin expression with age to stage 24 (Fig. 20b). The increased amounts intracellular α_4 in migrating neuroblasts and differentiating ganglion cells suggest a role in later NR differentiation. The strong expression for α_4 along the ILM at stage 22 may be consistent with

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early neurite extension along fibronectin to the ILM. The intense staining in RPE basement membrane at stages 22 to 24 may suggest a role in RPE stretching as the cells extend during growth. Cann and co-workers (1996) reported a steady increase of α_4 during this period but stated that its role in retina development remains inconclusive.

The post-optic cup distribution of α_5 integrin shows a steady increase through to stage 24 in both RPE and NR (Fig. 20a-b). An interesting finding was that the stage 22 differentiating ganglion cells of the NR at stage 22 showed strong intracellular staining which was concomitant with a decrease in α_3 expression; α_5 decreased by stage 24. The ganglion cells at this time were also producing fibronectin which appeared to be largely secreted by stage 24. It may be possible that the α_3 integrin mediates interactions of early migrating ganglion cells which then produce α_5 integrin once they have reached their final destination near the inner surface of the NR. The production of fibronectin at this time may be an indication of "pioneering" ganglion cells laying down fibronectin upon which other migrating neuroblasts can move. Taken together with the expression pattern in the lens fibres and RPE, the α_5 integrin may possibly be considered to play a role in cell migration in the developing eye since it diminishes upon lens fibre elongation, differentiation of ganglion cells, and is absent from RPE by E7 (Rizzolo et al., 1994).

The zipper-like staining pattern of α_v at the RPE-NR interface at stages 17 to 19 may indicate integrin-mediated interaction between the NR and RPE. The timing of this distribution corresponds to a number of differentiation events in the RPE and NR. The presumptive RPE at this time undergoes a rapid sequence of changes. At stage 16 the presumptive RPE begins to produce premelanosomes (Ide, 1972). This event is followed by

four significant changes in the RPE at stages 17 to 19: an increase in tyrosinase activity and melanosome formation (Ide, 1972; Stroeva and Mitashov, 1983); loss of NCAM reactivity (Rizzolo et al., 1994; present study); a marked decrease in DNA synthesis and mitoses (Stroeva and Mitashov, 1983; Zimmerman, 1975); establishment of a single layer of short columnar cells. These events mark the change in phenotype from neural to epithelial in the RPE. Experimental evidence for the role of contact of NR and RPE in influencing cell differentiation has been reported (Coulombre, 1955; Orts-Llorca and Genis-Galvez, 1960). Coulombre showed that insertion of a small glass rod into the optic vesicle of chicken embryos to relieve intraocular pressure prevented the developing retina from assuming its stretched state required for growth and resulted in folding of the NR. The areas of RPE subjacent to the folds where contact with the NR was lost transdifferentiated to NR. Orts-Llorca and Genis-Galvez (1960) showed that preventing contact by inserting a thread between the presumptive RPE and presumptive NR prior to formation of the optic cup also resulted in the transdifferentiation of RPE into NR. More recent experiments (Rizzolo et al., 1994) showed that loss of contact of NR and RPE resulted in changes in the apical distribution of β_1 integrins in the RPE. The finding was thought to indicate an integrin-mediated communication between the NR and RPE.

The pattern of expression of α_v in the present study suggests a role in both RPE differentiation at stages 17 to 19 and a role in NR differentiation at later stages. At stage 24 the distribution of α_v integrin contrasts with the pattern at stage 19. One possible explanation is that the difference in distribution may represent different α_v receptors being expressed at the different stages. Previous studies have indicated a role for α_v integrin and vitronectin in
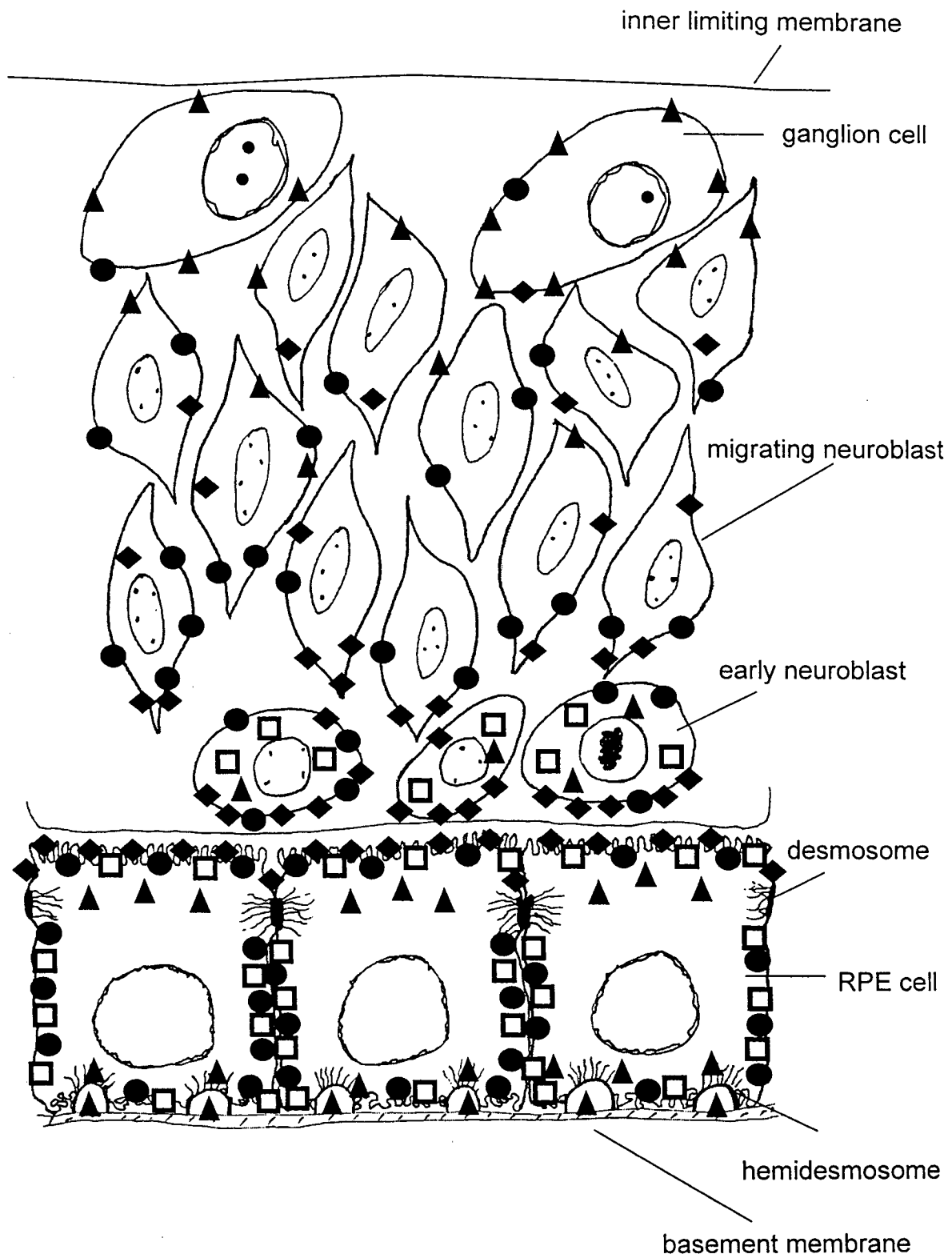
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retinal ganglion cell differentiation and neurite extension (Martinez-Morales et al., 1996; Cann et al., 1996; Yip et al., 1998). It was also speculated (Cann et al., 1996; Yip et al., 1998) that the interactions of the differentiating neurons with vitronectin were mediated by the $\alpha_v\beta_3$ integrin. The β_1 integrin results in the present study do not indicate the presence of $\alpha_v\beta_1$ at stage 24. Therefore the possibility exists that the α_v staining seen at stages 20 to 24 is largely $\alpha_v\beta_3$. While the possibility of the stage 19 α_v staining represents $\alpha_v\beta_3$ cannot be ruled out, the zipper-like staining pattern seen coincides with β_1 integrin expression. If that is indeed the case, then it may be possible that $\alpha_v\beta_1$ may be involved in NR-RPE interaction while $\alpha_v\beta_3$ is involved in later neuron differentiation in the NR.

Stage 19 represents a key point in differentiation events in the RPE and NR during which rapid changes in integrin subunit distribution occurs. This distribution is shown diagrammatically in Figure 21. The concentration of α_3 and β_1 integrin subunits surrounding RPE cells which are approaching the differentiated state appears to indicate a role in maintenance of the epithelial phenotype. The presence of α_6 integrin subunits in a polarized fashion within the RPE may point to interaction with the differentiating NR via $\alpha_6\beta_1$ integrins on the apical surface of the RPE and $\alpha_6\beta_4$ integrins within hemidesmosomes anchoring the basal cell membranes of the RPE to the basement membrane. The presence of increased amounts of α_3 and α_v integrin subunits on both sides of the interface of NR and RPE may indicate a role in communication between early migrating neuroblasts and RPE cells. Although the ligands involved are not clearly established, it is likely that fibronectin present in the area is being bound at this time, and in the case of α_v, perhaps vitronectin as well. Alpha 6 and β_1 integrin subunits are present in the NR at stage 19 and the steady increase in concentration

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Figure 21. Illustration of integrin subunit distribution in the stage 19 embryonic chicken retina.

The α_3 integrin subunit is heavily distributed surrounding the RPE cells and also appears intracellularly in early neuroblasts. The α_6 integrin subunit appears in a polarized fashion at the apices and basal areas of the RPE there is also α_6 integrin subunit located throughout the differentiating NR, particularly in the ganglion cells. In addition to the $\alpha_6\beta_1$ dimer, some of the α_6 integrin subunit seen at the basal extremity of the RPE may be $\alpha_6\beta_4$ dimers located in the hemidesmosomes. The α_v integrin subunit may be coexpressed with either β_1 or β_3 integrin subunits, and is mainly located at the interface of the outer NR and apical RPE in a zipper-like fashion. The β_1 integrin subunit expression is intense surrounding the RPE cells, likely indicating the presence of both $\alpha_3\beta_1$ and $\alpha_6\beta_1$ dimers. There is also expression at the apices of the RPE cells and in the NR where it may represent $\alpha_v\beta_1$ dimers as well.

($\square$) α_3 integrin subunit, ($\blacktriangle$) α_6 integrin subunit, ($\blacklozenge$) α_v integrin subunit, ($\bullet$) β_1 integrin subunit

inner limiting membrane

ganglion cell

migrating neuroblast

early neuroblast

desmosome

RPE cell

hemidesmosome

basement membrane

through the next several stages during which ganglion cells begin to differentiate and extend neurites along the ILM.

These novel findings, taken together with the distribution of α_4 subunits which increase through stage 24 and α_5 integrin subunits which are most prominent during cell migration events may provide information which can be utilized in further study of retina development. The distribution of fibronectin within and surrounding developing NE, RPE, and migrating ganglion cells is also a novel finding, and perhaps in conjunction with future studies on the distribution of vitronectin and FGF-2 receptors can elucidate important clues to key events in RPE and NR differentiation.

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