Changes in Extracellular Matrix Proteins and Actin During Corneal Endothelial Growth

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Basement membranes influence growth, shape and differentiation of cells and tissues. However, the role and influence of Descemet's membrane during corneal development is not understood. To address this question, the relationships between cell growth and fibronectin, laminin and actin distribution in the developing rat corneal endothelium in vivo has been examined. During fetal development, rat corneal endothelial cells undergo DNA synthesis and mitosis. However, at day 14 of gestation both processes begin to decline and neither can be detected in endothelium of 1-month-old animals. By this time cell number has increased to approximately 100,000 and tissue area has increased 25-fold. However, as the tissue area increased, cell density decreased, indicating that cell spreading occurred in order to maintain tissue integrity. Changes in endothelial growth were accompanied by changes in the distribution of laminin, fibronectin and actin. Laminin and fibronectin were diffusely localized within endothelial cells in newborn animals. By 4 weeks of age, no proliferation was demonstrated and both extracellular matrix proteins were localized in pericellular patterns. Actin, on the other hand, which appeared diffuse at 16 days in utero, was distributed at or near the cell membrane by 19 days in utero.

Thus, the reorganization of extracellular matrix glycoproteins and actin may indicate important roles for these components in regulating the growth and formation of the corneal endothelium in vivo. Invest Ophthalmol Vis Sci 31:94-101, 1990

The inner corneal surface of vertebrates is lined by the endothelium, which functions to maintain corneal hydration and transparency. Though endothelial cells of embryonic and young animals undergo DNA synthesis and mitosis, both processes abate in adult tissue, resulting in a noncycling or "G0" cell population. The time course of this mitotic cessation is unknown. Moreover, the reasons for mitotic arrest in young endothelia are not understood. During development, endothelial cells begin forming Descemet's membrane, their natural basement membrane. Once thought to serve only in the maintenance of tissue architecture, basement membranes are now known to be actively involved in the regulation of cell shape, growth, migration and differentiation. Studies by Fitch and Linsenmayer and Zak and Linsenmayer indicate that different basement membrane components become incorporated into Descemet's membrane at various developmental times. Because extracellular matrix (ECM) macromolecules influence many biological processes, their appearance within Descemet's membrane may regulate growth and development in the endothelium. Two widely studied ECM glycoproteins are fibronectin (FN) and laminin (LM), both of which play roles in cell adhesion and cell migration. Several in vitro studies have correlated active cell growth with losses of surface FN and LM. Both glycoproteins have been localized during corneal development, where they are detected in Descemet's membrane. In histological sections of the mature chick and rat cornea, FN is localized on the stromal side of Descemet's membrane. However, recent studies from this laboratory using flat mount preparations of rat endothelium, which allow for the visualization of the entire cell population, reveal that both glycoproteins colocalize pericellularly. Since the appearance and organization of these ECM glycoproteins may be correlated with the abatement of mitosis in this tissue, growth characteristics relative to FN, LM and actin distributions were investigated in the developing rat corneal endothelium in vivo.

Materials and Methods

Animals

Fourteen to 20-day-old fetal and day 1 to 4-week-old Sprague Dawley rats (Charles River Laboratories,
Portage, MI) were used in this study. Gestation times were verified according to Hebel and Stromberg. Rats were maintained on a 12 hr light/dark cycle. Animals received food and water ad libitum. The treatment of experimental animals in this study was in compliance with the ARVO Resolution on the Use of Animals in Research.

Immunocytochemistry

Isolated corneas were fixed in methanol (−10°C) for 5 min, extracted in acetone (−10°C) for 2 min and rehydrated to 0.1 M phosphate-buffered saline (PBS). FN was localized in tissues by incubating them in anti-FN antibody (ICN ImmunoBiologicals, Lisle, IL) at a 1:10 dilution in PBS for 1 hr at room temperature, washing in buffer and incubating in rabbit anti-sheep IgG conjugated to horseradish peroxidase (HRP; Boehringer-Mannheim Biochemicals, Indianapolis, IN) at a 1:50 dilution in PBS for 30 min. For LM localization, tissues were exposed to a 1:10 dilution of anti-LM antibody (GIBCO, Grand Island, NY) in PBS for 1 hr, washed in buffer and incubated in goat anti-rabbit IgG-HRP (Sigma Chemical Co., St. Louis, MO) for 30 min. Controls were performed as previously described, and consisted of: (1) omission of the primary antibody; (2) incubation of the tissues in adsorbed antibody; (3) incubation of the tissues in 5% nonimmune serum; or (4) incubation of the tissues in diaminobenzidine alone. An absence of immunoperoxidase staining was observed in all controls.

Visualization of Antibody Binding

Following antibody treatment, tissues were incubated in a cytochemical media containing diaminobenzidine to visualize HRP reactivity for 60–75 min at 37°C. Tissues were then washed, prepared as flat mounts, dehydrated and mounted in permount for light microscopic observation.

Actin Localization

Actin was localized using rhodamine conjugated phalloidin (Molecular Probes, Eugene, OR) as previously described for NBD-phallacidin. Briefly, corneas were fixed in 3.7% formaldehyde, extracted in acetone (−10°C) and exposed to phalloidin for 30–60 min at room temperature and mounted in 0.5 M carbonate buffer containing phenylenediamine and glycerol (pH 9.0) to retard fading. Photography was performed using a Leitz Laborlux-D epifluorescent microscope with Kodak tri-X-panatomic film.

Flat Mount Preparations

Following immunocytochemical staining, actin localization, or for histologically viewing the tissue, the endothelium was gently pulled away from the rest of the tissue. Preparations were placed endothelial side up on glass slides and, where necessary, radial incisions were made to allow them to lie flat. For histological observation, tissues were fixed overnight in Carnoy’s solution, rehydrated, flat mounted, stained with 1% cresyl violet acetate (Eastman Kodak, Rochester, NY) and mounted in permount.

Autoradiography

Isolated corneas were placed in basal medium Eagle (GIBCO) containing 5 μCi/ml 3H-thymidine (New England Nuclear, Boston, MA) for 2 hr at 37°C in a water-jacketed CO2 incubator with a humidified atmosphere of 5% CO2 and 95% air. Following fixation in Carnoy’s fluid, endothelia were separated from the rest of the cornea and prepared as flat mounts on glass slides. Slides were dipped into Kodak NTB-2 (Eastman Kodak) nuclear track emulsion, dried and exposed in the dark for 2 weeks at 4°C. Autoradiograms were developed in Kodak D-19, washed, fixed, counterstained in cresyl violet acetate and mounted.

Cell Count

In order to determine the average number of cells per endothelium at various times of development, cell counts were performed on flat mount preparations using a ×40 objective with a reticle mounted in a ×10 eyepiece. At least two 0.01 mm² areas/endothelium were counted, averaged and the values extrapolated to give cell density/mm². The X and Y axes of each preparation were measured and averaged to determine the value of the radius. Areas of flat mounts were calculated using the equation A = πr² and multiplied by the average cell density to yield the average number of cells/flat mount preparation. Values obtained from each preparation at a given time period (ie, 16 days in utero) were averaged to determine the number of cells present at that time of development.

Mitotic Index

Flat mounts were scanned and mitotic figures counted using a ×40 objective. For calculating indices, the average number of mitotic figures was divided by the average number of cells/flat mount and that resulting value was multiplied by 100.
Results

Cell and Tissue Growth During Development

During rat corneal development, endothelial cells actively undergo growth. However, as gestation proceeds, the mitotic indices drop from an average value of 1.2 at 14 days in utero to approximately 0.1 at birth and finally to 0.0 in 1-month-old animals (Fig. 1). Concurrently, the total cell number increases, from approximately 4000 to 100,000 cells/endothelium (Fig. 1) while the tissue area enlarges (Fig. 2). From 14 to 20 days in utero, the endothelium nearly triples in size and by 2 weeks after birth, the area reaches an average value of 7.68 mm². Finally, by 4 weeks of age the size of the endothelium increases to an average value of 17.75 mm². During this time, changes in cell density occur within the tissue (Fig. 2). At 14 days in utero, there are about 5200 cells/mm². By 16 days this value increases to 11,650 cells/mm², as a result of mitosis, and remains somewhat constant until 1 week following birth, at which time cell density begins to decline, so that the endothelium of 4-week-old animals averages 5600 cells/mm². The drop in cell density comes at a time when the endothelium more than doubles its area despite a decline in the mitotic index to nearly zero. Thus, endothelial cells spread to compensate for increased tissue size.

Histologically, endothelial cells of 14-day-old fetuses (Fig. 3) are small and compact and many are undergoing mitosis. By 1 week following birth very few mitotic figures are observed (Fig. 4). In 1-month-old animals (Fig. 5), endothelial cell density is decreased and mitosis is not evident. Autoradiograms show a decrease in ³H-thymidine incorporation to confirm the exit of endothelial cells from the proliferative cycle. Thus, thymidine incorporation, extensive in 18-day fetuses (Fig. 6), diminishes greatly in 2-week-old animals (Fig. 7) and completely abates in 1-month-old animals (Fig. 8).

FN and LM Distribution

During development light FN staining is detected intracellularly, and remains such in newborn rats (Fig. 9). In 2-week-old animals, the endothelial monolayer is more organized but FN staining continues to be detected only intracellularly (Fig. 10). As the cells spread and flatten on Descemet's membrane, the nonstaining nuclear region become visible. One month following birth (Fig. 11), FN is localized in pericellularly patterns. LM patterns (Figs. 12-14) closely resemble those seen for FN. In newborn animals, LM staining is intracellular. Again, 2-week-old...
Fig. 3-5. Flat mount preparations of rat corneal endothelium stained with cresyl violet acetate. All micrographs original magnification X340. Fig. 3. Cells of a 14-day fetal endothelium are small and compact. Several mitotic figures (M) can be observed. Bar = 50 µm. Fig. 4. One week after birth the number of mitotic figures (arrow) has greatly declined. Bar = 50 µm. Fig. 5. By 4 weeks after birth mitosis is absent from the flat mount preparation. Bar = 50 µm.

Animals show no indications of a pericellular distribution, only some intracellular staining. Nonetheless, by 1 month LM is localized pericellularly, thus codistributing with FN.

Actin Distribution
Rhodamine phalloidin staining of 16-day in utero endothelia (Fig. 15) revealed a somewhat nondistinct

Fig. 6-8. Autoradiograms of ³H-thymidine uptake in flat mounts of corneal endothelium counterstained with cresyl violet acetate. All micrographs original magnification X210. Fig. 6. At 18 days in utero, endothelial cells actively incorporate the isotope. Bar = 100 µm. Fig. 7. In 2-week-old animals isotope incorporation is greatly diminished and only a few cells show uptake. Bar = 100 µm. Fig. 8. By 4 weeks of age, no ³H-thymidine incorporation can be detected in the cells. Bar = 100 µm.
Figs. 9-11. Immunoperoxidase staining for fibronectin in endothelial flat mount preparations. All micrographs original magnification X530. Fig. 9. In 1-day-old animals, fibronectin is diffusely distributed within endothelial cells (En). Intercellular spaces (IS) can be seen throughout the tissue. Bar = 50 μm. Fig. 10. Two-week-old animals have a more organized monolayer and fibronectin staining can be seen within the cytoplasm (arrows). Nuclei (N) in flattened endothelial cells are devoid of immunoperoxidase activity. Bar = 50 μm. Fig. 11. In 4-week-old animals fibronectin is distributed pericellularly throughout the monolayer. Some cytoplasmic but no nuclear staining is observed. Bar = 50 μm.

Localization of actin. This pattern changes by 19 days in utero (Fig. 16), becoming more distinctly confined to the cell periphery as the monolayer becomes better organized. At this time the endothelium exhibits a pleomorphic appearance. In 1-month-old animals (Fig. 17), actin is observed very distinctly at or near

Figs. 12-14. Immunoperoxidase staining for laminin in endothelial flat mount preparations. All micrographs original magnification X550. Fig. 12. One-day-old animals exhibit diffuse intracellular staining for laminin in endothelial cells (En). Intercellular spaces (IS) are evident within the monolayer. Bar = 50 μm. Fig. 13. Two weeks following birth, laminin staining is observed within the cytoplasm (arrows). The nuclei (N) do not show any staining. Bar = 50 μm. Fig. 14. In 4-week-old animals, laminin is localized pericellularly in the endothelial monolayer. Some cytoplasmic but no nuclear staining can be seen. Bar = 50 μm.
Figs. 15–17. Fluorescent micrographs of endothelial flat mounts stained with rhodamine phalloidin to detect actin distribution. Fig. 15. At 16 days in utero, actin staining is nondistinctly localized in the cytoplasm (arrows) around the nonfluorescing nucleus. Intracellular spaces (IS) are also observed in the monolayer. Original magnification ×980, bar = 25 μm. Fig. 16. In 19-day fetuses actin fluorescence (arrows) is distributed at or near the cell membrane. The monolayer appears pleiomorphic. Original magnification ×490, bar = 50 μm. Fig. 17. Four weeks after birth, actin is distinctly localized circumferentially. The cell size of the monolayer appears more uniform. Original magnification ×490, bar = 50 μm.

the cell membrane and the endothelial monolayer is well defined with a more uniform cell size than that observed in 19-day fetuses.

Discussion

Basement membranes are known to regulate many cell processes, including growth. Nevertheless, the complex interplay existing between the developing endothelium and Descemet’s membrane has yet to be elucidated. Because the endothelium goes from a proliferative to a nonproliferative cell population, it is important to understand the contribution of Descemet’s membrane to endothelial growth and development.

Fetal and young rat corneal endothelial cells undergo DNA synthesis and mitosis as do endothelia of other young animals. The exit of young endothelial cells from the cell cycle is a programmed event and shortly after birth, both processes abate, resulting in a tissue comprised solely of noncycling cells. Although adult endothelium can be stimulated to divide by injury, the magnitude of the response varies with the species studied. Furthermore, H-thymidine, administered during the repair process, can be detected autoradiographically 4 years after wound repair, indicating that cell turnover in this tissue is virtually nonexistent.

Initial increases in cell number in early development result from mitotic activity. However, despite a decline in cell proliferation during gestation, the corneal endothelium increases in area. To compensate for decreased growth, endothelial cells spread out over Descemet’s membrane, thereby maintaining monolayer unity but decreasing cell density/mm². Thus, cell densities drop approximately 24% by 2 weeks of age and an additional 47% decrease occurs by 4 weeks after birth. Similar findings have been made in other species and are thought to result from a rapid postnatal enlargement in corneal size. After growth is completed, a progressive but much slower decline in cell density continues in noninjured adult tissue, as cells spread to fill in areas vacated by dead or dying cells. Therefore, in animals, including humans, the number of cells/mm² decreases as a function of age but monolayer integrity is sustained by endothelial cell spreading.

Honda and Eguichi speculated that membrane-associated microfilament bundles may act to maintain the integrity of epithelial cells as a sheet or monolayer. Such a circumferential actin distribution at or near the cell membrane corresponds to the classic terminal web of microfilaments and are characteristically seen in tissues such as intestinal and retinal pigment epithelium. In fetal endothelium, actin is at first diffusely seen within the cells and as the monolayer becomes more organized, a more distinct actin pattern at or near the cell membrane emerges.
Similarly, sparse cultures of corneal endothelial cells exhibit actin organized as stress fibers, but as the cultures become confluent, actin redistributes circumferentially. Furthermore, during endothelial wound repair, this actin distribution is lost and stress fibers appear in cells responding to injury. However, following the reestablishment of the monolayer, the circumferential patterns return. Thus, it appears that endothelial actin distributions correspond to the degree of monolayer organization rather than a cessation of mitosis.

Reasons for mitotic cessation in the endothelium are not well understood. In vitro studies by Goldminz and colleagues previously, Vlodavsky and Gospodarowicz demonstrated that LM mediated the adhesion of transformed cells, which only adhered and flattened in the presence of FN. Because carcinoma cells are of epithelial derivation, whereas sarcoma cells are derived from mesenchymal tissues, their responses to either LM or FN were thought to reflect that protein’s role in attaching to cell surfaces are associated with increased growth in transformed cells. Our previous studies noted that injury to the adult corneal endothelium initiates cell division, and results in the loss of pericellular FN and LM patterns, with increased intracellular immunoperoxidase staining for FN and LN. As the endothelium reestablishes itself, mitosis ceases and pericellular ECM patterns reappear. This series of injury-related changes in the adult corneal endothelium in vivo essentially parallels those changes described in this paper during corneal endothelial development in vivo. Thus, wound repair in the adult endothelium mirrors many events seen during its development.

Key words: corneal endothelium, growth, fibronectin, laminin, actin

Acknowledgments

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References