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Laminin and Fibronectin in Retinoid-Induced Keratolenticular Dysgenesis

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Acute embryonic exposure to isotretinoin during gastrulation (gestational day 7) in the mouse results in delay or failure of separation of the lens vesicle from the surface ectoderm. During normal lens vesicle detachment, laminin is localized within the lens, keratolenticular stalk and adjacent surface ectoderm. The mesenchyme surrounding the stalk stains positively for fibronectin. In contrast, isotretinoin-exposed embryos at the same stage of gestation exhibit reduced staining for both extracellular matrix components. Persistent keratolenticular attachment observed later in gestation in the exposed embryos is associated with increased production of laminin by the keratolenticular stalk and anterior lens epithelium. A delay in the sequence of production of extracellular matrix may be causally associated with persistence of the keratolenticular stalk. Invest Ophthalmol Vis Sci 31:751–757, 1990

The processes associated with lens induction have been extensively studied, including localization of laminin and fibronectin during lens placode formation and invagination[1] and their distribution within the developing lens capsule.[2] Parmigiani and McAvoy describe an adhesive network of fibrils within the space between the optic vesicle and surface ectoderm which may be involved in initiation of lens placode formation.[1]

In contrast to lens induction, the process of separation of the lens vesicle from the surface ectoderm has been relatively overlooked. Lens vesicle detachment is the initial event leading to formation of the chambers of the ocular anterior segment. This occurs on day 11 (40–44 somite pairs) in the mouse[3] and day 35 in the human.[4] This process is accompanied by active migration of epithelial cells out of the keratolenticular stalk,[5] cellular necrosis and basement membrane breakdown.[6] Following detachment, the lens vesicle is lined by a single cuboidal layer of cells surrounded by a basal lamina. This basal lamina is formed by the lens epithelium, previously continuous with the surface ectoderm.

Failure of separation of the lens from the surface ectoderm has been observed to occur spontaneously in the C57Bl/6J mouse strain.[3] Following exposure to teratogens including ethanol,[7,8] retinoic acid,[8] radiation[9] and ochratoxin A,[10,11] an increased incidence of anterior segment dysgenesis has been observed in this and other mouse strains. Following in utero exposure to ethanol in C57Bl mice, live offspring exhibit the characteristic features of Peters' anomaly: axial corneal opacity associated with defects in corneal stroma, endothelium and Descemet's membrane with or without associated anterior lenticonus and axial cataract.[7]

Although the constituents of the extracellular matrix are known to play a role in cell adhesion and migration, their distribution (and potential contribution) during the process of lens vesicle detachment has not been previously described.

Materials and Methods

These experiments conform to the ARVO Resolution on the Use of Animals in Research. Nulliparous female C57Bl/6J mice were placed with males of the same strain in a ratio of two females to one male for 1 hr and then examined for the presence of a copulation plug. The time of plug detection was denoted day 0, hour 0. On day 7, hour 0 of gestation, females were administered a single dose of 50 mg/kg maternal body weight isotretinoin suspended in sesame oil by gastric lavage. Control animals were administered an equal volume of sesame oil. Pregnant females were killed by cervical dislocation on day 10, 11, 12, 13 or 14 of gestation. The uterine tissue and extraembryonic membranes, except the amnion, were removed. Embryos to be evaluated using immunocytochemistry were fixed in Bouin's fixative. Those to be

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examined using SEM or TEM were fixed in 2.5% glutaraldehyde.

After 24 h, the Bouin's-fixed embryos were transferred to 70% ethanol to extract the picric acid. The tissues were further dehydrated in a graded ethanol series prior to infiltration and embedding in paraffin. Five micron serial sections were cut and mounted onto glass slides. Alternate sections were stained with periodic acid Schiff's reagent.

For the immunocytochemical studies, the primary antibodies, anti-laminin (dilution 1:5,000; E-Y #032301; Sam Mateo, CA) and anti-fibronectin (dilution 1:10,000; Cappell #2309; Philadelphia, PA) were used. Following extraction of the paraffin, the sections were incubated with the primary antibody for 24 hr at 4°C, rinsed in PBS and incubated with the secondary antibody (sheep anti-rabbit gamma globulin; dilution 1:100; Arnel Products Co., New York, NY), followed by the peroxidase anti-peroxidase (PAP; dilution 1:1000; Arnel Products Co.). The secondary antibody and PAP were repeated to result in an amplified end product.[12] Staining of the end product was accomplished with diaminobenzidine and hydrogen peroxide; sections were counterstained with hematoxylin.

Those tissues to be examined using SEM were microdissected in a horizontal plane through the eyes, rinsed in phosphate buffer, post-fixed in 2% osmium and dehydrated in a graded ethanol series prior to critical point drying from CO_2. Dried specimens were then mounted on aluminum stubs with silver paint and sputter-coated with platinum/palladium. SEM examination and photography was performed at 15 kV with a JEOL (Burlingame, CA) SM35 microscope.

After SEM analyses, selected specimens were further processed for TEM using the process described by Meller et al.[13] Sections 0.5 μm in thickness were stained with toluidine blue and evaluated by light microscopy. Ultrathin sections were obtained with a Sorvall MT2B ultramicrotome (Dupont, Newtown, CT) and collected on copper grids. Following staining with uranyl acetate and lead citrate, sections were examined on a JEOL-100 CX electron microscope at 60 kV.

Results

Exposure to isotretinoin on day 7 of gestation has been previously described to result in a deficiency in the neural plate, particularly the presumptive forebrain territory.[8] The lens vesicle in treated embryos was small and often failed to detach from the surface ectoderm. In more severely affected individuals, lens induction failed to occur, resulting in aphakia.[8] Fetuses exposed to isotretinoin have been shown to exhibit an incidence of 100% (73/73) eye malformations characterized by microphthalmia/anophthalmia and small (or absent) palpebral fissures.

Some degree of keratolenticular dysgenesis has been observed in 71% (22/31) of the eyes in which the cornea/lens relationship could be evaluated (Fig. 1A, B). The presence and/or degree of failure of separation of the lens from the surface ectoderm did not appear to directly correlate with the degree of microphakia. In eyes exhibiting persistent keratolenticular attachment, the surface ectoderm failed to reorganize into a continuous sheet with a zone of dysjunction present in the area of the former lens pore (Fig. 1C). The keratolenticular stalk prevented axial migration of neural crest cells destined to form the corneal stroma, endothelium, Descemet's membrane and anterior iris stroma (Fig. 1E, F). Basement membrane material was present around the persistent lens stalk and spanning the junction between the stalk and the anterior lens (Fig. 1G, H). At the periphery of the lens stalk, the basement membrane formed two layers, one of which separated the stalk and the anterior lens (Fig. 1H). This layer did not extend to the axial lens however, where the primary lens fibers directly adjoined the stalk cells (Fig. 1D). Examination of the cells making up the stalk revealed no evidence of cell pyknosis or degeneration (Fig. 1D, G).

Distribution of extracellular matrix during lens vesicle detachment is illustrated in Figure 2. Separation of the lens vesicle from the surface ectoderm normally occurs on gestational day 11 (40–45 somite pairs) (Fig. 2A). Immunocytochemical staining for extracellular matrix components at this stage revealed fibronectin between the posterior lens epithelial cells and around but not within, the lens stalk or surface ectoderm (Fig. 2C, E). Staining for laminin demonstrated a complementary distribution, concentrated within the anterior lens epithelium and primary fibers as well as the lens stalk and surface ectoderm (Fig. 2G, I). Studies on embryos exposed to isotretinoin on day 7 of gestation revealed markedly reduced deposition of laminin and fibronectin within and surrounding the microphakic lens and surface epithelium on day 11 (Fig. 2B, D, F, H, I).

Differences in extracellular matrix distribution on gestational day 14 are illustrated in Figures 3 and 4. By this time, staining for fibronectin was normally concentrated in the mesenchyme posterior to the optic cup, the anlage of the choroid and sclera; very little staining was observed within the lens or optic cup (Fig. 3A). Laminin was present within the lens and the basal lamina of the hyaloid vasculature (Fig. 3D). Staining for fibronectin was minimal within or surrounding the optic cup of the treated fetuses (Fig. 3B, C; Fig. 4C). Many of the isotretinoin-treated fe-

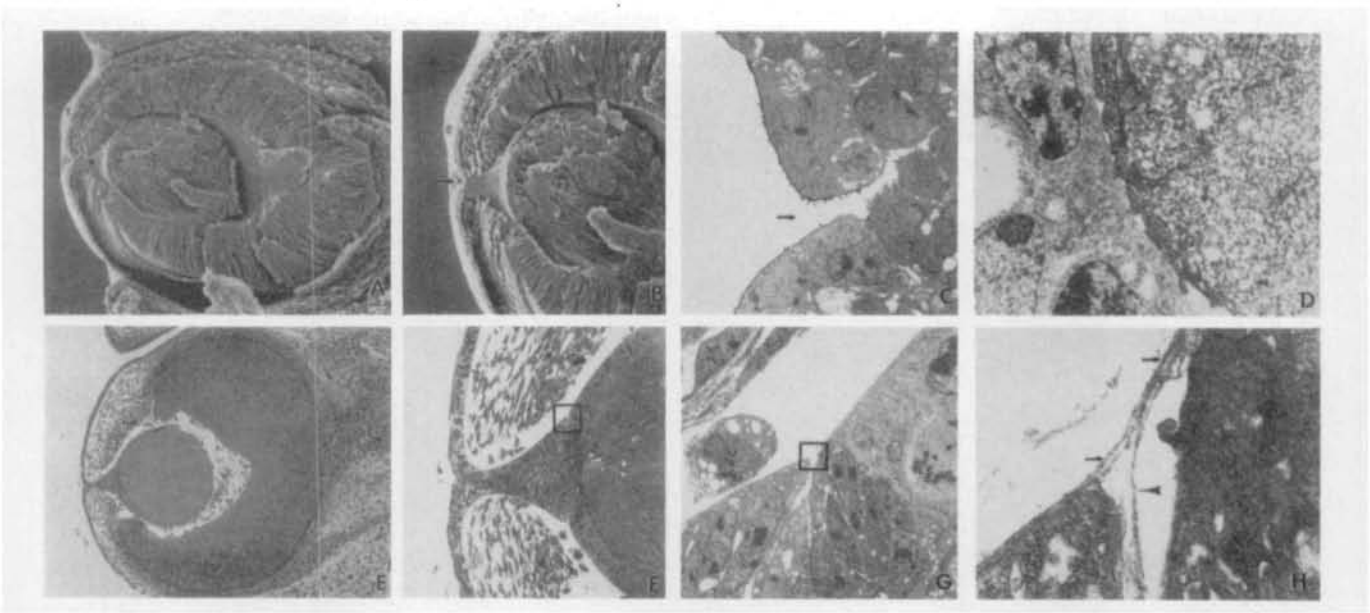

Fig. 1. Keratolenticular dysgenesis: SEM, LM and TEM analyses, day 14. **(A, B)** Persistence of the keratolenticular stalk (*). The arrow in **(B)** indicates the incompletely closed lens pore. L = lens; E = surface ectoderm, R = neural retina, N = neural crest mesenchyme (Original magnification A, ×100; B, ×144). **(C)** TEM of the lens pore seen in **(B)**. The surface epithelial layers have met but remain discontinuous. Surface microvilli which characterize the corneal epithelium are visible (Original magnification ×1600). **(D)** The axial junction between the anterior lens and the lens stalk is indicated by the arrowheads. The cell processes of the primary lens fibers (*) are directly adjacent to the cell membranes of the lens stalk. Note the absence of pyknosis or evidence of cellular degeneration in the stalk (Original magnification ×8333). **(E, F)** Light microscopy illustrates the mechanical obstruction to neural crest migration (N) created by the persistent lens stalk. E = surface ectoderm. (Toluidine blue; Original magnification E, ×120; F, ×400). **(G)** TEM view of area indicated in **(F)**. The abrupt transition between lens epithelium (LE) and stalk epithelium (S) can be seen. An adjacent phagocyte (M) exhibits cytoplasmic vacuoles (Original magnification ×1600). **(H)** Enlargement of area indicated in **(G)**. Two layers of basement membrane can be seen: (1) bridging the lens–stalk junction (small arrows); as well as (2) dividing the two zones peripherally (arrowhead). The second basal lamina may serve to create a plane of cleavage facilitating separation of the lens from the stalk, but is not continuous axially as can be seen in **(D)** (Original magnification ×16,667).

tuses exhibited failure of detachment of the lens from the surface ectoderm. The persistent keratolenticular stalk was surrounded by a PAS-positive basal lamina and exhibited increased staining for laminin as compared with the lens and surface ectoderm of the controls (Fig. 3E, F, Fig. 4A, B, D).

Discussion

The mechanics of normal lens vesicle detachment are not well understood. Previous investigations have indicated a variable sequence of cell death and breakdown of the surrounding basal lamina prior to detachment.[5,6,14] The distribution of specific extracellular matrix components during this process has not been previously described.

In addition to their structural role, the extracellular matrix has been demonstrated to influence cell metabolism, migration and differentiation.[15,16] Fibronectin is a glycoprotein to which neural crest cells preferentially adhere.[17] Laminin is a high molecular weight glycoprotein found exclusively in basal laminae.[18] It consists of a minimum of two subunits (220 and 440 kD) joined by disulfide bonds and has been shown to contribute to the maintenance of epithelial structure.[19] Basement membranes are formed and maintained by epithelial cells; their breakdown may precede or follow epithelial degeneration.

In the teratogen-induced persistent keratolenticular attachment, neither cell death nor basal lamina breakdown appear to occur. In the absence of normal cell death, cells of the persistent stalk appear to segregate from the anterior lens epithelium into a population which more closely resembles the surface ectoderm.

Deposition of basal lamina between the anterior lens and the stalk has been described to follow a plane of cleavage during normal lens vesicle detachment[20] and was observed to occur only at the stalk periphery in the abnormal eyes in this study. Remodeling of tissues during embryogenesis may be associated with simultaneous synthesis and degradation of supportive structures such as basal laminae. Collagenolytic activity has been demonstrated in the reorganization of corneal epithelium[21] and was theorized to be involved in lens vesicle detachment.[22]

In summary, there appears to be a teratogen-induced alteration in the developmental events which

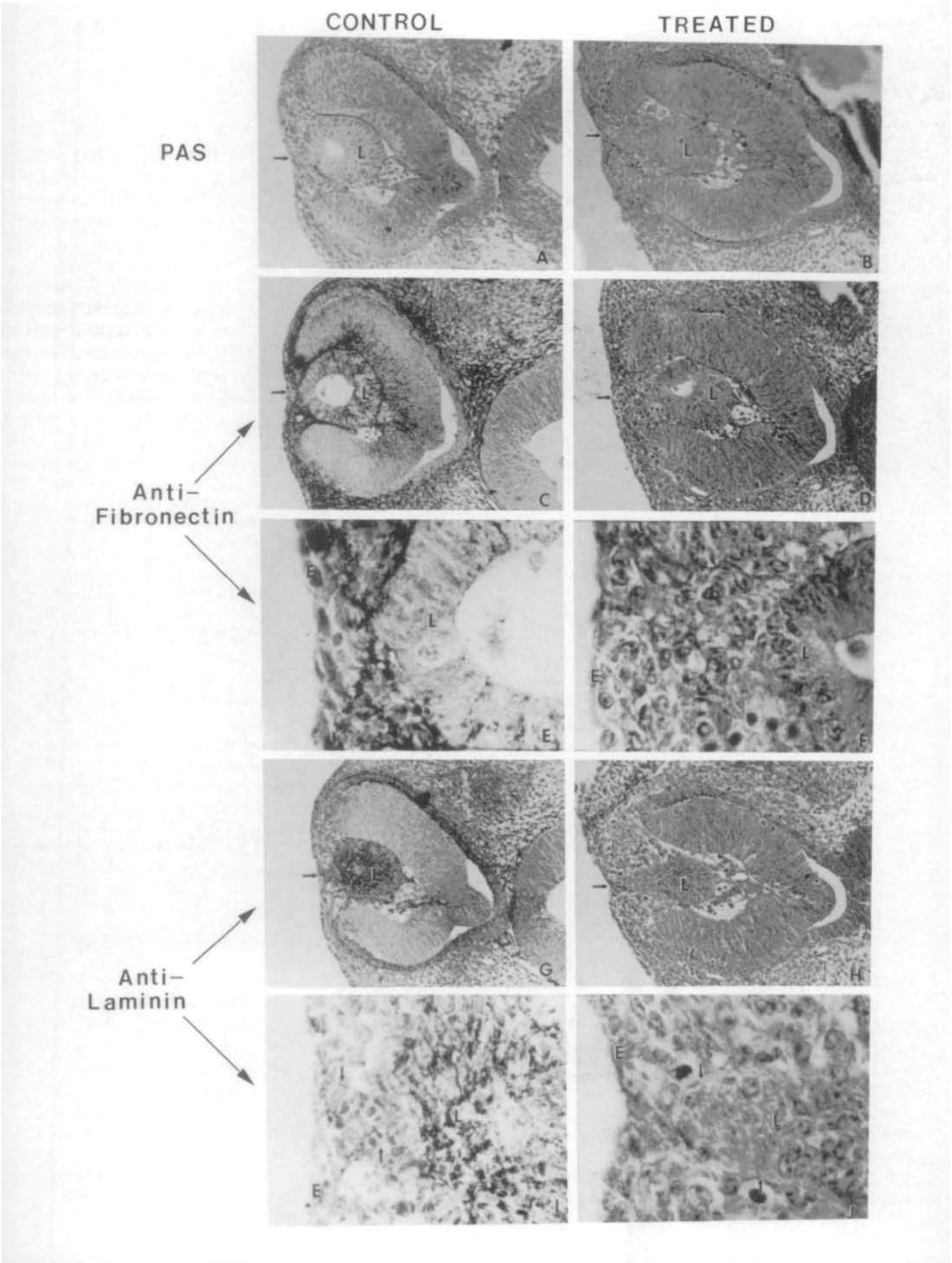

Fig. 2. Lens vesicle detachment: normal and isotretinoin-induced abnormalities; localization of laminin and fibronectin: day 11. **(A, C, E, G, I)** Control embryo (43 somite pairs); **(B, D, F, H, J)** isotretinoin-exposed embryo (43 somite pairs). **(A, B)** Periodic acid Schiff's staining demonstrates the area of the lens stalk (arrow), which is thicker and more elongated in the treated embryo **(B)**. L = lens vesicle (A, ×104; B, ×144). **(C–F)** Immunocytochemical staining for fibronectin. In the control eye, fibronectin is localized within the mesenchymal tissue adjacent to the lens stalk (arrows in **C, D**). Although there is some staining between the primary lens fibers in the posterior portion of the lens vesicle, the anterior lens epithelium and surface ectoderm (E) remain unstained. The eye of the isotretinoin-exposed embryo exhibits much less staining for fibronectin. L = lens vesicle (C, ×104; D, ×144; E, ×531; F, ×600). **(G–J)** Immunocytochemical staining for laminin. The entire lens (L) epithelium and, to a lesser degree, the stalk (arrows) and surface ectoderm (E) exhibit intense staining for laminin in the eye of the control embryo. The ocular anterior segment of the isotretinoin-exposed embryo demonstrates reduced staining (G, ×104; H, ×144; I, ×531; J, ×600).

occur during lens vesicle detachment. Cells within the lens stalk normally undergo programmed degeneration associated with breakdown of the surrounding basement membrane. Dying cells may release collagenase which leads to basement membrane degradation. Laminin and fibronectin may act to separate the stalk from the anterior lens by creating a plane of cleavage and facilitating the axial migration of neural crest cells between the surface ectoderm and the lens. The significance of these extracellular matrix components during this process is supported by the observation that their concentration within the developing anterior segment is reduced following lens detachment. Following teratogen exposure, deficiencies in

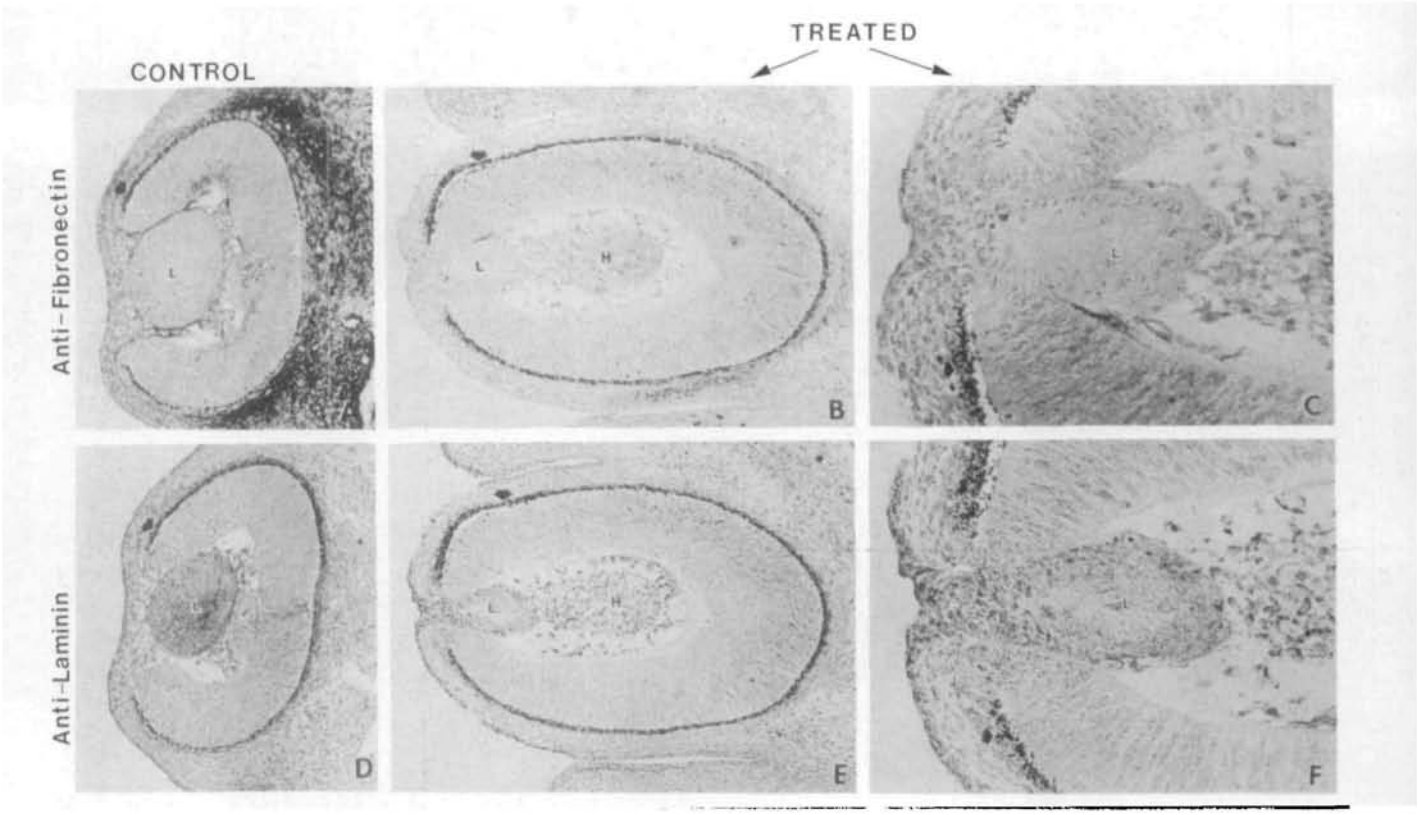

Fig. 3. Keratolenticular dysgenesis: localization of laminin and fibronectin, day 14. **(A, B, C)** Stained for fibronectin; **(D, E, F)** stained for laminin. **(A)** By day 14 of gestation, following detachment of the lens (L) from the surface ectoderm, there was very little staining for fibronectin within the optic cup of the control eyes. The retinal pigmented epithelium (arrowhead) should not be mistaken for positive staining. Intense staining was present posterior to the optic cup, in the area of formation of the choroid and sclera (Original magnification ×119). **(B, C)** Microphthalmia, microphakia, and keratolenticular dysgenesis induced by isotretinoin. Staining for fibronectin was negative within the optic cup; the mesenchyme posterior to the optic cup stained minimally. Arrow indicates the retinal pigmented epithelium. H = hyaloid; L = lens (Original magnification B, ×111; C, ×278). **(D)** Staining for laminin in the control eye was localized to a moderate amount within the lens (L). Arrow indicates the retinal pigmented epithelium (Original magnification ×119). **(E, F)** Persistence of the keratolenticular stalk induced by isotretinoin was associated with more intense staining for laminin within the anterior lens and stalk. The staining of the surface ectoderm rapidly diminished further from the lens pore. Arrow indicates the retinal pigmented epithelium. H = hyaloid; L = lens (Original magnification E, ×111; F, ×278).

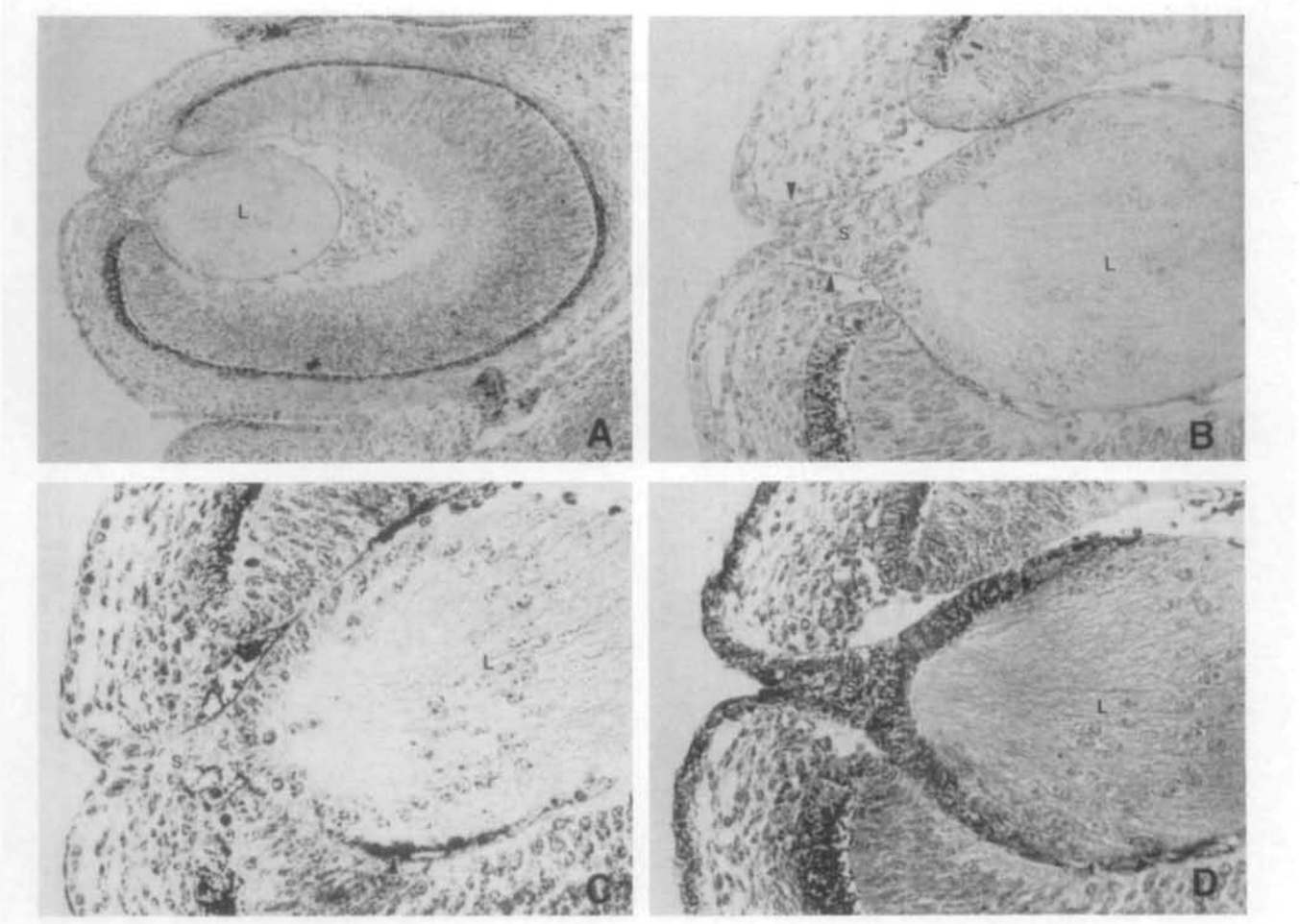

Fig. 4. Keratolenticular dysgenesis: localization of laminin and fibronectin, day 14 (continued). **(A, B)** Isotretinoin-induced persistent keratolenticular attachment illustrating the continuity of the Periodic acid Schiff's positive basement membrane (arrowheads) around the lens stalk (S). L = lens (A, ×133; B, ×343). **(C)** Staining for fibronectin reveals minimal staining in the area of the lens equator (future zonule fibers?). L = lens; S = keratolenticular stalk (×343). **(D)** Staining for laminin is intensely positive in the anterior lens epithelium, stalk (S) and adjacent surface ectoderm. L = lens (×343).

the size of the optic cup and lens vesicle may be associated with reduced production of laminin and fibronectin during the time of normal lens vesicle detachment. Subsequently, these glycoproteins are produced in apparent excess by the cells of the lens epithelium and persistent stalk, which do not exhibit normal cell death. This may be a reflection of a developmental delay or abnormal timing of other inductive influences. Although laminin is produced at this later stage there is failure to establish a basal lamina between the anterior lens and the stalk, possibly because the cells which have differentiated into lens epithelium have not established continuity across the anterior surface of the lens. Much of the laminin produced by the stalk epithelium is deposited around the stalk, presumably further complicating separation.

Key words: Peters' anomaly, anterior segment dysgenesis, basal lamina, teratogenesis, extracellular matrix

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