Age-Dependent Iris Abnormalities in Collagen XVIII/Endostatin Deficient Mice with Similarities to Human Pigment Dispersion Syndrome

Alexander G. Marneros and Bjorn R. Olsen

PURPOSE. Collagen XVIII is expressed in ocular basement membranes (BMs) and inactivating mutations cause Knobloch syndrome, with several ocular abnormalities. In this study we investigated ocular structures in collagen XVIII/endostatin (Col18a1-/-) deficient mice to elucidate the role of this extracellular matrix component in the eye.

METHODS. Eyes of Col18a1-/- and control mice were examined by light and transmission electron microscopy, laser scanning ophthalmoscopy, and fluorescence angiography. Immunohistochemical analysis of neural epithelial, epithelium, and immune cells in the eye was performed with antibodies against established cell markers.

RESULTS. Col18a1-/- mice showed a disruption of the posterior iris pigment epithelial (IPE) cell layer with release of melanin granules. The BM of the posterior IPE was attached to the lens and the nonpigmented epithelium of the ciliary body, which was flattened in mutant mice. In aged mutant mice a severe thickening of the stromal iris BM zone was found, and pigmented cells migrated out of the iris and covered the retina along the inner limiting membrane (ILM), sometimes penetrating into the retina. These cells resembled iris clump cells, and immunohistochemistry demonstrated that they were macrophage-like cells. Furthermore, morphologically abnormal retinal vasculature was seen by fluorescence angiography.

CONCLUSIONS. The abnormalities in the iris and ciliary body of Col18a1-/- mice demonstrate an important role of collagen XVIII for the function of ocular BMs. The absence of this collagen alters the properties of BMs and leads to severe defects in the iris, showing striking similarities to human pigment dispersion syndrome. In addition, loss of collagen XVIII creates changes that allow clump cells to migrate out of the iris. These cells have not been well characterized previously. In the current study we showed that they are macrophage-like cells and are able to penetrate the ILM in mutant mice. The disease mechanism of human pigment dispersion syndrome is not well understood, but Col18a1-/- mice may serve as a model and demonstrate the potential importance of alterations in extracellular matrix components in this disease. (Invest Ophthalmol Vis Sci. 2003;44:2367-2372) DOI:10.1167/iovs.02-1180

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out mice, which were further bred to generate homozygous offspring. To ensure uniformity of genetic backgrounds of littermate wild-type, heterozygous, and homozygous mice, the knockouts were backcrossed with C57BL/6 mice for 15 generations. For all experiments described herein, Col18a1−/− mice and wild-type littermates were used under the same conditions. Animal protocols adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Transmission Electron and Light Microscopy
For histologic examination, eyes were enucleated and fixed for 24 hours in 2.5% formaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After postfixation in 4% osmium tetroxide and dehydration steps, the eyes were embedded in Epon (TAAB; Marivac, Ltd., St. Laurent, Quebec, Canada) overnight. For light microscopy serial sections of 0.5 μm were stained with toluidine blue or azure II, and 85-nm thin sections were used for standard transmission electron microscopy.

Immunohistochemistry
Frozen, 7-μm-thick sections of eye tissue fixed for a few hours in 4% paraformaldehyde were used. Immunofluorescence experiments were performed using antibodies against cellular retinaldehyde binding protein (CRALBP), a marker for retinal and iris pigment epithelium; anti-pan-keratin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), which recognize epithelial cells; and anti-F4/80 antibodies (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada), which recognize murine macrophages. Primary antibodies and FITC-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) were used in serial dilutions.

Immunoelectron Microscopy
Polyclonal antibodies recognizing the N terminus of murine collagen XVIII were raised in rabbit, and the specificity was confirmed by the absence of labeling in Col18a1−/− tissue sections. Immunolabeling was performed as previously described.3

Fluorescence Angiography and Laser Scanning Ophthalmoscopy
The video fluorescein angiography (VFA) system used for these studies has been described.14

RESULTS
Attachment of the Posterior Iris BM to the Ciliary Body and to the Lens with Rupture of the IPE and Pigment Dispersion
Eyes of Col18a1−/− mice and wild-type littermates between 1 week and 22 months of age were embedded in plastic and investigated by light and transmission electron microscopy. All mutant mice displayed separation within the IPE (Fig. 1a). This separation was variable, but commonly the posterior IPE cell layer was detached from the iris. The detachment was often seen to be within the posterior IPE cell layer with rupture of the pigment cells and dispersed pigment granules (Fig. 1b). The separated part of the posterior IPE layer adhered with its BM to the posterior ciliary body BM (Fig. 2). The histologic changes suggest that strong adhesion between these BMs occurs with early onset, and that mechanical force through iris sphincter contractions results in disruption of the IPE. Pharmacological dilation of the pupil and subsequent constriction was maintained in the mutant mice, showing that the iris sphincter is functional. Examination of the lens, removed from eyes not previously fixed in formalin, revealed that the detached IPE layer adhered not only to the ciliary body, but also to the lens (Fig. 3a). Examination of the anterior eye chamber with a dissection microscope in aged mutant mice showed pigmented areas at the pupil site (Fig. 3b).

Collagen XVIII Immunoelectron Microscopy Labeling and Ciliary Body Epithelium Abnormalities
Normal nonpigmented ciliary body epithelium has a well-developed secretory surface with infoldings at its apical side. This epithelium is covered by a thin basement membrane, an ex-
were visible in the pupil (arrow) when compared with wild-type littermates (Fig. 5b).

This abnormal epithelium could already be detected in young (Fig. 5a) 2-month-old mutant mice when assessed by transmission electron microscopy. No structural abnormalities in 2-month-old mutant mice when compared with wild-type littermates (Fig. 6a). Amorphous material and disorganized collagen fibrils along that BM zone resulted in a thickened appearance, which was clearly visible, even by light microscopy. Thus, in addition to changes in the BMs along the vitreous–retina surface, lack of collagen XVIII resulted also in changes of the stromal BM zone, implying a role of this collagen in the structural organization of this BM zone.

**Abnormal Migration of Iris Pigmented Cells along the ILM and Penetration into the Retina**

Laser-scanning ophthalmoscopy was performed in a group of young mice (2 months old) and a group of aged mice (16–18 months old) to assess the retinal structures in the living animal. The retinal surface of young mutant mice showed no pigmentation abnormalities. In aged Col18a1−/− mice, distinct pigmented spots were identified on the retinal surface (Fig. 7a). Some of these spots were also visible in front of the retinal vessels. When ophthalmoscopy was combined with fluorescence angiography these pigmented spots were clearly seen to block the light from retinal vessels (Fig. 7b), demonstrating that these spots are pigmented structures on the surface of the retina and not within the retina. Histologic examination of these eyes revealed pigmented cell clusters and single cells that covered the surface of the retina (Fig. 7c), ciliary body, and iris. These cells were frequently observed to migrate out of the iris stroma (Fig. 6b), whereas no migration through the ciliary body was noted. The cell clusters were also visible with a dissection microscope in the anterior eye chamber of aged mutant mice (Fig. 3b). Examination of the iris–pupil area showed no typical iris ruff in mutant mice, and pigmented spots covered the corneal endothelium, similar to the situation in patients with pigment dispersion syndrome. The pigmented cells commonly appeared as a string of cells, elongating from the iris along the retina and to the optic nerve. The cells showed a rounded appearance when seen in the vitreous (Fig. 8b), but flattened when attached to the ILM along the retinal surface. Most of these pigmented cells were visible at the vitreous–retina border (Fig. 7c). However, some of the cells were seen within the neural retina, suggesting that they can penetrate the ILM of Col18a1−/− mice (Fig. 8a).

Ultrastructurally, the pigmented cells contained small pigment granules similar in size to those of iris stroma melanocytes, compared with wild-type littermates. This BM zone is known to thicken with age in some strains of mice, including the C57BL/6 mouse strain we investigated. However, in Col18a1−/− mice, thickening of the anterior iris BM zone was severely increased in aged mice when compared with wild-type littermates (Fig. 6a). Amorphous material and disorganized collagen fibrils along that BM zone resulted in a thickened appearance, which was clearly visible, even by light microscopy. Thus, in addition to changes in the BMs along the vitreous–retina surface, lack of collagen XVIII resulted also in changes of the stromal BM zone, implying a role of this collagen in the structural organization of this BM zone.

**Age-Dependent Thickening of the Anterior Iris Basement Membrane Zone**

The BMs of the iris and ciliary body, which showed strong adhesion to each other, did not show major structural alterations when assessed by transmission electron microscopy. No thickening or disruption of these membranes was seen even in aged mutant mice. The anterior iris BM zone showed no major structural abnormalities in 2-month-old mutant mice when compared with wild-type littermates.

**Figure 4.** Immuno-electron microscopic labeling of the iris BM zones in a normal C57BL/6 control mouse, using an antibody that recognizes the N-terminal region of collagen XVIII and gold-conjugated secondary antibodies. (a) Labeling of the matrix subjacent to the lamina densa of the BM (arrow) between the iris stroma and the IPE cell layers. (b) Labeling along the posterior iris BM (arrow) at the posterior IPE-vitreous border. Scale bar: (a) 0.15 μm; (b) 0.3 μm. Magnification: (a) ×40,000; (b) ×20,000.

**Figure 5.** Transmission electron micrographs of the ciliary body of a 2-month-old Col18a1−/− mouse eye (a) and the eye of a wild-type littermate (b). (a) Reduced secretory surface and apical infoldings of the nonpigmented ciliary body epithelium in a Col18a1−/− mouse (arrow), (b) Wild-type ciliary body nonpigmented epithelium showing extensive apical infoldings (arrow). Scale bar: (a) 1.5 μm; (b) 2.8 μm. Magnification: (a) ×4000; (b) ×2100.

**Figure 3.** (a) Lens of a 22-month-old Col18a1−/− mouse eye that was not fixed in formalin. Parts of the detached posterior IPE layer are adherent to the anterior lens capsule. (b) View of the iris (arrow) and pupil of a 22-month-old Col18a1−/− mouse eye. Clusters of pigmented spots were visible in the pupil (arrow). Magnification: (a) ×4; (b) ×8.
cytes, but also larger oval-shaped pigment granules as seen in the IPE. Their surface showed plasma membrane protrusions, resembling villi or pseudopods (Fig. 8b). The ultrastructural morphology of the pigmented cells is very similar to the morphology of iris clump cells,13 which can normally be seen in the iris stroma and are usually not found outside the iris.

Characterization of Pigmented Cells as Macrophage-like Cells

Ultrastructural analysis suggested that the abnormally located pigmented cells in the mutant mice are clump cells. It has been suggested that clump cells may be macrophage-like cells, but this has not been shown.13 We performed immunohistochemical analysis of these cells using antibodies against established cell markers of IPE cells, melanocytes, or immune cells. No labeling of these pigmented cells with antibodies against CRALBP or keratins was observed by immunofluorescence, whereas labeling of IPE cells in the iris was noted. Distinct labeling at the cell membrane of these pigmented abnormally migrating cells was observed when using antibodies against the murine macrophage marker F4/80, a 160-kDa transmembrane glycoprotein (Fig. 8c). In conclusion, the absence of collagen XVIII results in an age-dependent abnormal migration of pigmented macrophage-like cells that originate from the iris stroma and have the ability to penetrate the ILM.

Abnormal Retinal Vasculature in Collagen XVIII Deficient Mice

Col18a1−/− mice show a developmental delay in hyaloid vessel regression, affecting postnatal levels of VEGF expression in the neural retina.3 We assessed the vasculature in adult mice between 2 and 18 months of age by fluorescein angiography. All mutant mice displayed an abnormal pattern of retinal vessels, with irregular bending of major retinal arteries (Fig. 9b). The perfusion of the retina revealed neither areas with significant reduction of blood supply, nor any significant leakage of fluorescein. Thus, despite the irregular appearance of retinal vessels, they allow for a proper perfusion of the retina. Consistent with this finding, no cell loss or atrophy of the retina was detected.

DISCUSSION

Col18a1−/− mice have ocular abnormalities, most strikingly affecting the iris. The rupture of the posterior IPE with pigment dispersion, most likely due to mechanical force after adhesion of the posterior iris BM to the surface of the ciliary body and to the lens, shows many similarities to human pigment dispersion syndrome. In patients with this syndrome, which in part shows autosomal-dominant inheritance,15 pigment granules from the iris are released and deposited within the eye.16 It has been hypothesized that pigment dispersion involves mechanical damage to the posterior IPE resulting from iridozonular friction during physiologic pupillary movement.17,18 However, the molecular basis of this disease remains unknown. The prevalence of pigment-dispersion syndrome has been shown to be much higher than previously appreciated, with a prevalence of approximately 2.45% in a white population.19 Dispersion of pigment can lead to occlusion of the ocular drainage structures with elevation of the intraocular pressure, a condition called pigmentary glaucoma. Several studies indicate that in up to 50% of individuals with pigment dispersion glaucoma will eventually develop.20–22 A mouse model for pigmentary glaucoma is the DBA/2J(D2) mouse, with mutations in melanosomal proteins, encoded by the genes for Tyrp1β and Gpmmb.23 The increased intraocular pressure results in a degeneration of the optic nerve with loss of vision. However, no mouse model for pigment dispersion syndrome exists that shows IPE damage with pigment dispersion and normal intraocular pressure. Col18a1−/− mice showed no morphologic change of the neural retina or optic nerve that would result from glaucoma. Measurements of the intraocular pressure in Col18a1−/− mice also provided no evidence of glaucoma in these mice (Pihlajaniemi T, personal communication, November 2002). In conclusion, mice deficient in collagen XVIII represent the first mouse model with similarities to human pigment dispersion syndrome and no glaucoma and suggest a role for the extracellular matrix and collagen XVIII/endostatin in this syndrome.

Pigment dispersion syndrome frequently affects young individuals and is commonly associated with myopia and a high risk of retinal detachment.24–26 Very similar to patients with Knobloch syndrome who have inactivating collagen XVIII mutations in which high myopia and retinal detachment are major clinical features.11 Clinical examination of the eyes of such patients has revealed, in some cases, structural iris abnormalities with atrophy and synechiae.27,28 These reports suggest that the histopathological changes observed in Col18a1−/− mice might be found in patients with Knobloch syndrome as well. However, because no donor eyes are available from...
Knobloch syndrome patients, the histologic abnormalities resulting from the absence of collagen XVIII in humans remain unknown.

In addition to the IPE abnormalities, we find in aged Col18a1−/− mice pathologic migration of cells originating from the iris stroma and containing melanin granules. We characterized these pigmented iris cells by transmission electron microscopy and immunohistochemistry and identified them as macrophage-like cells with the ultrastructural appearance of clump cells. These cells have not been well characterized, but they have been described as pigmented round cells in the iris stroma, with unknown function. The clump cells of Koganei have been hypothesized to be macrophage-like cells, mainly based on results from photocoagulation experiments. The observation of such macrophage-like cells outside the iris stroma in aged Col18a1−/− mice is intriguing.

Immunoelectron microscopy of iris with antibodies against collagen XVIII showed no labeling within the iris stroma, but...
instead a distinct localization of collagen XVIII at the BM zones of the iris. Therefore, the absence of collagen XVIII may not directly affect anchorage of these pigmented cells within the iris stroma. Instead, the observed abnormal migration of these cells may be a secondary, age-dependent consequence of functional abnormalities in ocular BMs of Col18a1−/− mice.

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