Structurally altered basement membranes and hydrocephalus in a type XVIII collagen deficient mouse line

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Type XVIII collagen/endostatin is known to be crucial for the eye, as witnessed by severe eye defects in Knobloch syndrome patients with mutations in this collagen and in Col18a1⁻/⁻ mice. We show here that in a specific C57BL background, 20% of the Col18a1⁻/⁻ mice developed hydrocephalus, and dilation of the brain ventricles was observed by MRI in all of the mutant mice. Significant broadening was observed in the epithelial basement membrane (BM) of the choroid plexuses (CP), its width being 86.4 ± 10.52 nm, compared with 61.4 ± 6.05 nm in wild-type mice. The CP epithelial cell morphology was balloon-shaped rather than cuboidal, and the microvilli of the apical surface of the CP epithelium contained more vacuoles in the null mice than in the wild-type, as also did the CP epithelial cells, which is suggestive of alterations in cerebrospinal fluid production. Analysis of BMs elsewhere in the body revealed a broadened epidermal BM in the Col18a1⁻/⁻ mice, but this did not result in any apparent functional deficiencies. Moreover, markedly broadened BMs were found in the atrioventricular valves of the heart and in the kidney tubules, whereas the glomerular mesangial matrix of the kidneys was expanded in the mutant mice and serum creatinine levels were elevated, indicating alterations in kidney filtration capacity. We thus suggest that type XVIII collagen is a structurally important constituent of BMs, and that its absence can result in a variety of phenotypic alterations.

INTRODUCTION

Type XVIII collagen is a non-fibrillar collagen with heparan sulphate side chains that occurs widely in the body in association with basement membranes (BMs) (1–4). It has a large non-collagenous N-terminal domain with a thrombospondin-1-like motif and an interrupted collagenous domain (5). Its globular C-terminal NC1 domain contains a fragment called endostatin, which strongly inhibits endothelial cell proliferation and migration, and reduces tumor growth (6). The mechanism of action of endostatin has been addressed in many studies, including demonstration of its binding to cell-surface glypican (7), its interaction with integrins implicated in angiogenesis (8,9) and its blocking of VEGF-mediated signalling by binding to VEGFR-2 (10). It can inhibit certain matrix metalloproteinases (11,12) and cyclin D1 (13), and it may be associated with lipid rafts and inhibit cell migration by inducing reorganization of the actin cytoskeleton (14). One member of the collagen family, type XV collagen, is structurally homologous to type XVIII collagen, and the highest homology involves precisely this C-terminal endostatin-like region, which can also affect angiogenesis in the case of type XV collagen (15,16).

Mutations in the human type XVIII collagen gene can cause Knobloch syndrome (17,18), a recessive disorder with high myopia, vitreoretinal degeneration and an occipital
encephalocele. The symptoms associated with the Knobloch syndrome show great variation between patients, however, and can include defects in the heart, kidney and central nervous system, for example (17,19). In mice, a lack of type XVIII collagen has been shown to result in ocular abnormalities that include delayed regression of the hyaloid vessels along the surface of the retina and abnormal outgrowth of the retinal vasculature (20). The critical role of type XVIII collagen in the eye was further emphasized by the identification of disruption of the iris, atrophy of the ciliary body and retinal pigment epithelial cell degeneration in null mice (21,22). Studies with Caenorhabditis elegans have suggested that type XVIII collagen may affect cell migration and axon guidance via its NC1/endostatin domain and that this collagen is important for synapse formation (23,24).

As the tissues responsible for the production of the ocular and cerebral fluids, the ciliary processes and choroid plexuses (CPs), respectively, are developmentally, structurally and functionally comparable (25), we propose here that in addition to malformation of the ciliary body of the eye (21), lack of type XVIII collagen in mice also results in ultrastructural changes in the BMs of the CPs in the brain and leads to dilation of the brain ventricles and susceptibility to hydrocephalus. We also present data on the broadening of BMs in the epidermis, heart atrioventricular valves and kidney proximal tubules of a Col18a1−/− mouse line, and on an increase in the glomerular mesangial matrix.

RESULTS

Col18a1−/− mice manifest hydrocephalus

When Col18a1−/− mice in a C57BL/6J genetic background were bred with the C57BL/6JOlaHsd strain, we noticed that some of them developed a significant enlargement of the skull compared with the wild-type mice (Fig. 1A and B). Removal of the skin showed massive bleeding under the skull of these null mice and findings indicative of severe hydrocephalus (Fig. 1C and D). Sagittal sections of the head revealed contraction of the brain and displacement of the skull bones due to pathologically elevated intracranial pressure, and also an extensively dilated lateral ventricle compared with the wild-type (Fig. 1E and F). The Col18a1−/− mice were born without any signs of dilation of the brain ventricles, and the first cues to significant enlargement were usually seen at the age of 3 weeks to 2 months.

The incidence of overt hydrocephalus in the Col18a1−/− mice was investigated by crossbreeding heterozygous Col18a1+/− mice and observing the offspring for 2 months. Severe hydrocephalus were seen in nine out of 42 Col18a1−/− mice during this period, whereas only five of the 130 heterozygous littermates and none of the 33 wild-type littermates showed any signs of dilated ventricles.

Magnetic resonance imaging of the brain

Only extensive dilation of the brain ventricles is detectable by traditional histology, whereas small differences in ventricle sizes remain undetected owing to sample processing artefacts. We thus resorted to magnetic resonance imaging (MRI), which allowed high-resolution investigation of the ventricles of live control and mutant mice at ages of 3 weeks (three controls and three knockout mice), 2 months (three controls and two knockout mice), 2.5 months (six controls and six knockout mice) and over 1 year (two controls and two knockout mice). It is important to note that none of these 13 null mice used in the MRI analysis had overt hydrocephalus with conspicuous skull enlargement and behavioural changes. However, two of them had a mildly enlarged skull but not visibly manifested hydrocephalus. The brain ventricles of the wild-type mice

![Figure 1](image-url)
showed only minor variations in size (Fig. 1G and H), whereas the lateral, third and fourth brain ventricles of the Col18a1−/− mice varied from mildly dilated (Fig. 1I and J) to massively enlarged (Fig. 1K and L). To test this effect statistically, the volumes of the ventricles of the 2.5-month-old mice were determined from the MRI images. Although one mouse with a mildly enlarged skull and clear MRI changes was excluded, the remaining ones still had slightly larger ventricles, 31.1 ± 5.1 mm³ (n = 5), than the control animals, 25.6 ± 2.8 mm³ (n = 6), the difference being statistically significant (P = 0.026). Thus, enlargement of the brain ventricles appeared to be a highly penetrant feature in the knockout mice.

Localization of collagen XVIII/endostatin in the brain

In order to relate the observed hydrocephalus phenotype to the occurrence of type XVIII collagen, we used immunofluorescence to examine the localization of this collagen in the brain. The pial BM and endothelial BMs of most blood vessels showed clear staining with antibodies against type XVIII collagen (Fig. 2A and B), the strongest staining being seen in the epithelial BM of the CP (Fig. 2C). Analysis of type XVIII collagen expression at 13.5 dpc of fetal development revealed a comparable expression pattern to that in the adult animals (Fig. 2D and E). Immunoelectron microscopy of the CP with an antibody against the N-terminal non-collagenous domain shared by all three type XVIII collagen variants (ELQ) revealed clear signals in the endothelial and epithelial BMs that could be localized to the border of the BM with the adjacent matrix (Fig. 2F and G).

Ultrastructural changes in the CP epithelium

As type XVIII collagen was expressed in tissues involved in both the secretion and absorption of cerebrospinal fluid (CSF), it is apparent that malfunction of either end of the CSF circulation pathway can lead to dilation of the brain ventricles and the development of hydrocephalus in Col18a1−/− mice. To avoid secondary changes caused by elevated intracranial pressure, only mice with a very mild phenotype were used to study the cause of this hydrocephalus.

The CP develops properly in the Col18a1−/− mice, and no difference was seen between the mutant and wild-type CP or ependyma at the light microscopy level (Fig. 3A and B). Transmission electron microscopy nevertheless revealed that the mutant CP epithelial cells were round in shape rather than cuboidal, with individual cells making protrusions into the ventricle (Fig. 3C and D). In addition, instead of being apical, the tight junctions were located more closely on the basal side of the epithelial cells in the CPs of the null mice (Fig. 3C and D), and there were more vesicles inside the mutant CP epithelial cells than in the wild-type (Fig. 3C and D), possibly indicating alterations in the CSF outflow. The apical microvilli were abnormally dilated and contained numerous vacuoles (Fig. 3E and F).

The most remarkable finding was that the BM underlying the CP epithelial cells was broader in the null mice than in the wild-type (Fig. 3G and H). The thickness of the epithelial BM in the 2-month-old Col18a1−/− mice was found to be

86.4 ± 10.52 nm (n = 6, 19 measurements/mouse), as compared with 61.4 ± 6.05 nm in their wild-type littermates (n = 6, 19 measurements/mouse) (Fig. 4A). Thus, the mutant BMs were significantly broader (P < 0.001) and varied significantly in thickness (P < 0.001).

Lack of clear defects in other brain structures

Although the endostatin domain of type XVIII collagen is known to possess antiangiogenic activity (6), the brain blood
vessels of the null mice were found to develop properly. The BMs of the fenestrated capillaries inside the CP connective core were somewhat broadened (Fig. 3G and H), but this difference was less conspicuous than in the epithelial cells. The massive bleeding seen in the mutant mice with severe hydrocephalus (Fig. 1D) is most probably secondary to the pathologically elevated intracranial pressure, which will inevitably lead to disruption of the brain capillaries. The pial BM, which is known to be important for cortical histogenesis (26), was not morphologically altered in the absence of type XVIII collagen (data not shown). Moreover, the ciliated ependymal cell layer and the subcommissural organ in the Col18a1<sup>-/-</sup> mice were morphologically similar to the wild-type (data not shown), and no changes were seen in the structure of the cerebral aqueduct in the cases of mild dilation of the brain ventricles, whereas in the mutant mice with severe hydrocephalus the cilia of the ependymal cell layer were atrophic, the cerebral aqueduct was sometimes narrowed and the CP epithelium was ruptured (data not shown). These changes are likely to be secondary to the elevated intraventricular pressure and massively enlarged ventricles.

**Broadened epidermal BM in Col18a1<sup>-/-</sup> mice**

As the broadened BMs in the CP raised the possibility that structurally abnormal BMs may also exist elsewhere in the body, we examined the BM at the epidermal–dermal junction carefully by electron microscopy, as the cell–BM–fibrillar matrix interphase is particularly well characterized in this location (27). Interestingly, the epidermal BM was also found to be broadened in the null mice (Fig. 5A and B), the measured thicknesses at 12 days and 7–15 months being 59.2 ± 8.3 and 77.8 ± 8.95 nm, respectively, as compared with 46.4 ± 3.77 and 56.8 ± 5.81 nm, in wild-type mice of the same ages (<i>P</i>, 0.001 in both the age groups) (Fig. 4B). The BM thickness also varied significantly in the mutants (<i>P</i> < 0.001 in both the age groups).

**Broadened atrioventricular valve BMs in Col18a1<sup>-/-</sup> mice**

We have recently determined the localization of collagen XVIII in the cardiac valves during mouse development by light and electron microscopy (L.S. Carvalhaes et al., manuscript in preparation). This study was extended here to ultrastructural analysis of the atrioventricular valves of the Col18a1<sup>-/-</sup> mice. The leaflets of the tricuspid valve are composed of an endothelial layer overlying a central core of connective tissue that contains fibroblast-like cells (Fig. 5C). At higher magnifications the collagen fibres are seen to be well organized, and are in general oriented parallel to the base-to-tip axis (data not shown). Collagen fibres are more abundant on the ventricle side of the leaflet, whereas elastin fibres are more common on the atrial side (data not shown). In adult animals, the N-terminal antibody ELQ gives a clear staining on the matrix side of the BM underlying the endothelial cell layer (Fig. 5D).

No obvious differences were observed in the overall morphology of the tricuspid valve leaflets or in the endothelial cells of the Col18a1<sup>-/-</sup> mice, but at higher magnification the
BM underlying the endothelial cell layer of the mutant mice was clearly broader than that of the wild-type littermates, 128.44 ± 19.13 nm on the atrial side and 132.04 ± 23.66 nm on the ventricular side, as opposed to 64.22 ± 2.54 and 62.9 ± 3.04 nm, respectively (Fig. 5E and F). This broadening was statistically significant \((P \leq 0.001)\) on both the atrial and ventricular sides of the \(Col18a1^{-/-}\) leaflets (Fig. 4C and D). In addition, both mutant BMs varied significantly in thickness \((P \leq 0.01)\).

Ultrastructural and functional defects in the kidney

As type XVIII collagen is known to occur in the epithelial and endothelial BMs of both developing and adult kidneys \((4,16,28–30)\), we studied its ultrastructural localization in the mouse kidney by immunoelectron microscopy. The N-terminal antibody ELQ gave clear staining on the matrix side of the kidney tubular BM (Fig. 6A) and in the BM of Bowman’s capsule (Fig. 6B), although some additional staining was detected on the lamina lucida side of the latter (Fig. 6B). In the glomerular BM, which is a specialized structure consisting of fused podocyte and vascular endothelial BMs, a diverse staining pattern was observed, immunogold labelling being located on both sides (Fig. 6C). Faint staining was also detected in the mesangial matrix (Fig. 6D).

Although light microscopic studies of the \(Col18a1^{-/-}\) kidneys did not reveal any abnormalities (data not shown), electron microscopy showed clear broadening of the tubular BM (Fig. 7A and B). To confirm this finding, the thickness of the proximal tubular BM was measured in control and mutant animals at ages of 12 days, 2 months and 7–11 months (adult) (Fig. 4E). The BMs of the knockout mice proved to be significantly broader in all the three age groups \((P \leq 0.001)\), whereas the mutant BM thickness varied significantly in the two oldest age groups \((P \leq 0.05\) at 2 months, \(P \leq 0.001\) at 7–11 months).

Careful studies of the BMs of the Bowman’s capsule and the glomerulus of the \(Col18a1^{-/-}\) mice did not reveal any structural changes (data not shown), but the mesangial matrix was expanded in some of the mutant glomerulae (Fig. 7C and D) and its structure had altered from clearly defined to somewhat indefinite (Fig. 7E and F). Despite an increase in the amount of extracellular matrix in the mesangium, type IV collagen staining did not give an increased immunosignal in the mutant glomerulae compared with the wild-type (Fig. 7E and F).

In view of the changes detected in the kidneys of the \(Col18a1^{-/-}\) mice, we measured serum creatinine levels in the mutant and control animals, and found these to be significantly elevated in the knockout mice \((10.11 ± 1.59 \text{ mol/l})\) compared with the wild-type \((8.42 ± 2.07 \text{ mol/l})\) \((P \leq 0.025)\), indicating alterations in kidney filtration capacity.

DISCUSSION

Hydrocephalus in the \(Col18a1^{-/-}\) mouse line is background-dependent

We backcrossed C57BL/6J inbred \(Col18a1^{-/-}\) mice once with C57BL/6JolaHsd mice and maintained the ensuing line by means of heterozygotic \((Col18a1^{+/-})\) matings. Surprisingly, about 20% of these mice developed severe hydrocephalus, and dilation of the brain ventricles was observed in all of
the mutant mice by MRI. Thus, crossing of the two substrains led to increased heterozygosity in our mouse strain, and after several brother–sister matings we now have an ‘Oulu’ C57BL/6J mouse strain presenting phenotypic features not previously observed in Col18a1<sup>2/2</sup> mice (20). In fact it has recently been suggested that differences exist between the C57BL/6 strains and substrains (31). To circumvent this sub-strain complexity, we used wild-type littermates as controls in all the analyses, so that our findings showed associations with a lack of type XVIII collagen. Where the inbred C57BL/6 strain is known to be susceptible to hydrocephalus, with an incidence of 4–6% (32), the ‘Oulu’ C57BL/6J mice showed a 3.8% incidence of overt hydrocephalus in heterozygous Col18a1<sup>+/−</sup> mice and 21.4% in Col18a1<sup>−/−</sup> individuals, compared with 0% in controls. Thus, the incidence of severe hydrocephalus was significantly increased in the Col18a1<sup>−/−</sup> mice (P < 0.001), whereas the low occurrence of hydrocephalus in the heterozygous mice closely corresponds to the rate of spontaneous hydrocephalus development reported previously (32). A deletion in the Nr2e1 gene has been previously reported to lead to hydrocephalus formation that is detected only in the C57BL/6 background and not when backcrossed with other mouse lines (33). As shown here, a lack of type XVIII collagen also enhances the vulnerability of inbred C57BL/6 mice to dilation of the brain ventricles and the development of hydrocephalus.

**Role of type XVIII collagen in the CP BM**

Hydrocephalus is a complex disorder of CSF dynamics in which an accumulation of CSF results in pathological dilation of the brain ventricles. Its incidence in humans is 1–1.5 per 1000 births (34). The genetic factors responsible for its development are not known, but it is often associated with developmental defects such as the CRASH, Arnold-Chiari and Dandy Walker malformations. The main sites of secretion of the intracerebral fluid are the CPs, which are located within the brain ventricles. The development of hydrocephalus may be caused by overproduction of CSF, obstruction of its free flow or its impaired absorption in the subarachnoid space.

Analysis of the mice produced for this study, combined with earlier work, indicates that type XVIII collagen expression in the brain takes place in the BMs of the blood vessels, the pia...
and the CPs (3, 35), and that the staining observed in 13.5 dpc fetuses is comparable to that in adult mice. Attempts to investigate the mechanism lying behind the development of hydrocephalus revealed no differences in the pial BM, in the amount and localization of the brain vessels, or in the composition of the cortex between the wild-type mice and those with mildly dilated brain ventricles. However, significant broadening was observed in the epithelial BM of the CPs, which are composed of a tight epithelium with tight apical junctions that provide a basis for the blood–CSF barrier (36). The Col18a1<sup>−/−</sup> mice have tight junctions in their CPs, but the cell morphology is balloon-shaped rather than cuboidal, so that the tight junctions seem to be localized closer to the basal side. Moreover, the microvilli of the apical surface of the CP epithelium in the null mice contain more vacuoles than in the wild-type, as also do the CP epithelial cells, possibly indicating alterations in CSF production. Investigations into whether the abnormal BM of the Col18a1<sup>−/−</sup> mice affects the expression of Na<sup>+</sup>K<sup>+</sup>-ATPase and aquaporin 1, which are involved in active Na<sup>+</sup> transport and water flux from the blood across the CPs and into the ventricles (37, 38), revealed no differences in the immunolocalization of these molecules between the wild-type and Col18a1<sup>−/−</sup> samples (data not shown). It has been suggested that the BM may promote the organization of the CP epithelial cells into a functional epithelium and thus maintain the integrity of the blood–CSF barrier (39). We consider it likely that the morphological changes detected in the CP epithelial cells and the alterations in the production of CSF in the Col18a1<sup>−/−</sup> CPs are due to changes in the epithelial BM, which lead to altered filtration of the brain fluid components from the CP capillaries into the epithelial cells, and not to changes in the major fluid transporters.

Hydrocephalus caused by overproduction of CSF is rare in humans and is mostly associated with CP papillomas (34). We are aware of two reports on mice that may have developed hydrocephalus on account of CP abnormalities, i.e. deficiencies in the transcription factors Otx and E2F-5, leading to hydrocephalus with morphological changes in the CP epithelial cells (40, 41).

**Type XVIII collagen in hydrocephalic disorders**

The Col18a1<sup>−/−</sup> mice are similar to Knobloch patients in terms of their eye abnormalities (17, 18, 20, 21), but encephalocele seems to be typical only of human patients. Kliemann et al. (19) have recently reported on four patients with central nervous system defects, two of whom also had ventricular dilation with subependymal heterotopic nodules. In the light of these reports and our new findings with regard to Col18a1<sup>−/−</sup> mice, dilation of the brain ventricles seems to be a new, additional phenotype caused by type XVIII collagen deficiency.

A point mutation in the forkhead transcription factor foxc1 causes the congenital hydrocephalus (ch) mouse mutant (42), and mice that are heterozygous for a null mutation of this gene have multiple anterior segment disorders closely resembling...
many of the abnormalities seen in the \textit{Col18a1}^{−/−} mice (43). Mutations in the human \textit{FOXC1} gene are found in some cases of the Axenfeld–Rieger syndrome and in Peters’ anomaly patients, who suffer from defects in the anterior segment of the eye, the hyaloid system, teeth, periumbilical skin and heart, and in some rare cases also hydrocephalus (44,45). In addition, dilation of the brain ventricles is a fairly common symptom in Peters’-Plus syndrome, involving other developmental abnormalities as well as Peters’ anomaly (46). Interestingly, Moog \textit{et al.} (47) reported on two sisters with recessive Axenfeld–Rieger syndrome who also had hydrocephalus and leptomeningeal calcifications. The close similarities in eye phenotypes between the \textit{Col18a1}^{−/−} mouse line and the Axenfeld–Rieger syndrome, Peters’ anomaly and Peters’-Plus syndrome patients indicate a possible role for type XVIII collagen in these human defects, and the development of hydrocephalus seems to be one additional phenotype connecting this collagen type with the human syndromes.

\textbf{Abnormal epidermal and atrioventricular valve BMs with no apparent phenotypic consequences}

The width of the epidermal BM was 1.4-fold greater in the adult \textit{Col18a1}^{−/−} mice than in the wild-type mice, and the BM underlying the endothelial cells that sheath the atrioventricular valves was over 2-fold wider in the \textit{Col18a1}^{−/−} mice. This BM must be able to resist the continuous stresses imposed on it during repeated opening and closing of the valves over the entire lifespan of the individual. Another BM that is similarly affected by mechanical stress is the iris, which is markedly expanded in the mutant mice (21). Nevertheless, neither the skin nor the heart was functionally affected, possibly due to these animals’ sedentary life style.

\textbf{Kidney abnormalities in the \textit{Col18a1}^{−/−} mice}

Type XVIII collagen has been shown to be involved in intra-organ patterning processes during kidney development in organ cultures (48,49). It is found in Bowman’s capsule, the glomerular and tubular BMs and the mesangial matrix of the kidney in both humans (4,29) and mice (16,28,30). Our immunoelectron microscopy results indicating type XVIII collagen staining along the tubular, Bowman’s capsule and glomerular BMs and in the mesangial matrix agree well with the previous findings. Moreover, though endostatin immunolabelling in the kidney tubular BM has been reported previously (30), we demonstrate here the occurrence of the N-terminal non-collagenous domain on the matrix side of the tubular BM. Thickenning of the glomerular and tubular BMs and expansion of the mesangial matrix are characteristic changes seen in diabetic nephropathy, a major complication of diabetes in humans (50). Among the extracellular matrix proteins, mutations in perlecan have been shown to be associated with diabetic nephropathy in humans (51). The kidneys of the \textit{Col18a1}^{−/−} mice developed normally, but ultrastructural analyses revealed broader BMs in the proximal tubulus and the glomerular mesangial matrix was expanded. These abnormalities are mild compared with the renal changes seen in diabetic nephropathy and resemble its early stages. The serum creatinine levels of the mutant mice are nevertheless elevated, indicating impaired kidney filtration capacity. In the light of the findings in the \textit{Col18a1}^{−/−} mouse line, we propose that type XVIII collagen may also have a role in the susceptibility to renal damage associated with diabetes, for instance.

\textbf{Role of type XVIII collagen in BMs}

A lack of type XVIII collagen seems to affect BMs in a tissue-specific manner, so that some show no obvious structural abnormalities, whereas others are clearly broadened. The BM abnormalities in the eye lead to defoliation of the posterior portion of the iris and atrophy of the ciliary body and retinal pigment epithelial cells (21,22), whereas those in the CP and the kidney tubules can cause hydrocephalus and altered kidney filtration, respectively. In most tissues, however, the altered BMs do not lead to obvious symptoms. It is significant that many of the patients with typical Knobloch syndrome symptoms have other, additional clinical findings, including heart and kidney defects. The first report of Knobloch syndrome included one patient with cardiac dextroversion (52), and one of the patients described by Czeizel \textit{et al.} (53) developed pyelonephritis caused by a duplicated collecting system and bifid ureter in the left kidney. As in human patients, the most crucial defects in mice lacking type XVIII collagen are detected in the eyes (20,21), but as shown here, abnormalities may also occur in other tissues. We consider it likely that, by rendering BM structures weaker, the lack of type XVIII collagen may predispose the individual to a variety of symptoms, the severity of which is greatly dependent on the particular genetic environment. This is clearly shown in the development of hydrocephalus in the \textit{Col18a1}^{−/−} mouse line in the present specific genetic background and in human patients with a variety of symptoms (17).

In summary, we propose that type XVIII collagen has a structural role in BMs. It has been shown that its absence leads to broadening of the epithelial BM of the iris and ciliary body (21), and we show here that broadening of the BM is not confined to the eye, as the analysis of CPs, skin, atrioventricular valves and kidney proximal tubules from null mice revealed that BMs were significantly broader than that in the controls. It is possible that the broadening of the BMs in the \textit{Col18a1}^{−/−} mice seen at the electron microscopy level may be due to a minor increase in the amount of other BM components not detectable by immunostainings, or that some other BM components not tested so far may have altered the staining intensities in the mutant BMs. On the other hand, it is conceivable that type XVIII collagen could have a role in binding to other BM components, as demonstrated by the binding of its C-terminal NC1 and endostatin domains to laminin-1, perlecan, heparin and fibulin-1 and -2 (54). Type XVIII collagen has a polarized location in the skin BM, with its C-terminal endostatin domain embedded in the BM and the N-terminal portion located at the BM–matrix interface (22, H. Elamaa \textit{et al.}, manuscript in preparation). A similar polarized location is suggested here by the occurrence of the type XVIII collagen N-terminal epitopes at the BM–matrix interface in the BMs of the CP epithelial cells, heart valve endothelial cells and kidney tubular cells. The occurrence of type XVIII collagen at the fibrillar matrix
interface is suggestive of an anchoring function, and consequently the lack of this collagen entails loosening of the BMs. This seems to cause the critical consequences in the eye, whereas it only predisposes other tissues to pathological conditions.

**MATERIALS AND METHODS**

The Col18a1−/− mouse line

Heterozygous Col18a1−/− mice (20) were backcrossed to the C57BL/6J mouse strain (Jackson Laboratory) for more than 15 generations to produce an inbred C57BL/6J Col18a1−/− mouse line. This strain was then crossed once with the C57BL/6JolaHsd (Harlan) mouse strain and the resulting line was maintained by means of heterozygous Col18a1+/− matings. Wild-type littermates were used as controls in all the experimental analyses. The mouse genotypes were confirmed by polymerase chain reaction as described previously (55).

**Magnetic resonance imaging**

For high-resolution MRI, the animals were anaesthetized with a subcutaneous injection of a 1:4 dilution of fentanyl-fluanisone (Hypnorm, Janssen-Cilag) and midazolame (Dormicum, Roche) in sterile H2O (0.05 ml/10 g) and externally fixed to a custom-built animal holder. Warm air was blown through the magnet bore to maintain physiological body temperature during the experiments. *T*2-Weighted horizontal and coronal images (time-to-repetition 3 s, time-to-echo 50 ms, 8 averages per scan, field of view 25.6 × 12.8 mm2, data matrix 256 × 64 zero-filled to 512 × 128 prior to 2D Fourier transformation, slice thickness 0.75 mm, no gaps between slices) were acquired at 9.4 T using a single loop surface coil (diameter 27 mm). The total volume of the brain ventricles was estimated by visually delineating areas containing ventricles in 11 corresponding planes in the wild-type and mutant mice using UTHSCSA ImageTool Version 3.0 and multiplying the summed ventricular area by the slice thickness.

**Histological examination and immunostainings**

For histological examination of the brains, head samples from control and mutant mice sacrificed using carbon dioxide were fixed in 20% dimethylsulphoxide–water at 8 °C for 7 days, deparaffinized, blocked with 3% BSA–PBS, pH 7.3, for 60 min at room temperature and stained overnight at 4 °C. Heads of mutant and control mice were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon Embed 812 (Electron Microscopy Sciences, Fort Washington, PA, USA) for transmission electron microscopy. The skulls/meninges of the control and mutant mice were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, incubated in 5% EDTA in 0.1 M phosphate buffer with 1% glutaraldehyde and processed further as described earlier. Thin sections were cut with a Reichert Ultracut ultramicrotome and examined in a Philips CM100 transmission electron microscope. Images were captured and the thicknesses of the BMs measured using a CCD camera equipped with Tietz Video and Image Processing Systems GmbH (Gaunting, Germany). The BM measurements included both the lamina lucida and the lamina densa. The number of CP measurements available for statistical analysis was 114 (19 measurements/mouse) for both the control and mutant mice, and the number of skin measurements 84 (21 measurements/mouse) for both the 12-month-old wild-type and Col18a1−/− mice, 47 for the 7–15-month-old wild-type mice and 210 for their Col18a1−/− counterparts (21 measurements/mouse in each case). The number of measurements of the atrioventricular valves on both the atrial and ventricular side was 20 in both the wild-type and Col18a1−/− mice (five measurements/mouse), and the number of measurements of kidney proximal tubus was 96 for both the control and mutant mice at age 12 days, 168 for the controls and 120 for the mutant mice at 2 months and 168 and 96, respectively, at 7–11 months (24 measurements/mouse). The variances and averages of the BM measurements were calculated using the F and t-tests, respectively.

**Immunoelectron microscopy**

Samples from wild-type and Col18a1−/− mice for immunoelectron microscopy were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer with 2.5% sucrose for 2 h at room temperature, the CP samples also being embedded in gelatine, following immersion in 2.3 M sucrose, and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT microtome.

Sections for immunolabelling were first incubated in 0.05 M glycine in PBS, followed by incubation in 5% BSA with 0.1% cold water fish skin (CWFS) gelatin (Aurion, Wageningen, Netherlands) in PBS. Antibodies and gold conjugate were recognizing the N-terminal part of type XVIII collagen (J. Saarela et al., unpublished data), rabbit anti-mouse endostacin polyclonal antibody (Chemicon), rabbit anti-mouse collagen IV polyclonal antibody (Chemicon), monoclonal anti-heparan sulphate proteoglycan antibody recognizing mouse perlecan (Seikagaku Corporation), monoclonal rat anti-laminin B2 chain monoclonal antibody (Chemicon), polyclonal goat anti-Na+K+-ATPase antibody (Santa Cruz Biotechnology) and polyclonal rabbit anti-aquaporin 1 antibody (Chemicon).
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Serum creatinine measurements

Orbital blood was collected from 12 wild-type and 11 Coll18a1−/− mice aged 6–16 months after culling using carbon dioxide. The serum was separated out by centrifuging for 10 min at 2000g and creatinine levels were determined on a Cobas Integra 700 analyser (Hoffmann-La Roche Ltd, Diagnostic Division, Basel, Switzerland), using an enzymatic, colorimetric creatinine method (cat. no. 0766127, Roche Diagnostics, Switzerland).

REFERENCES


