Human Amniotic Membrane, Like Corneal Epithelial Basement Membrane, Manifests the α5 Chain of Type IV Collagen

Ken-ichi Endo, Takahiro Nakamura, Satoshi Kawasaki, and Shigeru Kinoshita

Purpose. To reexamine whether the α5 chain of type IV (α5[IV]) collagen, thought to be absent, is in fact present in human amniotic membrane.

Methods. Cryosections of human amniotic membrane obtained at Cesarean section were immunohistochemically examined for the presence of α5(IV), with or without inclusion of the denaturing step. Amniotic membrane was digested with collagenase to release the noncollagenous NC1 domain from the α-chain. The NC1 domain of α5(IV) was then assayed on Western blot analysis. Identical experiments were performed with human corneas and conjunctivae obtained from an American eye bank.

Results. The basement membrane of denatured samples of amniotic membrane and cornea stained positive for α5(IV). Without the denaturing step, only corneal samples were positive. With or without denaturing, conjunctival epithelium did not stain. Western blot analysis detected NC1 domains of α5(IV) in amniotic membrane and corneal samples.

Conclusions. The basement membrane of amniotic membrane resembles that of corneal epithelium but not conjunctiva. Amniotic membrane may be an excellent substrate for corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2004;45:1771–1774) DOI:10.1167/iovs.03-0952

The basement membrane is a continuous sheet of specialized extracellular matrices (e.g., collagen types IV and VII, laminins, entactin, and heparin sulfate proteoglycan). It separates epithelial and endothelial cells from the underlying connective tissue, serves as a molecular filter in capillaries and glomeruli, prevents the passage of proteins, and provides the scaffolding that maintains normal tissue architecture during regeneration and growth.[1] The function of the basement membrane is closely related to its composition, and in different areas of the body, the ratio of its components varies. Furthermore, each component is made up of a class of several isoforms.

The major structural component, type IV collagen, is a helical trimer of the α(IV) chain with a globular NC1 domain at the carboxyl termini. The trimer further combines to form supramolecular networks by dimerization at the carboxyl terminus through the NC1 domains and by forming tetramers at the amino terminus. To date, six different α(IV) chains, α1(IV) to α6(IV), have been identified.[2,3] Whereas the α1(IV) and α2(IV) chains are ubiquitous components of all basement membranes, α3(IV) to α6(IV) exhibit restricted tissue distribution.

The amniotic membrane, which comprises the innermost placental layer, consists of a single layer of epithelial cells, a thick basement membrane, and an avascular stromal matrix. Amniotic membrane has been widely used as a graft in ocular surface transplantation since Kim and Tseng reintroduced its use in 1995.[4] In their immunohistochemical study, Fukuda et al.[5] demonstrated that α5(IV) is absent in the basement membrane of amniotic membrane and conjunctiva, but found it in that of the corneal epithelium. Consequently, the basement membrane of the amniotic membrane is thought to be similar to that of the conjunctival epithelium but different from that of the corneal epithelium.

However, we and others[6–11] have used intact or epithelially denuded amniotic membrane as a substrate for cultivating corneal limbal epithelial cells. The cells proliferate well and form a three-dimensional multilayered structure closely resembling corneal epithelial tissue. Moreover, ocular surface reconstruction in which cultivated cells were used with the membrane as a graft has succeeded in the treatment of acute and severe ocular surface disorders such as chemical burns and Stevens-Johnson syndrome.[12–14] This led us to question the assumed incompatibility of amniotic membrane and corneal epithelial cells and to reexamine the presence of α5(IV) chains in amniotic membrane and corneal and conjunctival basement membranes. Our findings that the α5(IV) chain was in fact present in the amniotic membrane but not in the conjunctival basement membrane, encouraged a revision of earlier assumptions and suggest amniotic membrane as a useful substrate for growing corneal epithelial cells.

Tissues and Materials

Prior informed consent to harvest amniotic membrane was obtained, in accordance with the tenets of the Declaration of Helsinki for research involving human subjects, from women scheduled for Cesarean section. Our study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. The membranes were washed with phosphate-buffered saline (PBS) and preserved with 50% glycerol containing Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA) at −80°C until use. Corneas and conjunctivae were obtained from corneal–scleral rim after removal of donor corneal material (Northwest Lions Eye Bank, Seattle, WA) that was used in penetrating keratoplasty, by careful cutting under a binocular microscope.

Monoclonal antibodies against α5(IV) were H52 (Shigei Medical Research Institute, Okayama, Japan) for immunohistochemical and MAB5 (Wieslab, Lund, Sweden) for Western blot studies. For the detection of entactin (nidogen) we used monoclonal antibody JF6 (Chemicon International, Temecula, CA). Other reagents were of the highest grades.

From the Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

Submitted for publication September 2, 2003; revised November 10 and December 9, 2003, and February 1, 2004; accepted February 13, 2004.

Disclosure: **K. Endo**, None; **T. Nakamura**, None; **S. Kawasaki**, None; **S. Kinoshita**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Ken-ichi Endo, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kawaramachi Hirokoji Kajii-cho, Kamigyo-ku, Kyoto 602-0841, Japan; kendo@ophth.kpu-m.ac.jp.

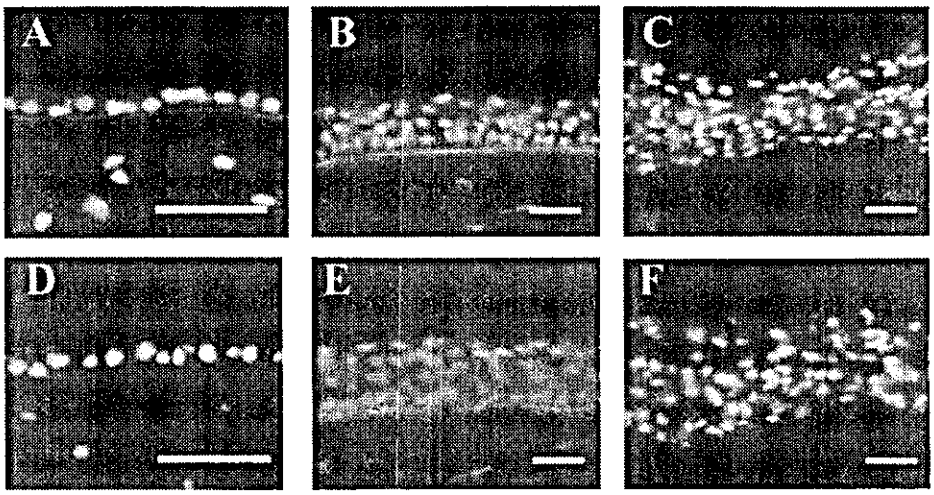

FIGURE 1. Immunohistochemical staining for α5(IV) in amniotic membrane (A, D), cornea (B, E), and conjunctiva (C, F). There is obvious staining (*green*) just beneath the epithelium of amniotic membrane and corneal samples that had been subjected to glycine-urea pretreatment (A–C). Without pretreatment (D–F), amniotic membrane did not stain. Conjunctival samples were negative for α5(IV), with or without urea pretreatment. Scale bar: 50 μm.

Immunohistochemistry

Semithin (6 μm) frozen sections were obtained from unfixed tissue embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles, Inc., Elkhart, IN). After a 20-minute fixation with cold acetone, they were or were not, exposed for 10 minutes to 6 M urea-0.1 M glycine-HCl (pH 3.5) solution and incubated for 30 minutes with 10% goat serum. Then they were exposed for 1 hour to diluted (1:200) H52, washed three times with PBS, and incubated for 1 hour with Alexa Fluor 488–conjugated anti-rat IgG antibody (Molecular Probes, Inc., Eugene, OR). After three PBS washes, the sections were mounted on glass slides with antifade medium containing propidium iodide (Vectashield; Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope.

Western Blotting

The NC1 fraction of type IV collagen was obtained as described previously.[15] Briefly, amniotic membrane, conjunctiva, and cornea without Descemet's membrane were homogenized into 50 mM Tris-HCl buffer containing protease inhibitors and then incubated with collagenase type IV (Invitrogen) at 37°C for 48 hours. Insoluble materials were removed by 15-minute centrifugation at 10,000*g* and the supernatant was subjected to reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amounts of protein applied ranged from 14 to 60 μg. Because the level of contamination of proteins derived from epithelia and adjacent tissues varied among the samples, they were adjusted so that they manifested comparable entactin bands. Resolved proteins were electrically transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) and blocked in PBS containing 5% nonfat milk, followed by a 1-hour incubation with the primary antibody (MAB5 or JF6) at room temperature. A combination of biotinylated anti-mouse IgG (secondary antibody; Vector Laboratories) and the an alkaline phosphatase assay kit (Amplified Alkaline Phosphatase Immun-Blot; Bio-Rad) was used for chromogenic staining according to the manufacturer's instructions. Band intensities were quantified on computer (Image PC software; Scion Corp., Frederick, MA).

RESULTS

Immunohistochemistry

In the absence of glycine-urea treatment, none of the three different human amniotic membranes we examined manifested immunoreactivity for α5(IV) (Fig. 1D). On the other hand, all samples pretreated with glycine-urea were strongly and evenly fluorescent for α5(IV) just below the epithelium (Fig. 1A). In human cornea, fluorescence was observed on Bowman's membrane even without glycine-urea treatment (Fig. 1E); it was intense in urea-pretreated samples (Fig. 1B). Conjunctival samples were negative for α5(IV) fluorescence regardless of whether they had, or had not, been pretreated (Figs. 1C, 1F).

Western Blot Analysis

On Western blot analysis of amniotic membrane- and corneal samples, the α5(IV) chain was detected as a 25.5-kDa band and 52- to 60-kDa bands (Fig. 2). Reportedly, the low molecular weight band is a monomeric NC1 subunit of α5(IV), whereas the high molecular weight bands are the dimeric forms.[16–18] Of note, in the cornea we detected an 82-kDa immunoreactive substance against anti-α5(IV) antibody in addition to dimers and monomers. As the sample resisted further digestion, we concluded that this was not the result of incomplete digestion. To our knowledge, ours is the first report of this complex. Based on its molecular weight, we posit that it represents a trimer of the NC1 subunits. Densitometric analysis demonstrated that in the cornea, 20.5% of total α5(IV) was incorporated into the trimerlike complex; in amniotic membrane it was less than 0.5%. No bands were detected in the conjunctival samples. The presence of bands for entactin, a component of basement membrane, confirmed that the protein content in these samples was the same as in basement membrane.

DISCUSSION

Our immunohistochemical findings in urea-pretreated samples demonstrated the presence of the α5 chain of collagen type IV on amniotic membrane as well as corneal basement membrane. However, it was absent on conjunctival basement membrane. Western blot analysis confirmed these findings. Our results are contrary to those reported by others.[5] As shown in Figure 1, our immunohistochemical detection of α5(IV) is due to our pretreating the samples with glycine-urea.

Most immunohistochemical studies using antibodies against α5(IV) require that the tissue be pretreated with urea,[17,19–22] because the epitope these antibodies recognize is apt to be masked in normal nondenatured tissue. Glycine-urea treatment opens up the NC1 domain in such a way that the antigenic epitope is presented, thus enabling antibody recognition. H52 is no exception and the supplier recommends adding acidic

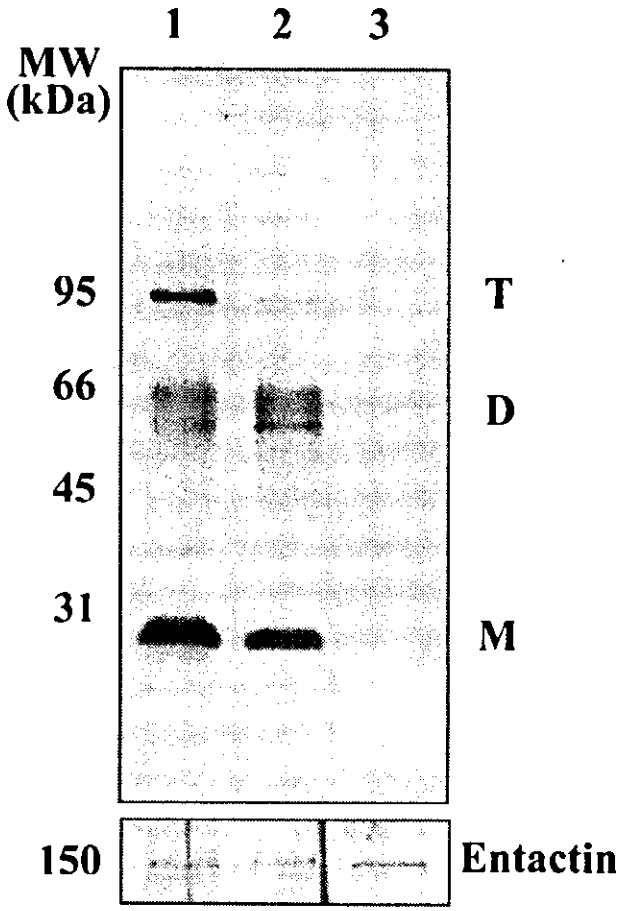

FIGURE 2. Identification of α5(IV) by Western blot analysis. Several forms of α5(IV) were detected in corneal (*lane 1*) and amniotic membrane (*lane 2*) but not in conjunctival samples (*lane 3*). T, D, and M represent a trimerlike complex and dimer and monomer forms of α5(IV), respectively. Entactin bands demonstrate that the samples contained almost equal amounts of basement membrane fraction.

urea pretreatment to the regular immunohistochemical protocol. At present, we do not know why acidic urea pretreatment was not necessary for the detection of α5(IV) in corneal epithelial basement membrane. We posit that the trimerlike complex we observed consists of the NC1 domain of α5(IV) (Fig. 2). The NC1 domain of α5(IV) in the undigested trimerlike complex may serve to maintain a state in the corneal basement membrane in which the antibodies have easy access without denaturing. Such an α5(IV) complex may be peculiar to corneal epithelial basement membrane because, to our knowledge, it has not been observed in other tissues. Studies are underway in our laboratory to address this question.

In the normal physiology of the corneal epithelium, α5(IV) constitutes a highly important component. The recurrent corneal erosion in patients with Alport's syndrome may reflect mutation(s) in the gene for α5(IV).[23–26] The basement membrane of patients with dysplastic corneal epithelium reportedly lacks α5(IV).[20] Because α5(IV) is present in amniotic membrane and remains even after epithelial denudation with EDTA, we suggest that it is a suitable substrate for cultivating corneal epithelial cells. Our work extends existing observations regarding the collagen composition of human amniotic membrane. The presence of the α5(IV) chain in amniotic- and corneal basement membrane may recommend the former as an excellent substrate for corneal epithelial cells.

References

1. Martin GR, Timpl R. Laminin and other basement membrane components. *Ann Rev Cell Biol.* 1987;3:57–85.
2. Hudson BG, Reeders ST, Tryggvason K. Type IV collagen: structure, gene organization, and role in human diseases: molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. *J Biol Chem.* 1993;268:26033–26036.
3. Yurchenco PD, Ruben GC. Basement membrane structure in situ: evidence for lateral associations in the type IV collagen network. *J Cell Biol.* 1987;105:2559–2568.
4. Kim J, Tseng S. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea.* 1995;14:473–484.
5. Fukuda K, Chikama T, Nakamura M, Nishida T. Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva. *Cornea.* 1999;18:73–79.
6. Yam HF, Pang CP, Fan DS et al. Growth factor changes in ex vivo expansion of human limbal epithelial cells on human amniotic membrane. *Cornea.* 2002;21:101–105.
7. Cohen EJ. Use of autologous limbal epithelial cells cultured on amniotic membranes for unilateral stem cell deficiency. *Arch Ophthalmol.* 2001;119:123–124.
8. Hernandez Galindo EE, Theiss C, Steuhl KP, Meller D. Gap junctional communication in microinjected human limbal and peripheral corneal epithelial cells cultured on intact amniotic membrane. *Exp Eye Res.* 2003;76:303–314.
9. Koizumi N, Cooper LJ, Fullwood NJ, et al. An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. *Invest Ophthalmol Vis Sci.* 2002;43:2114–2121.
10. Pan Z, Zhang W, Wu Y, Sun B. Transplantation of corneal stem cells cultured on amniotic membrane for corneal burn: experimental and clinical study. *Chin Med J (Engl).* 2002;115:767–769.
11. Ti SE, Anderson D, Touhami A, Kim C, Tseng SC. Factors affecting outcome following transplantation of ex vivo expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. *Invest Ophthalmol Vis Sci.* 2002;43:2584–2592.
12. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. *Arch Ophthalmol.* 2001;119:298–300.
13. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology.* 2001;108:1569–1574.
14. Shimazaki J, Aiba M, Goto E, et al. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. *Ophthalmology.* 2002;109:1285–1290.
15. Rodgers H, Irvine C, van Wezel I, et al. Distribution of the a1 to a6 chains of type IV collagen in bovine follicles. *Biol Reprod.* 1998; 59:1334–1341.
16. Gunwar S, Bejarano PA, Kalluri R, et al. Alveolar basement membrane: molecular properties of the noncollagenous domain (hexamer) of collagen IV and its reactivity with Goodpasture autoantibodies. *Am J Respir Cell Mol Biol.* 1991;5:107–112.
17. Reddy G, Gunwar S, Kalluri R, Hudson B, Noelken M. Structure and composition of type IV collagen of bovine aorta. *Biochim Biophys Acta.* 1993;1157:241–251.
18. Ries A, Engel J, Lustig A, Kuhn K. The function of the NC1 domains in type IV collagen. *J Biol Chem.* 1995;270:23790–23794.
19. Borza D, Bondar O, Ninomiya Y, et al. The NC1 domain of collagen IV encodes a novel network composed of the alpha 1, alpha 2, alpha 5, and alpha 6 chains in smooth muscle basement membranes. *J Biol Chem.* 2001;276:28532–28540.
20. Saika S, Kawashima Y, Okada Y, et al. Immunohistochemical and ultrastructural analysis of dysplastic epithelium of human ocular surface: basement membrane and intermediate filament. *Cornea.* 1999;18:343–352.

21. Simoneau A, Herring-Gillam F, Vachon P, et al. Identification, distribution, and tissular origin of the alpha5(IV) and alpha6(IV) collagen chains in the developing human intestine. *Dev Dyn*. 1998;212:437–447.
22. Yoshioka K, Hino S, Takemura T, et al. Type IV collagen alpha 5 chain: normal distribution and abnormalities in X-linked Alport syndrome revealed by monoclonal antibody. *Am J Pathol*. 1991; 144:986–996.
23. Rhys C, Snyers B, Pirson Y. Recurrent corneal erosion associated with Alport's syndrome: rapid communication. *Kidney Int*. 1997; 52:208–211.
24. Huismans H. Alport's syndrome. *Klin Monatsbl Augenheilkd*. 1978;172:775–778.
25. Huck D, Meythaler H, Rix R. Alport-syndrome with participation of the cornea and alterations of ERG. *Klin Monatsbl Augenheilkd*. 1976;168:553–556.
26. Colville DJ, Savige J. Alport syndrome: a review of the ocular manifestations. *Ophthalmic Genet*. 1997;18:161–173.