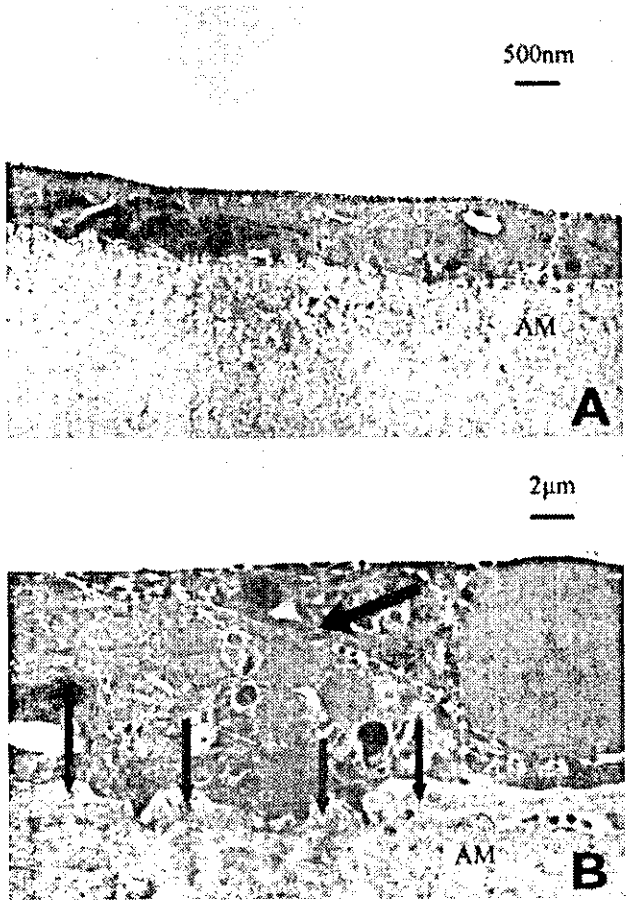

FIGURE 3. Transmission electron micrographs of cultivated HCECs cultivated on denuded AM for 2 weeks. (A) A continuous monolayer of these cells covered the surface of denuded AM. These cells had a few pits and vacuoles, but they kept normal endothelial features. (B) Adjoining cells either overlapped each other slightly or abutted to maintain good contact. *Thick arrow*: cell–cell junction; *thin arrows*: cells producing basement membrane material.

nificantly thinner when compared with both the stripped Descemet's membrane and AM control groups in daily pachymetry measurements ($P < 0.05$). The average value for rabbit corneal thickness before the operations was 350.7 ± 10.2 μm (mean ± SD). After the operation, average values for corneal thickness were always greater than 800 μm in the acellular AM and stripped Descemet's control groups. However, in the HCEC group, the average values for corneal thickness was consistently below 500 μm from days 5 to 7 after the operation (day 5: 460.7 ± 100.6 μm; day 6: 436.0 ± 100.5 μm; day 7: 460.7 ± 117.7 μm, mean ± SD). The average counts in the HCEC group were not significantly different from those of the TO group (Fig. 6).

Light and electron microscopy of the HCEC sheet 7 days after transplantation showed a continuous monolayer of HCECs on AM. The cells were polygonal, fairly uniform in size, and in close contact with one another (Figs. 7A, 7B). A transmission electron microscopy image of the HCECs shows that a continuous monolayer of cHCECs covered the surface of the denuded AM. Apparent complexity of cell–cell junctions and cell-AM substrates were observed. Many mitochondria and lysosomes were present in the cytoplasm of cHCECs on AM (Fig. 7C).

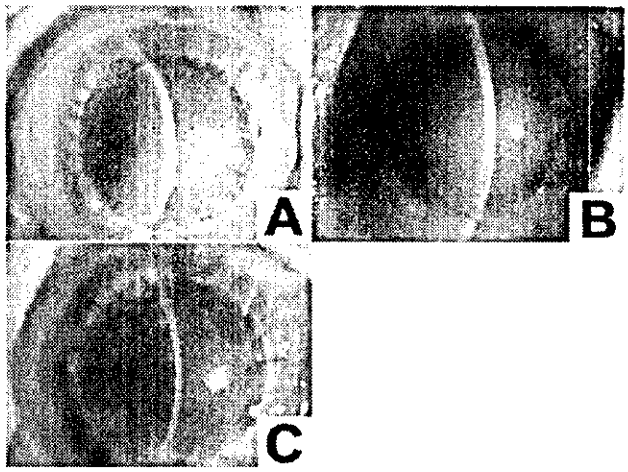

FIGURE 4. At 4 days after the operation, the control corneas consisting of stripped Descemet's membrane (A) and acellular AM (B) became highly edematous. The grafts consisting of HCECs on AM (C) had little edema and excellent transparency.

Assessment of Cultured HCECs Transplanted In Vivo

Before transplantation, cHCECs were present on AM at high density (Fig. 8A). One week after transplantation, the cells were still present on the AM carrier and maintained a high density (2410.0 ± 31.1 cells/mm^2; Fig. 8B). In addition, no proliferation or migration of cHCECs was observed beyond the edge of the AM (Fig. 8C). Alizarin red stain of the cHCEC sheet with corneal button showed that there were no endothelial cells between the cHCEC sheet and the host-graft junction. Therefore, rabbit corneal endothelial cells did not seem to have migrated into the corneal button at the time examined (Fig. 8D).

DISCUSSION

We made cHCEC sheets using denuded acellular AM as a nonsynthetic carrier and transplanted them in vivo in our

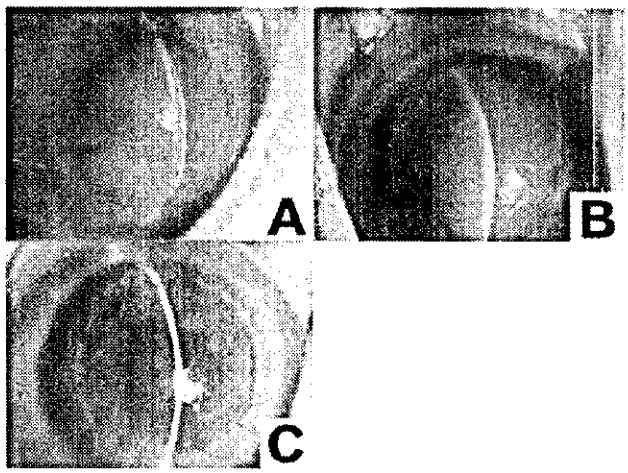

FIGURE 5. At 7 days after the operation, the control corneas consisting of stripped Descemet's membrane (A) and acellular AM (B) were highly edematous. The grafts consisting of HCECs on AM (C) had little edema and corneal transparency.

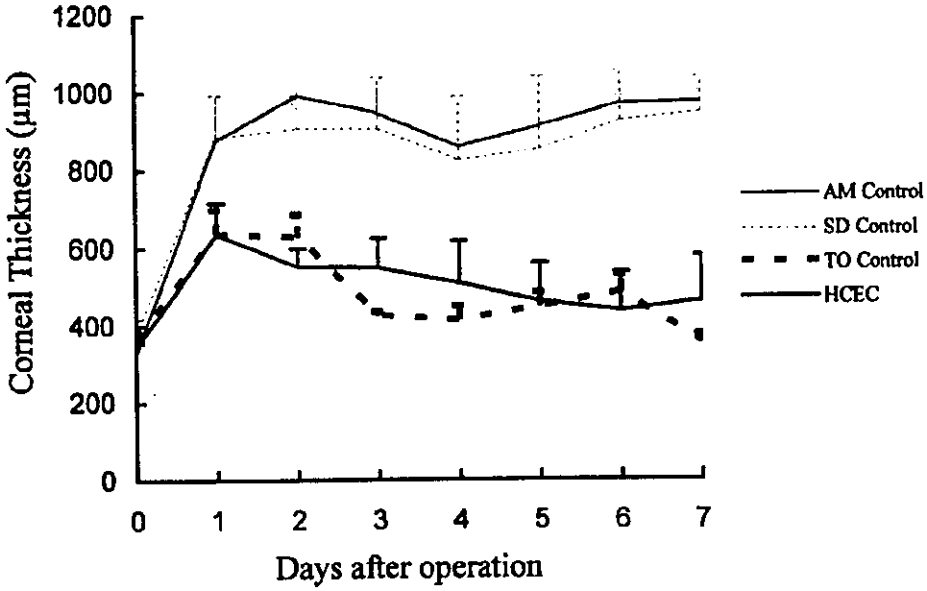

FIGURE 6. Average corneal thickness (mean ± SD) after transplantation. The average corneal thickness was always greater than 800 μm in both the stripped Descemet's membrane and the acellular AM control groups. In the cHCEC transplant recipients, the average corneal thickness was consistently less than that in both the control and AM groups. Between the TO control and cHCEC groups, there was no significant difference in the average corneal thickness.

present study. Cultivated HCEC transplantation on Descemet's membrane in vitro has been reported,[14-16] and the cells were reported to form a stable monolayer and keep their characteristic construction. We think AM is one of the most feasible candidates for cHCEC transplantation, because AM can be obtained more easily than any other nonsynthetic carriers such as Descemet's membrane, and it has been used for cultured corneal epithelial cell transplantation with good clinical results. There have been several studies[4,8,17-19] of corneal endothelial cell transplantation in vivo, but most of these do not involve corneal endothelial cells from humans, but instead cultured corneal endothelial cells from animals: rabbit, bovine, and cat. To our knowledge, in one study,[19] human neonatal corneal endothelial cells were cultivated and seeded onto the Descemet's membrane of the corneal button and then the cells transplanted into African green monkeys. Therefore, this is the first report of cHCECs transplanted in vivo using adult cHCECs.

In our study, the morphology and structure of cHCECs transplanted on denuded AM were evaluated by vital staining, as well as scanning and transmission electron microscopy. We found that the ultrastructure and density of these cells was very similar to that of normal corneal endothelial cells ex vivo. It is also important in cHCEC transplantation to obtain higher cell density. Clinically, it has been proposed that donor corneal tissue with endothelial density of more than 2500 cells/mm^2 are ideal to be transplanted in patients with bullous keratoplasty. Using centrifugation, we obtained cHCEC sheets with a density of more than 3000 cells/mm^2. These cHCEC sheets seem to have high enough cell density to be used in patients for bullous keratopathy.

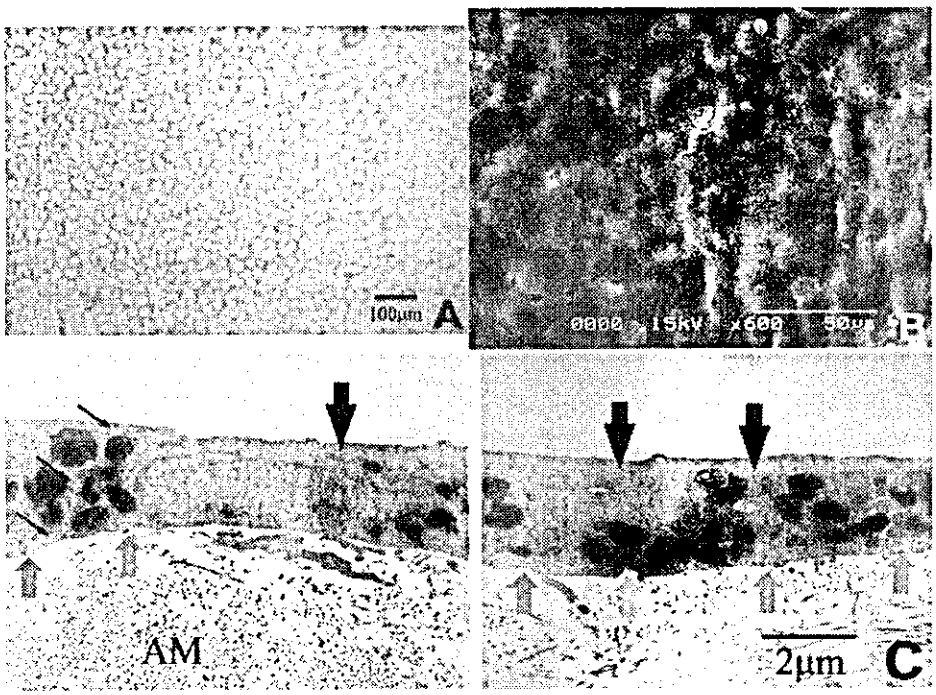

FIGURE 7. Light and electron micrographs of HCECs 7 days after transplantation. (A) A fairly continuous monolayer of HCECs. (B) Scanning electron micrograph also showing that HCECs formed a continuous monolayer layer on the AM. They were polygonal, fairly uniform in size, and in close contact with one another. (C) A transmission electron micrograph of HCECs showing that a continuous monolayer of cHCECs covered the surface of denuded AM. *Thin arrow*: designate cell–cell junction. *Gray arrows*: close association of basal plasma membrane and AM; *thick arrows*: mitochondria in cytoplasm.

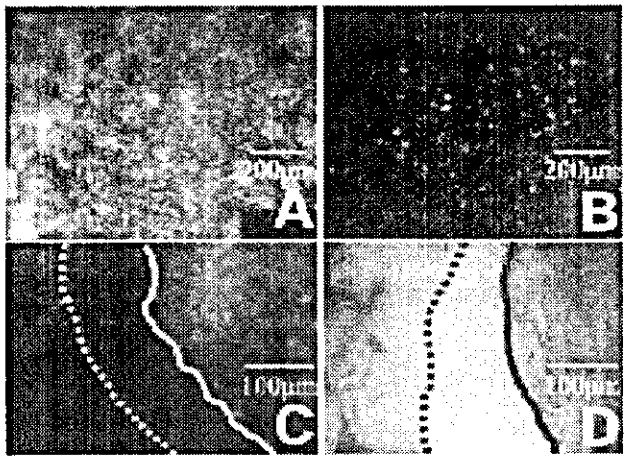

FIGURE 8. Dil-labeled cHCECs on AM. (**A**) At day 0, before transplantation in vivo, the cHCECs were present on the AM at a high density. (**B**) At day 7 after transplantation, the cultured HCECs were present on AM. (**C**) At day 7 after transplantation, the cHCECs showed no evidence of proliferation or migration beyond the edge of the AM. (**D**) Between cHCEC sheet and host-graft junction in the Descemet-stripped area, there were no endothelial cells. Neither donor nor host endothelial cells migrated from the original region. (**C**, **D**) *Solid line*: edge of cHCEC sheet; *dotted line*: host-graft junction. Magnification: (**A**, **B**) ×100; (**C**, **D**) ×40.

In our in vivo study, we also found that the rabbit corneas with transplanted cHCECs on AM retained their thickness and transparency compared with the controls for 1 week, and we also found, but in only one rabbit, that this situation continued for 4 weeks (data not shown). Because rabbit corneal endothelial cells have been known to proliferate in vivo, we wished to investigate the extent of survival of cHCECs transplanted in vivo by using DiI labeling. The results of the DiI labeling showed that the cHCECs remained on the denuded AM transplanted onto the corneal button at least 4 weeks. These results show that transplanted cHCEC sheets remained and were functional for at least 4 weeks. We intend to further investigate the long-term consequences of cHCEC transplantation and the functions of the cHCEC sheet. The HCEC density in the transplants showed 27% reduction 7 days after transplantation, either because of the tissue damage at the time of surgery or the short life of some cultivated endothelial cells. To investigate the long-term consequences of cHCEC transplantation, it is not suitable to use rabbits as recipients, because their corneal endothelial cells proliferate in vivo. It may be better to use cats or monkeys as recipients, because their corneal endothelial cells more closely mimic HCECs in having little or no mitotic activity and a limited regenerative capacity.

The transplanted cHCEC corneas using AM as a carrier are clearer and thinner than either corneas transplanted with AM only or corneas with removed Descemet's membranes and endothelial cells. Furthermore, the transplanted cHCEC corneas are as thin as corneas with trephination only. These results indicate that HCECs on AM function as well as that of normal endothelium at least until 7 days after transplantation. The transplanted cHCEC corneas with AM regained partial transparency after transplantation. Normal corneal stromal clarity depends on the regular arrangement of collagen fibers. AM does not have such a characteristic structure. However, we think thinning of AM after transplantation increased its transparency, as seen in the eyes with cultured corneal epithelial cells transplanted for corneal epithelial diseases.[19] There was a gap of 0.1 to 0.2 mm between donor human endothelial cells and host rabbit endothelial cells, but the transplanted corneas retained their corneal thickness and transparency. We think that the gap had some influence on the pump-leak balance. The water permeating into the corneal stroma through the nonendothelial area may pump out through the adjacent human and rabbit endothelial area. Because an area of central cornea more than 6 mm in diameter is covered by transplanted HCECs, it is reasonable to speculate that endothelial cells pump water from corneal stroma, although other possible factors such as evaporation may contribute to this to some extent.

In our present study, we transplanted cHCEC sheets by trephining the central corneas, removing Descemet's membranes with corneal endothelial cells, placing cHCEC sheets on the stroma of corneal buttons, and suturing them. A technique termed posterior lamellar keratoplasty, an operation for the treatment of bullous keratopathy, has been reported by Melles et al.[20] In this method the full-thickness cornea is not transplanted, just the posterior lamella of the cornea, and the method could be adapted for cHCEC transplantation. We have now investigated a cHCEC transplantation technique to remove corneal endothelial cells with Descemet's membrane and transplant a cHCEC sheet through a corneoscleral incision, similar to posterior lamellar keratoplasty. cHCEC sheet transplantation by this technique would be expected to have the same advantages as a posterior lamellar keratoplasty: fewer problems with sutures after they are in place, lower astigmatism, and more efficient use of donor tissue. In addition cHCEC transplantation may well have the advantage that scheduled operations could be performed, because we would not be dependent on the availability of corneoscleral discs. This possibility therefore has many advantages for both patients and health professionals.

cHCEC transplantation has the potential to be performed, not only as an allogeneic transplantation procedure but also as an autotransplantation procedure, if a small number of corneal endothelial cells from a healthy eye were cultivated, expanded, and transplanted to the contralateral endothelial damaged eye of the same patient. Moreover, in regenerative medicine, the potential of some pluripotent stem cells[21-25] for use in clinical treatments has been noted. Therefore, if pluripotent stem cells (e.g., hematopoietic stem cells and mesenchymal stem cells obtained from bone marrow) could be obtained from patients who undergo bullous keratoplasty and these stem cells could be induced to differentiate into corneal endothelial cells, it would be possible to transplant autologous corneal endothelial sheets without any risk of rejection.

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ERRATUM

Erratum in: In "Quantitative Analysis of Retinal Ganglion Cell (RGC) Loss in Aging DBA/2NNia Glaucomatous Mice: Comparison with RGC Loss in Aging C57/BL6 Mice" by Danias et al. (*Invest Ophthalmol Vis Sci.* 2003;44:5151–5162), the authors discovered an error in Table 1 of the above manuscript. The numbers in the fourth column are incorrect. The ratio of columns 3 and 4 should result in the fifth column numbers, but does not. Below is the corrected table.

TABLE 1. Mean RGC Counts, Retinal Area and Average RGC Density for Each Age Group in Both Strains

Strain	Age (mo)	RGC Count (n)	Retinal Area (mm^2)	RGC Density per Retina (RGC/mm^2)
DBA2/NNia	3	89,492 ± 5,746.0	16.59 ± 0.17	5,396 ± 362
	6	73,834 ± 4,698.8	15.33 ± 0.31	4,805 ± 243
	9	86,363 ± 9,995.3	19.46 ± 0.82	4,403 ± 417
	12	72,322 ± 5,192.7	18.69 ± 0.50	3,882 ± 295
	15	30,769 ± 8,877.0	19.32 ± 0.32	1,583 ± 440
	18	25,106 ± 13,087.3	19.22 ± 0.65	1,248 ± 605
C57/BL6	3	84,027 ± 2,171.6	15.97 ± 0.37	5,268 ± 136
	6	83,925 ± 5,549.8	16.82 ± 0.26	4,980 ± 279
	9	85,168 ± 6,118.7	17.14 ± 0.44	4,954 ± 280
	12	69,824 ± 7,943.6	16.43 ± 0.85	4,212 ± 323
	15	69,002 ± 7,419.0	17.61 ± 0.59	3,916 ± 389
	18	45,890 ± 3,549.8	16.90 ± 0.17	2,724 ± 232

Density was calculated as total RGC count divided by total retinal area. Data are expressed as the mean ± SEM.

The online version of this article was corrected on January 28, 2004, in departure from print.

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.

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

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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.

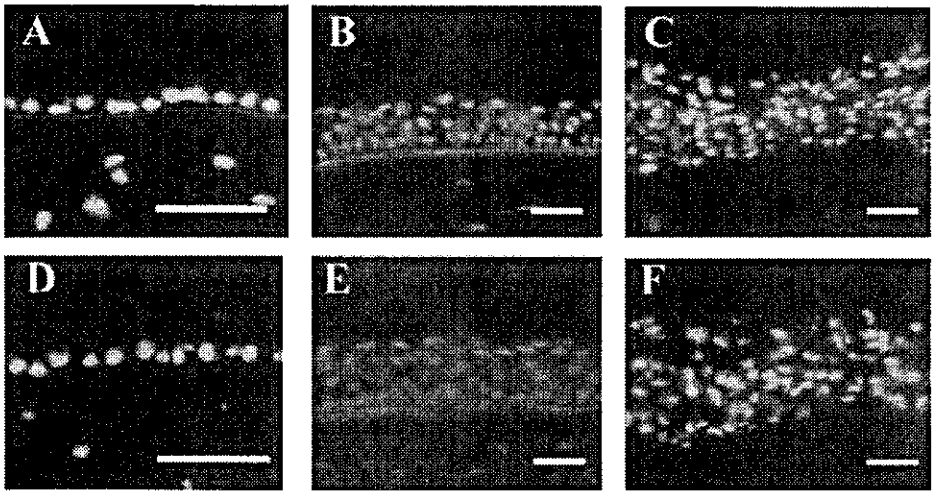

FIGURE 1. Immunohistochemical staining for α5(IV) in amniotic membrane (A, D), cornea (B, E), and conjunctiva (C, F). There is obvious staining (*green*) just beneath the epithelium of amniotic membrane and corneal samples that had been subjected to glycine-urea pretreatment (A–C). Without pretreatment (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

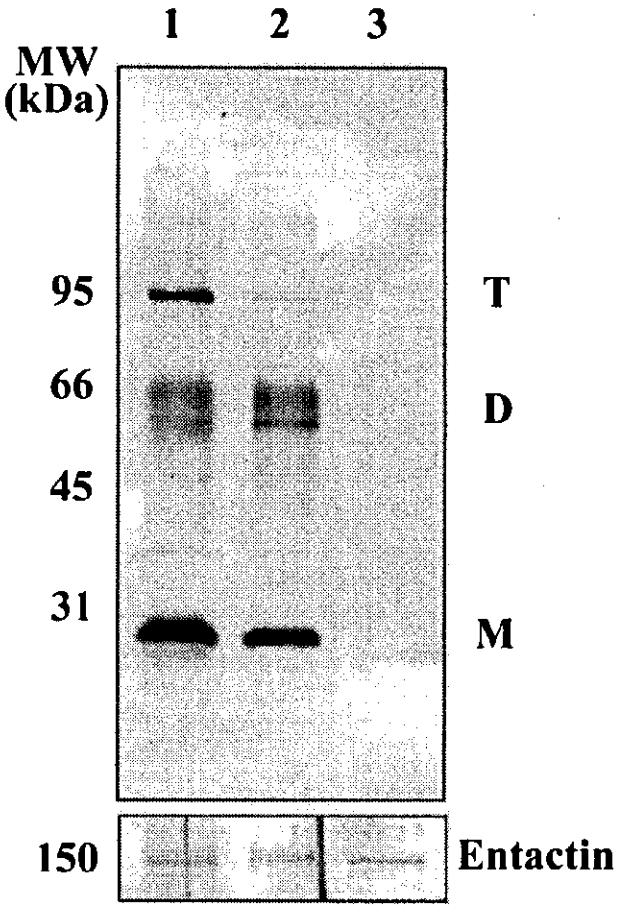

FIGURE 2. Identification of α5(IV) by Western blot analysis. Several forms of α5(IV) were detected in corneal (*lane 1*) and amniotic membrane (*lane 2*) but not in conjunctival samples (*lane 3*). T, D, and M represent a trimerlike complex and dimer and monomer forms of α5(IV), respectively. Entactin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

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