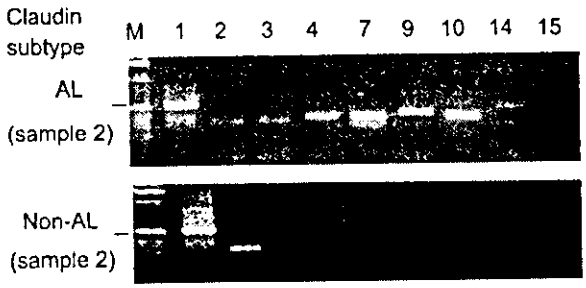

Fig. 8. Claudins expressed in cultured human corneal limbal epithelial cells detected by RT-PCR. Results of sample 2 from the AL and Non-AL groups are presented.

3.7. Claudins expressed in cultured human corneal limbal epithelial cells detected by RT-PCR

To date, 24 subtypes of the claudin gene family have been characterized; some are distributed in a tissue-specific manner.

Yi et al. (2000) reported the expression of claudin subtypes in simian virus (SV) 40-immortalized human corneal epithelial (THCE) cells; we reported (Ban et al., 2003) the expression of claudin isotypes in in vivo human corneal epithelial cells. In in vivo human corneal epithelium, the transcripts for claudin-1 and several other claudin isotypes, such as -2, -3, -4, -7, -9 and -14 were identified. The results differed from those for THCE cells, from which claudins-1, -2, -3, -7, -9, -14 and -15 were amplified.

We used PCR primers designed by Yi et al. (2000) for human claudins-1 to -4, -7, -9, -10, -14 and -15, and determined the presence of transcripts of these nine species in cultured human corneal limbal epithelial cells, and change in claudin species with AL.

In the AL group, claudins -1, -2, -3, -4, -7, -9, -10 and -14 were always expressed (Fig. 8, Table 1). This result was very close to the in vivo results (Ban et al., 2003). Claudin-10, an exception, was not expressed in vivo, though it was expressed in the AL group.

In the Non-AL group, some samples expressed claudin as in the AL group, but there were cases in which claudin subtype expression was strongly restricted, as in sample 2 (Fig. 8, Table 1).

4. Discussion

The AL method was originally developed to make skin cell culture sheets for transplantation. In this method, the culture is submerged in the medium, then raised to an air–liquid interface by lowering the medium level. Differentiation of epidermal cells, organized stratification, advanced cornification, elaborated lamellar bodies deposited in intercellular domains, lipid profile typical of cornifying epidermis, etc. are further promoted when the cells are at an air–liquid interface. Skin cell cultures made using the AL method appear morphologically to be more similar to in vivo tissues than to tissues grown submerged beneath culture medium (Prunieras et al., 1983; Williams et al., 1988; Nolte et al., 1993). In general, the research of many investigators has shown that the closer the culture conditions are to the natural tissue environment, the more closely the cultured epithelium mimics in vivo tissue. Therefore, epidermal cell differentiation is further promoted when the cells are raised to an air–liquid interface.

Minami et al. (1993), who used the AL method for bovine corneal cell culture in three-dimensional collagen gel culture condition, pointed out that this method promoted corneal epithelial cell proliferation and differentiation. For example, epithelial cells were positively immunostained by the antibody against keratin3, which is specific for in vivo corneal epithelium.

Table 1
Claudins expressed in cultured human corneal limbal epithelial cells detected by PCR in comparison with in vivo cornea and HCE. In the air-lifted culture (AL group): the claudin subtypes expressed remained constant and close to that of in vivo cornea. In the non air-lifted culture (Non-AL group): although some samples expressed the same claudin subtypes as in the AL group, in the majority of cases claudin subtype expression was strongly restricted, as in sample 2

Claudin subtype	1	2	3	4	7	9	10	14	15
AL sample									
1	+	+	+	+	+	+	+	+	−
2	+	+	+	+	+	+	+	+	−
3	+	+	+	+	+	+	+	+	−
4	+	+	+	+	+	+	+	+	−
5	+	+	+	+	+	+	+	+	−
Non-AL sample									
1	+	+	−	+	+	+	−	+	−
2	+	+	−	−	−	−	−	−	−
3	+	+	+	+	+	+	+	+	−
4	+	+	−	+	+	+	+	+	−
5	+	+	+	+	+	+	+	+	−
In vivo	+	+	+	+	+	+	−	+	−
HCE	+	+	+	−	+	+	−	+	+

Zieske et al. (1994) first exposed rabbit cultured corneal epithelial cells to a perfect 'dry' environment. In the dry model, the cornea epithelium grew out more evenly and stratified to up to 20 cell layers, with multiple layers of enucleated squamous-like cells in the apical surface of the culture, resembling cornified cells in the epidermis. These authors' next model was with the level of the medium lowered to just meet the surface of the culture, enabling the medium to wet the surface and the tissue construct to remain moist on its apical surface. They found that a rabbit corneal epithelial culture sheet maintained by this new AL method differentiated more than a culture submerged or in a perfectly dry environment. This result seems very reasonable, because in vivo cornea is not in a dry environment but is always kept wet by the tear film.

In this study, we investigated whether AL is useful in human corneal epithelial cell culture on AM. In our AL method, the culture sheet surface is not perfectly dry but slightly wet, like the second method of Zieske et al. (1994). The primary purpose of creating this new culture system was to use this culture sheet for ocular surface reconstruction in ocular surface disorders with stem cell deficiencies, like Stevens–Johnson syndrome, chemical burn or ocular cicatricial pemphigoid. In these severe clinical conditions, a culture sheet that is morphologically closer to in vivo and has a good barrier function should be better. In one of our preliminary experiments, we reported that the transplantation of rabbit corneal epithelial sheet cultured on AM with AL persisted longer than that of sheet without AL (Koizumi et al., 2000b). This is partially due to the fact that the TJ work against cell loss caused by blinking. Also desmosomal junctions give the epithelial sheet increased mechanical strength. Therefore, we paid careful attention to TJ and desmosome development with the AL method.

The electron microscopic study yielded considerable information on the morphological changes induced by AL. The intercellular spaces between neighboring cells are decreased by AL, and, by contrast, the number of desmosomes was increased (Fig. 2). These changes were highly statistically significant. Scanning electron microscopic examination revealed that the intercellular junctions of the superficial cells are more tightly opposed with AL (Fig. 4).

An important result was the change in HRP permeability as a result of AL. Before AL, the HRP penetrated the space to a depth of 3–4 cell layers, but did not penetrate the most apical cell-cell contact after AL (Fig. 1). This shows that the barrier function of the epithelium is promoted by AL.

TJ in the superficial layer of corneal epithelium play a vital role in barrier formation. Occludin and claudin are the integral transmembrane proteins in the TJ. The amount of occludin and claudin-1 did not change with AL in either mRNA or protein level (Figs. 6 and 7). However, the distributions of these two transmembrane proteins, and ZO-1, changed similarly to those of in vivo epithelium after AL (Fig. 5). These results suggest that many TJ-related proteins are already present in culture cells before AL, and that they are recruited to the TJ itself by AL.

Claudins form the TJ strands and to date 24 claudins have been identified (Tsukita et al., 2001). In addition to strand number, network pattern complexity may also be an important factor in determining the TJ barrier properties. Some reports speculate that TJ strand network complexity is determined by the combination and mixing ratio of the expressed claudin species (Furuse et al., 1998, 1999). Furuse et al. (2001) reported that when claudin-2 was introduced into MDCK 1 cells, in which claudin-1 and claudin-4 are primarily expressed, the cells' transepithelial electrical resistance (TER) value fell to the same level that is present in MDCK 2 cells in which large amounts of claudin-2 are expressed in addition to claudin-1 and claudin-4. Importantly, there was no change in the number of TJ strands in this experiment. These results tell us that claudins not only form the backbone of TJ strands, but also form extracellular aqueous pores, and that the combination and mixing ratios of claudin species determine the tightness of individual TJ strands. In our culture, some samples in the Non-AL group expressed the same claudin sub-types as the AL group, but there were more cases in which claudin subtype expression was strongly restricted (Fig. 7 and Table 1). At this stage it is difficult to explain the variation in expression of claudin subtypes in the Non-AL group. If we could quantify the amount of the claudin subtypes at the protein or mRNA level, we might have a better understanding. However this is not practical due to limited available of antibodies to the claudin subtypes.

Recently, Toropainen et al. (2001) demonstrated that the TER values and permeation of ^{3}H-mannitol of HCE culture sheets increased with AL. Their findings support our results, even though their culture consisted of HCE. Taking these results together with our own, we conclude that AL helps make cell-cell attachment tighter, helps create the superficial epithelial cell barrier, and is useful in culturing corneal epithelial cells for use in ocular surface reconstruction.

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Ocular Surface Reconstruction Using Cultivated Mucosal Epithelial Stem Cells

Takahiro Nakamura, MD, PhD and Shigeru Kinoshita, MD, PhD

Abstract

Purpose:
To investigate the possibility of using cultivated oral epithelial cells in ocular surface reconstruction.

Methods:
An ocular surface injury was created in adult albino rabbits by a lamellar keratectomy. Oral mucosal biopsy specimens taken from both adult albino rabbits and human volunteers were cultivated for 2–3 weeks on a denuded amniotic membrane (AM) carrier. The cultivated epithelium was examined by histologic and immunohistochemical analysis. At 3–4 weeks after the ocular surface injury, the rabbit conjunctivalized corneal surfaces were surgically reconstructed by transplanting both the rabbit and human cultivated oral epithelial cells on the AM carrier.

Results:
Both the rabbit and human cultivated oral epithelial sheets had 5 or 6 layers of stratified, well-differentiated cells. Histologic examination revealed that the cultivated epithelial cells were similar in appearance to those of in vivo normal corneal epithelium. Immunohistochemistry confirmed the presence of the keratin pair 4 and 13 and keratin 3 in the cultivated oral epithelial cells. Corneas that were grafted with rabbit and human cultivated oral epithelial cells on an AM carrier were clear and were epithelialized 10 and 2 days after surgery, respectively.

Conclusions:
We have generated confluent cultures of both rabbit and human oral epithelial cells on AM expanded ex vivo from biopsy-derived oral mucosal tissues. We have successfully carried out xeno- and autologous transplantation of these cultivated oral epithelial cells onto the ocular surfaces of keratectomized rabbit eyes. We believe that xeno- and autologous transplantation of cultivated oral epithelium is a feasible method for ocular surface reconstruction.

Key Words: amniotic membrane, corneal epithelium, ocular surface reconstruction, oral epithelium, stem cell

(*Cornea* 2003;22(Suppl 1):S75-S80)

The normal ocular surface is composed of two types of epithelial cells: corneal epithelium and conjunctival epithelium.[1] The limbal epithelium, which is located in the transitional zone between the cornea and bulbar conjunctiva, consists of corneal epithelial stem cells.[2,3] Severe limbal epithelial cell deficiency (LECD), such as occurs in Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and thermal and chemical burns, often leads to the covering of the corneal surface by invading neighboring conjunctival epithelial cells with ingrowth of fibrous tissue, stromal scarring, and neovascularization.[4] This severely compromises the ocular surface and seriously affects visual acuity.

Many attempts have been made to establish a surgical treatment of LECD, including corneal epithelial transplantation (keratoepithelioplasty, limbal transplantation, and amniotic membrane (AM) transplantation).[5–11] The most recently developed treatment involves the use of cultivated corneal epithelial stem cell transplantation.[12–14] Because LECD is usually bilateral, we have developed a surgical system for allocultivated corneal epithelial stem cell transplantation using AM as a carrier.[15,16] We have also shown that, in cases where the initially transplanted cultivated epithelium becomes opaque, it is possible to repeat the allotransplantation process with new cultivated epithelium on AM.[17] However, intensive, prolonged, postoperative immunosuppressant therapy is necessary to prevent inflammation and allograft rejection. This markedly reduces patients' quality of life and severely affects the clinical results. These drawbacks prompted us to investigate

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whether human ocular surface could be reconstructed by autologous mucosal epithelium of nonocular surface origin including tissue-specific stem cells. We attempted to overcome the problems of allogeneic transplantation by using oral epithelial cells as a substitute for corneal epithelial cells.[18]

METHODS

Cultivation of Rabbit Oral Epithelial Cells

We cultivated albino rabbit oral epithelial cells using our previously reported culture method for limbal epithelial cells with several modifications.[19] With informed consent and approval by the institutional review board of the Kyoto Prefectural University of Medicine, human AM was obtained at the time of cesarean section, and the amniotic epithelium was removed by EDTA treatment. Oral mucosal biopsy specimens, each measuring 4-6 mm^2, were obtained from albino rabbits (2-2.5 kg) under anesthesia. These specimens were then incubated at 37°C for 1 hour with 1.2 IU dispase and treated with 0.25% trypsin-EDTA solution for 30 minutes at room temperature to separate the cells. The culture medium used was Dulbecco Modified Eagle Medium and Ham F12 medium (1:1 mixture) with 10% fetal bovine serum, 5 μg/mL insulin, 0.1 nmol/L cholera toxin, 10 ng/mL human recombinant epidermal growth factor, and 50 IU/mL penicillin-streptomycin. The oral epithelial cells (1×10^5 cells/mL) were cultivated for 3 weeks on denuded AM carrier with 3T3 fibroblast coculture and airlifting.

Cultivation of Human Oral Epithelial Cells

We cultivated human oral epithelial cells using our culture method for rabbit oral epithelial cells with several modifications.[18] With informed consent and approval by the institutional review board of the Kyoto Prefectural University of Medicine, normal oral mucosa was provided by five patients as superfluous tissue debrided during oral surgery. The oral epithelial cells were cultivated for 2-3 weeks on denuded AM carrier with 3T3 fibroblast coculture and airlifting.

Histologic and Immunohistochemical Analysis

Oral epithelial cells cultivated on AM were examined using electron microscopy as previously described.[18] We used immunohistochemical analysis to determine the presence of keratins in the cultivated oral epithelial sheets using our previously described method.[20]

Xeno- and Autologous Cultivated Oral Epithelial Transplantation

Ocular surface injury was created by lamellar keratectomy as previously described.[18] Xeno- (using human cells) and autologous (rabbit) oral epithelial transplantation was then performed using methods and modifications of methods previously described.[18]

RESULTS AND DISCUSSION

We cultured both rabbit and human oral epithelial cells on AM expanded ex vivo from biopsy-derived oral mucosal tissues. We then transplanted both cultivated oral epithelial sheets on to the ocular surfaces of keratectomized rabbit eyes and evaluated the survival of these cultivated sheets.

Histologic and Immunohistochemical Analysis

Oral epithelial cells cultivated on AM began to form colonies within 3 days. At 2-3 weeks, the cultivated oral epithelial cells showed 5 or 6 layers of stratification and appeared similar to in vivo normal corneal epithelium (Fig. 1). Immunohistochemical analysis showed that epidermal keratinization-related keratin pair 1 and 10 was not expressed in any layers of the oral epithelial cells (Fig. 2A,B). The presence of nonkeratinized, stratified-specific keratin pair 4 and

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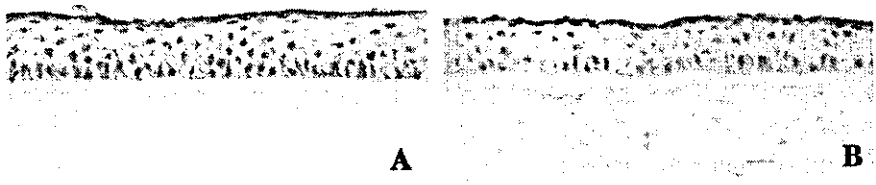

FIGURE 1. Histology of the cultivated oral epithelial cells on AM (A) and normal corneal epithelial cells (B). The cultivated oral epithelial sheet had 5 or 6 layers of stratified cells and appeared similar to normal corneal epithelium. Original magnification ×400. Partly reproduced with permission of the Association for Research in Vision and Ophthalmology from Nakamura et al. *Invest Ophthalmol Vis Sci* 2003;44:109.[18]

13 (Fig. 2C,D) and cornea-specific keratin 3 (Fig. 2E) was revealed. Cornea-specific keratin 12 was not expressed in any layers (Fig. 2F).

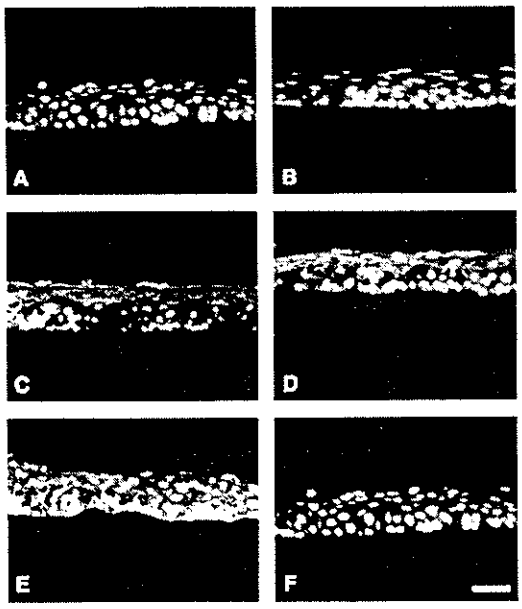

FIGURE 2. Representative immunohistochemistry of keratins 1 (A), 10 (B), 4 (C), 13 (D), 3 (E), and 12 (F) in cultivated oral epithelial cells. Keratin 1 and keratin 10 were not expressed in any layers of the cultivated oral epithelial cells (A,B). In contrast, keratin 4 and keratin 13 were expressed in the superficial and intermediate layers (C,D). Keratin 3 was expressed in all epithelial layers of the cultivated oral sheet (E), whereas cornea-specific keratin 12 immunostaining was not observed (F). Scale bar: 100 μm. Partly reproduced with permission of the Association for Research in Vision and Ophthalmology from Nakamura et al. *Invest Ophthalmol Vis Sci* 2003;44:110.[18]

The oral epithelial cells cultivated on denuded AM have the characteristics of nonkeratinized but not keratinized mucosa. We suggest that they might have the ability to differentiate into cornea-like epithelium under our culture conditions.[18]

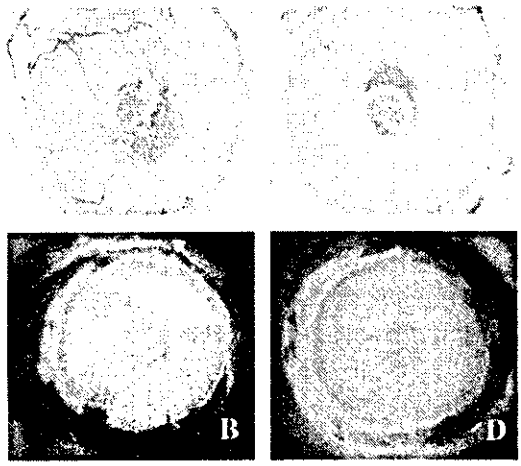

FIGURE 3. Representative slit-lamp photographs of eye of one rabbit taken before transplantation (A), 48 hours after transplantation with fluorescein (B), and 10 days after transplantation with (D) and without fluorescein (C). Before transplantation, the eye showed total limbal stem cell destruction (A). At 48 hours after surgery, most of the corneal surface was covered with transplanted cultivated oral epithelial cells, which showed no fluorescein staining (B). Ten days after surgery, the central epithelialized area had spread outward, and the corneal surface was covered with clear cultivated oral epithelium (C,D). Partly reproduced with permission of the Association for Research in Vision and Ophthalmology from Nakamura et al. *Invest Ophthalmol Vis Sci* 2003;44:114.[18]

Autologous Cultivated Oral Epithelial Transplantation

After the successful culture of rabbit oral epithelial cells on denuded AM, we tried to reconstruct damaged ocular surfaces using autologous cultivated oral epithelial cells. At 3-4 weeks after keratectomy, rabbit conjunctival epithelium completely covered the damaged corneal surface with considerable neovascularization and subconjunctival inflammation (Fig. 3A). After removal of conjunctivalized tissue, we reconstructed the ocular surface with a cultivated oral epithelial sheet on denuded AM (day 0). In the early stages (day 2) after transplantation, the eyes that had received autologous cultivated oral epithelial sheets all possessed an epithelialized area (Fig. 3B). Ten days after transplantation, the ocular surface covered by the epithelium had expanded outward and was connected with healing conjunctival epithelium in some areas (Fig. 3C,D). Moreover, the rabbit corneal surfaces were clear, smooth, and completely covered with transplanted autologous cultivated oral epithelial cells.

Cultivation of Human Oral Epithelial Cells

A confluent primary culture of human oral epithelial cells was established on acellular AM after 7 days. After 2-3 weeks in culture, the cultivated oral epithelial sheet had 5-7 layers of stratified, well-differentiated cells. Light micrography revealed that the human cultivated oral epithelial cells were similar in appearance to normal corneal epithelial cells (Fig. 4A). Immunohistochemistry confirmed the presence of keratin pair 4 and 13 and keratin 3 in the cultivated oral epithelial cells as in the animal model.[18]

Xenotransplantation of Human Cultivated Oral Epithelial Cells

After the successful culture of human oral epithelial cells on denuded AM, we tried to reconstruct superficial keratectomized corneas of albino rabbits by xenotransplanting human oral epithelial cells cultivated on AM to evaluate the physiologic functions of these sheets in an early stage. We observed them during only the first 2 days after the operation because acute epithelial rejection often occurs, especially in the case of xenotransplantation, despite intensive immunosuppression. The rabbit corneas were clear and were all epithelialized at 48 hours after surgery (Fig. 4B). Although the long-term outcome of such transplantation is not yet clear, from these results we believe that human cultivated oral epithelium sheets can function as an ocular surface epithelium and that culti-

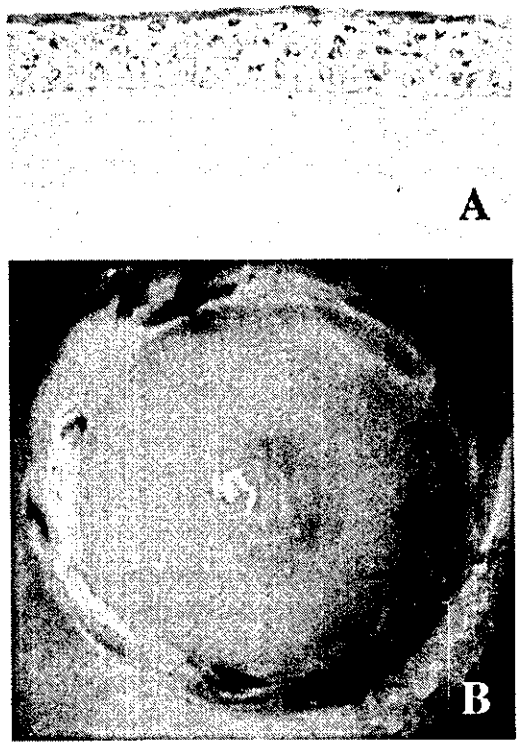

FIGURE 4. Histology of the human cultivated oral epithelial cells on AM (A). Human cultivated oral epithelial sheet with 5 or 6 layers of stratified, well-differentiated cells (original magnification ×400). Representative slit-lamp photograph of rabbit eye 48 hours after xenotransplantation of human cultivated oral epithelial cells (B). The corneal surface was covered with transplanted human cultivated oral epithelial cells, which showed no fluorescein staining.

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vated human oral epithelium transplantation is a feasible method of ocular surface reconstruction.

CONCLUSION

Transplantation of tissue-engineered, cultivated, corneal epithelial sheets has long been a dream of ophthalmologists, especially corneal surgeons. We investigated whether it was possible to reconstruct the human corneal surface using autologous mucosal epithelium of nonocular surface origin. We have successfully generated, on AM, confluent cultures of both rabbit and human oral epithelial cells expanded ex vivo from biopsy-derived oral mucosal tissues. We have successfully transplanted these cultivated oral cells onto keratectomized rabbit corneas. We believe that transplantation of cultivated oral epithelial cells represents an effective technique for ocular surface reconstruction in patients with severe limbal stem cell deficiency. In addition, it has been reported that holoclones have been identified as stem cells for surface epithelium such as epidermis, ocular surface, and hair follicles.[21-23] However, oral epithelial stem cells have not been clearly identified. We should investigate whether cultivated oral epithelial sheets contain stem cells and how long these cultivated oral epithelial stem cells can maintain the ocular surface.

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Induction of Epithelial Progenitors In Vitro from Mouse Embryonic Stem Cells and Application for Reconstruction of Damaged Cornea in Mice

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Purpose. Severe ocular surface diseases and injuries cause loss of the corneal limbal epithelium, leading to re-epithelialization by bulbar conjunctival cells, resulting in vascularization of the cornea, conjunctival scarring, and loss of visual acuity. In this study, the optimal culture condition for induction of differentiation of epithelial progenitor cells from embryonic stem (ES) cells was determined for use in transplantation to damaged cornea in mice.

Methods. Mouse ES cells were cultured on Petri dishes coated with several extracellular matrix proteins, and the markers for epithelial cells were analyzed with RT-PCR and Western blot analysis. The optimal condition for induction of epithelial progenitor cells was determined, and the progenitors were transplanted onto mouse eyes with corneal epithelia that had been damaged by exposure to *n*-heptanol.

Results. Epithelial progenitors were successfully induced by culturing mouse ES cells on type IV collagen for 8 days. These progenitors expressed keratin (K)12, which is specific to corneal epithelial cells, and cell surface CD44 and E-cadherin, both of which are essential in corneal epithelial wound healing. Complete re-epithelialization of the corneal surface occurred within 24 hours after transplantation. The resultant corneal epithelial cells expressed markers of the grafted cells, and no teratomata were observed during the follow-up period.

Conclusions. Epithelial progenitors were successfully induced in vitro from ES cells and were applicable as grafts for treating corneal epithelial injury. ES cells may become an unlimited donor source of corneal epithelial cells for corneal transplantation and may restore useful vision in patients with a deficiency of limbal epithelial cells. This is an important first trial toward assessing the use of ES cells to reconstruct corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2004;45: 4320–4326) DOI:10.1167/iovs.04-0044

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The corneal surface is covered by corneal epithelial cells that form the anterior ocular surface, together with limbal and conjunctival epithelial cells and tear film. Corneal epithelial cells are maintained by the centripetal migration of corneal epithelial stem cells, one of the region-specific epithelial progenitor cells that are also called limbal cells. The limbal cells play a role in the palisades of Vogt, located in the limbus—the narrow transitional zone of the ocular surface between the cornea and the bulbar conjunctiva.[1,2] In development, corneal epithelial stem cells originate from the surface ectoderm that supplies various kinds of region-specific epithelial progenitor cells. Embryonic stem (ES) cells are derived from pluripotent cells within preimplantation embryos and have the pluripotentiality to differentiate into ectoderm, mesoderm, or endoderm cells, assuming the form of any cell lineage, including epithelial progenitors.

Severe and widespread damage of the cornea in ocular surface diseases and injuries, such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, severe microbial infection, and chemical or thermal burn, lead to loss of corneal and limbal epithelial cells. Limbal cell deficiency is manifested by chronic inflammation and vascularization, resulting in conjunctival scarring in the cornea and loss of visual acuity. The therapeutic strategies for these diseases involve two major approaches: one is the transplantation of the limbal graft taken from the healthy contralateral eye (limbal allograft),[3–5] and the other is regeneration of sheets of corneal epithelium in vitro and their transplantation in vivo.[6–17] Recently, limbal cells obtained by biopsy were cultured in the appropriate condition, or on the amniotic membrane to induce differentiation into corneal epithelial cells for transplantation to the damaged cornea.[7–17] More recently, successful culture and autologous transplantation of oral mucosal epithelial cells on the amniotic membrane has been reported in rabbit.[18] It should be noted that transplantation of corneal epithelial cells requires allogeneic donors and carries a risk of immunologic rejection. Although the successful reconstruction of the cornea by transplantation of autologous limbal epithelial cells has been reported,[5,8,11] such a procedure is impossible in severe ocular surface diseases such as Stevens-Johnson syndrome, because the effects of the diseases are usually bilateral, and often the oral mucosa is damaged.

In this study, we report a new strategy for generating corneal epithelial cells from mouse ES cells in vitro and successful reconstruction of damaged corneas by transplantation of the ES-cell–derived epithelial progenitor cells. This is one of the first steps toward using ES cells to reconstruct the corneal epithelium.

Materials and Methods

Cell Culture and Induction of ES Cell Differentiation

Undifferentiated ES cells (R-CMTI-1A; passages 12–18) were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). They originated

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from a 129SV/EVJ mouse and had a normal karyotype and characteristics of prevalent undifferentiated ES cells, including the expression of alkaline phosphatase and OCT-4, a transcription factor essential for pluripotency. The cells were maintained in the presence of mitomycin-C–treated mouse fetal fibroblasts on gelatin-coated dishes.[19,20] The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1× nonessential amino acids, 1× pyruvate, 15% fetal calf serum (FCS), and 1000 U/mL recombinant mouse leukemia inhibitory factor (LIF; Invitrogen-Gibco, Grand Island, NY), to maintain the undifferentiated state of the ES cells. For induction of epithelial cell differentiation, ES cells were first cultured on bacterial-grade Petri dishes in Iscove's modified Dulbecco's medium (Invitrogen-Gibco) containing 10% FCS. After the 4-day culture, floating cell aggregates, called embryoid bodies (EBs), were transferred to plates coated with type IV or VI collagen, poly-L-lysine, or fibronectin (Iwaki, Tokyo, Japan) and cultured in the same medium for 8 days. The cells adhering firmly to the dishes were recovered, and other cell types, including floating cell aggregates and those adhering weakly, were removed by aspiration. The resultant cell population was used for analysis and transplantation.

In some experiments, to trace the ES-cell–derived epithelial progenitor cells, the cells were labeled with $Fe3^-$. The cells were incubated in culture medium supplemented with 20 μg/mL iron oxide (Tanabe, Osaka, Japan) and 20 μg/mL transfection reagent (Roche, Basel, Switzerland).[21–23] We used 2×10^6 cells labeled with $Fe3^-$ as grafts for each damaged cornea.

Transplantation to Recipient Mice

C57BL/6 female mice aged 6 weeks (Charles River, Kanagawa, Japan) served as recipients. All subsequent experiments were conducted in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and received the approval of the Animal Care Facility of St. Marianna University School of Medicine. Mice were anesthetized and placed in a stereotaxic frame (Narishige, Tokyo, Japan). Each mouse cornea was enclosed with a glass tube (Iwaki) to keep the eye open after emplacement, and paper wiper (Kimwipe S-200; Crecia, Tokyo, Japan) with a 2-mm diameter, soaked in 2 μL of *n*-heptanol, was placed on the center of the cornea for 1 minute after anesthesia to injure the corneal epithelium.[24] The graft cells (2×10^6 cells/tube) were put into the tube and allowed to adhere to the damaged cornea for 1 hour. Each group of mice with or without transplantation included 10 mice, and both eyes of all mice were injured. The graft cells were transplanted to both eyes of all the mice in the transplant-recipient group, and the results were compared to the control eye corneas without transplantation.

Flow Cytometric Analysis

Cell surface antigen expression was studied by a flow cytometer (EPICS XL; Beckman Coulter, Tokyo, Japan) using anti-mouse CD44 (BD-PharMingen, Lexington, KY) and anti-mouse E-cadherin (Takara, Ohtsu, Japan). The single cell suspension was incubated with each primary antibody or isotype control IgG, followed by incubation with FITC-conjugated secondary antibody and analysis by flow cytometry.[19]

Reverse Transcription–Polymerase Chain Reaction

Total RNA extraction and cDNA synthesis were conducted as reported previously.[19] Briefly, total RNA was extracted, and then 1 μg of total RNA was reverse transcribed, and complementary DNA (cDNA) was synthesized. β-Actin was used for detection of housekeeping gene expression in all RT-PCRs. The primers used and the expected size of amplified polymerase chain reaction (PCR) products were as follows: β-actin (440 bp), sense gatgacgatatcgctgcgctg and antisense gtacgaccagaggcatacagg; pax-6 (206 bp), sense aacaacctgcctatgcaacc and antisense cttggacgggaactgacact; K12 (437 bp), sense cgagagtggtatgaaaca and antisense tggctctcatttcattg; and K14 (199 bp), sense ggtcgatttgatggatttgg and antisense gttcagtgttggcctcttcc. Cycling parameters were as follows: a hot start at 94°C for 2 minutes, denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 60 seconds. The reaction was repeated for 30 cycles and followed by elongation at 72°C for 10 minutes.

Immunoblot Analysis

Immunoblot analysis was conducted as described previously.[19] Briefly, cells were lysed in 2× SDS sample buffer, and the lysates were electrophoresed on 5% SDS-polyacrylamide gels. Proteins were electrically transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Tokyo, Japan). The membrane was incubated with anti-Pax-6 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-keratin 12 (Santa Cruz Biotechnology), and anti-E-cadherin (Chemicon International, Temecula, CA) antibodies, followed by incubation with a biotin-conjugated secondary antibody (Dako, Kyoto, Japan) and streptavidin-conjugated horseradish peroxidase (HRP). Detection was performed by chemiluminescence (Amersham Pharmacia Biotech).

Histologic Analyses

Cryostat sections (5 μm) of the mouse eyes were mounted on slides. For hematoxylin and eosin (H&E) staining, the sections were fixed with 20% formaldehyde in methanol, washed extensively, and stained with H&E. For the detection of intracellular $Fe3^-$ of colloidal iron-transfected donor cells, we performed Berlin blue staining. Briefly, frozen sections were fixed with 10% formaldehyde solution, extensively washed, and stained with Berlin blue solution, followed by counterstaining of the nucleus with fast red solution. For immunostaining, the samples were incubated with primary antibody followed by a peroxidase-conjugated secondary antibody. Reactivity was visualized with 3-amino-9-ethylcarbazole.

Sry Gene PCR

PCR was used to detect the male-specific sequence (*Sry* gene of ES cells) in genomic DNA extracted from the cornea of recipient female mice by a conventional method.[25,26] The reaction was performed with primers specific for the *Sry* gene on chromosome Y (sense, gttttggactggtgacaattg; antisense, gtcttgcctgtatgtgg). We know that the 402-bp band (*Sry*) identifies the male genotype, since we confirmed the DNA sequence of the amplified product by TA cloning and subsequent DNA sequencing (data not shown). β-Actin–specific primers were used to monitor the correct amplification of the template DNA (data not shown). This method allowed the identification of the ES-cell–derived epithelial cells in the recipient female cornea.

Results

Establishment of Optimal Culture Condition for ES Cells to Differentiate into Epithelial Progenitor Cells

Based on the evidence that (1) type IV collagen is the basic structural component of all basement membranes (BMs)[27]; (2) the cornea is initially covered with an epithelium that attaches to normal BM in the development of eyes[27]; (3) the conjunctival, limbal, and corneal BMs each include diverse collagen components[28,29]; and (4) the limbal epithelial cells can be successfully cultured on amniotic membrane[17] that consists of collagen and BM, we attempted to culture ES cells on several extracellular matrices, including type-IV and -VI collagen, poly-L-lysine, and fibronectin, to induce differentiation into the epithelial lineage. Undifferentiated ES cells were recovered from the maintenance culture consisting of LIF and mouse fetal fibroblasts. The ES cells were cultured in noncoated dishes for 4 days to form EBs. Then the EBs were transferred to a plate coated with each extracellular matrix. The cells cultured in the presence of type IV collagen began to grow and form an epithelium-like monolayer at day 8 of culture (Fig. 1), suggest-

FIGURE 1. Induction of differentiation of epithelial progenitor cells from ES cells in vitro. ES cells were cultured in noncoated dishes for 4 days to form EBs, and the EBs were cultured on type-IV collagen for 8 days. The adhering cells had an epithelial-cell-like appearance. Staining, H&E. Scale bar, 50 μm.

ing that the ES cells had differentiated into cells committed to an epithelial lineage. To confirm and characterize the differentiation of ES cells into corneal epithelial cells, we investigated the expression pattern of cytokeratins in addition to the eye development marker *pax-6*. As shown in Figure 2A, RT-PCR demonstrated that ES-cell-derived epithelial cells cultured on type-IV or -VI collagen expressed K12, a specific marker of corneal epithelial cells. They did not express K14, which was expressed in the squamous epithelial basal layer. Moreover, these cells expressed *pax-6*, which is necessary for early development of eyes (Fig. 2A). As shown in Figure 2B, immunoblot analysis confirmed the expression of K12 in ES-cell-derived epithelial cells cultured with type IV collagen. Pax-6 appeared in the early stage of differentiation and was hardly detected at day 8 of culture (Fig. 2B). These results indicated that ES-cell-derived cells cultured on type-IV-collagen-coated plates had the characteristics of epithelial cells necessary for eye development.

Cell Surface Expression of E-Cadherin and CD44 on ES-Cell–Derived Epithelial Progenitor Cells

We established epithelial progenitor cells from mouse ES cells cultured on plates coated with type-IV or -VI collagen. We next evaluated whether the cells were appropriate for transplantation to the damaged cornea. We focused on studying the expression of cell adhesion molecules. E-cadherin has been deemed to have an important role in wound healing after corneal epithelial ablation.[30] Similarly, an earlier increase of CD44 transcription is observed during corneal epithelial wound healing.[31] We investigated the expression of E-cadherin and CD44 on ES-cell-derived epithelial progenitor cells. The cells cultured on type IV collagen expressed E-cadherin, and its expression level gradually increased along with epithelial differentiation (Fig. 3A). We next analyzed the cells by flow cytometry. We detected cell surface expression of CD44 and E-cadherin on the ES-cell-derived epithelial progenitor cells. The percentage of CD44 and E-cadherin–positive cells was 33.6% and 24.3%, respectively (Fig. 3B). The adhesion molecules expressed on the ES-cell-derived progenitor cells may facilitate their adhesion to the injured corneal surfaces. These findings suggested that these cells could be used for transplantation and corneal wound healing.

Successful Transplantation of ES-Cell–Derived Epithelial Progenitor Cells to Damaged Cornea

After corneal surface injury with *n*-heptanol, the ES-cell-derived epithelial progenitor cells cultured on type IV collagen for 8 days were transplanted to the injured cornea. At 1, 12, and 24 hours after transplantation, histologic examination of the eyes disclosed that the epithelial progenitor cells adhered well to the recipient corneal stroma and completely covered the damaged corneal surface (Figs. 4C–F, 4J, 5A). After injury, almost all epithelial cells were gone from the corneal surface, the stroma shrank, and inflammatory cells infiltrated the stroma and anterior chamber (Fig. 4B), compared with normal cornea (Fig. 4A). The stromal shrinkage was inhibited by the transplantation of ES-cell-derived epithelial progenitor cells, and subepithelial infiltration of inflammatory cells was greatly reduced after transplantation (Figs. 4C–F). At a higher magnification, normal corneal epithelium showed complex layering consisting of basal cells, wing cells, and superficial cells (Fig. 4G). In contrast, transplanted cells formed a monolayer (in places, two layers) on the stroma; however, they had nuclei, and some of them showed a basal or wing-cell-like appearance (Fig. 4H, arrows), indicating that transplanted cells had characteristics of nonkeratinized corneal epithelial cells. Cells were not observed on the stroma, however, either in the center (Fig. 4I) or in the limbus (Fig. 4J) of injured cornea without transplantation 24 hours after the injury, suggesting that the epithe-

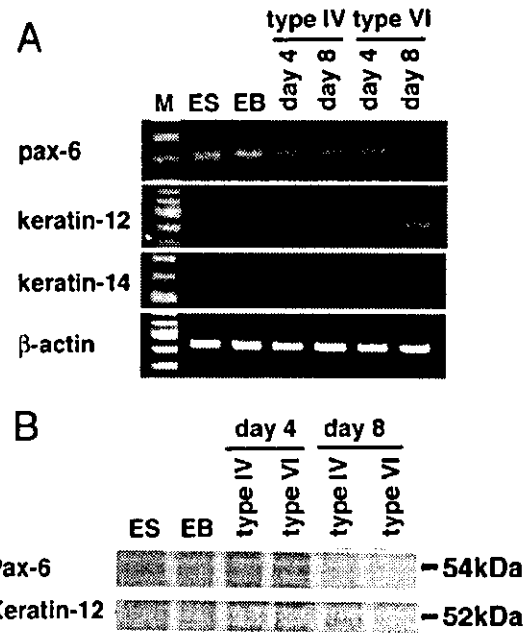

FIGURE 2. Expressions of keratins and *Pax-6* in ES-cell-derived cells. (A) ES cells were induced to differentiate into epithelial progenitor cells. Total RNA was extracted at each differential stage, reverse transcribed, and PCR amplified. The product was electrophoresed on 1.5% agarose gel and visualized with ethidium bromide. Data shown are representative of results in three independent experiments. (B) At each stage of differentiation of ES-cell-derived cells cultured on plates coated with type-IV or -VI collagen, cell lysates were extracted. Then immunoblot analyses with Abs to K12 and Pax-6 were performed. *Arrows*: bands corresponding to the size of Pax-6 and K12. Data are representative of results in three independent experiments.

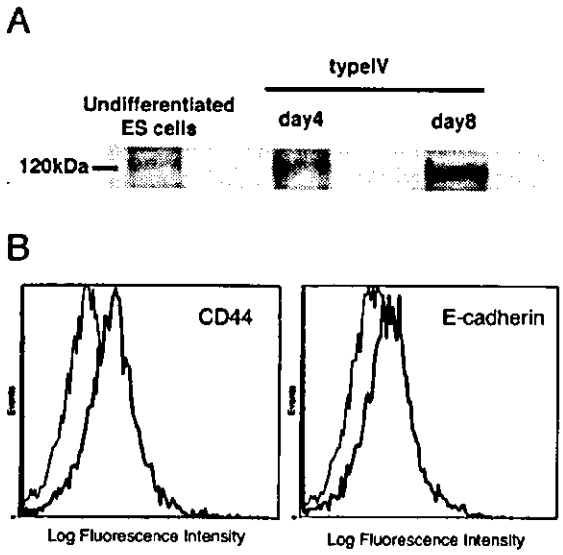

FIGURE 3. Expressions of E-cadherin in ES-cell-derived epithelial progenitor cells. (A) ES cells were induced to differentiate into epithelial progenitor cells by culture on type-IV collagen-coated plates. At each stage of differentiation, cell lysates were prepared, and expression of E-cadherin was analyzed by immunoblot analysis. *Arrow*: a band corresponding to the size of E-cadherin. (B) Cell surface expressions of CD44 and E-cadherin on the epithelial progenitor cells induced from ES cells were examined by flow cytometry. The results using control IgG (*thin line*) and each Ab (*thick line*) are shown. A representative of three independent experiments is shown in each panel.

lial cells covering the corneal surface after transplantation originated from grafted cells, not host-derived epithelial progenitor cells. Moreover, immunochemical staining for E-cadherin, one of the epithelial markers, demonstrated that the cells covering the corneal surface were of E-cadherin–positive epithelial lineages but not other lineages, such as myofibroblasts.

Origin of Corneal Epithelial Cells Covering the Damaged Corneal Surface

To examine further whether the epithelial cells covering the damaged cornea after transplantation originates from ES-cell-derived epithelial progenitor cells, we used two different approaches. First, we introduced colloidal iron ($Fe3^+$) into the grafted cells by using a transfection reagent.[21–23] We detected the cells labeled with colloidal iron by Berlin blue staining after corneal transplantation. Our labeling procedure with colloidal iron was simple and seemed essentially to make no change in the characteristics and viability of epithelial progenitor cells, as reported in earlier studies.[21–23] The epithelial cells covering the damaged corneal surface were stained well with Berlin blue (Fig. 5A, 5B; blue spots) at 24 hours after transplantation, suggesting that these cells originated from the ES-cell-derived epithelial progenitor cells, not the host. To confirm this fact, we next examined the expression of the *Sry* gene, which is located on the Y chromosome of ES cells. Recipient female mice were negative for the gene. At 1 and 24 hours after transplantation, the epithelial cells on the cornea had *Sry* gene expression (Fig. 5C). The epithelial cells covering the damaged cornea may have originated from the ES cells.

DISCUSSION

Severe and wide ocular surface diseases and chemical or thermal burn are the most difficult problems for ophthalmologists. In recent years, surgical reconstruction after ocular surface damage, even in the acute phase of inflammation or injury, has been advanced. Application of a limbal graft taken from the healthy contralateral eye,[3–5] transplantation of amniotic membrane,[7,9,15,16] transplantation of cultured epithelial cells in vitro,[7–11,17] and autologous transplantation of oral mucosal epithelial cells on amniotic membrane[18] have been reported. However, the most important key to making the reconstruction possible is the provision of sufficient materials for transplantation. Thus, we focused on developing a method for corneal epithelial transplantation using epithelial progenitor cells derived from ES cells.

ES cells are derived from the pluripotent cells of early embryos and can maintain a normal karyotype infinitely on culture in vitro and can differentiate into any cell type under appropriate conditions. Recently, human ES cells have been established, and the production of any kind of cell and tissues derived from ES cells for transplantation has logically become a reality.[32–38] This experimental therapeutic approach has already been reported in the nervous system.[20,39–41] In addition, some differentiation of epithelial lineages, such as cutaneous epithelial cells,[42] lung alveolar epithelial cells,[43] epithelial islets of thymus,[44] and pigmented epithelial cells,[45] has been induced from ES cells in vitro. In this report, we established a system to induce differentiation into epithelial progenitor cells by culturing ES cells on plates coated with type IV collagen for transplantation and further differentiation into corneal epithelial cells in vivo. Previous reports have demonstrated that mouse ES cells cultured without LIF on plates coated with type IV collagen differentiate into Flk-1-positive cells and are approximately 40% of the total ES cells after culturing for 4 days.[46,47] In addition, all the Flk-1-positive cells further differentiate into either vascular endothelial cells or vascular smooth muscle cells in the presence of vascular endothelial growth factor (VEGF).[46,47] In this study, a considerable portion of the ES cells cultured without LIF on the plate coated with type-IV collagen differentiated into epithelial cells in vitro. They may have further differentiated into corneal epithelial cells in vivo. This means that the epithelial progenitor cells established in our study were not completely committed to becoming corneal epithelial cells and may have the plasticity to differentiate into other epithelial cells. Thus, further identification of our epithelial progenitor cells is warranted. However, the partial or incomplete differentiation of ES cells into some lineages has the advantage of avoiding the development of teratoma, which is an obstacle to the successful transplantation of ES-cell-derived cells. Moreover, we can directly observe the corneal surface, facilitating early detection of unwanted events on the cornea. Indeed, we have not noted development of teratoma to date.

Keratins are the intermediate filamentous proteins of epithelial cells. A large number of mammalian keratins have been identified and, based on their biochemical properties, have been divided into two groups. The type-I keratins are designated K1 to K8 and the type II, K9 to K20. The keratin proteins form heterodimers with one member from each group.[48] Specific members of type-I and -II keratins are characteristically associated with each other in different cell and tissue types. Simple epithelia, such as the gut, express predominantly K8 and K18. Stratified squamous epithelia express mainly K5 and K14 in their basal layers, whereas the suprabasal layers express K1 and K10 in skin and K4 and K13 in some other epithelia, such as the esophagus.[49]

In ocular epithelia, it is important to distinguish the corneal epithelia from conjunctival epithelia because they have inverse characteristics, and successful transplantation of pure corneal epithelium depends on the exclusion of conjunctival epithelium. Recent reports have suggested that K3 and K12 are

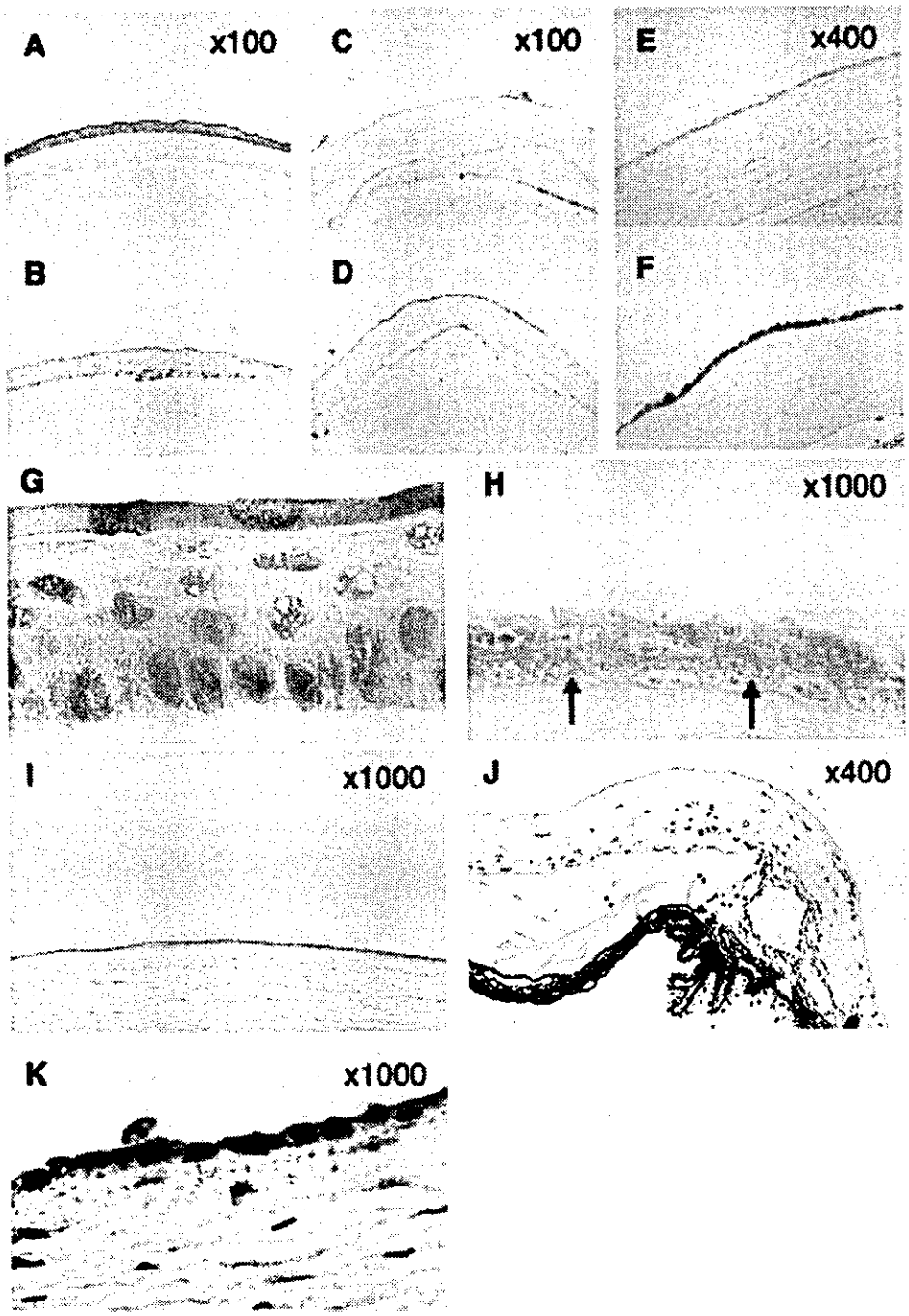

FIGURE 4. Histologic analysis of injured cornea, with or without transplantation of the ES-cell-derived epithelial progenitor cells. The ES-cell-derived epithelial progenitor cells (day 8 culture) were transplanted to *n*-heptanol-injured cornea of mice. (A) Normal mouse cornea. (B) *n*-Heptanol-injured cornea without transplantation. (C–F) Mouse eyes were injured with *n*-heptanol. At 1 hour (C, E) and 12 hours (D, F) after transplantation, the eyes were enucleated. Cryostat sections were fixed with 20% formaldehyde in methanol, stained with H&E, and compared with those of normal cornea. (G) Higher magnification of the normal corneal epithelium shown in (A). (H) Higher magnification of another preparation of the ES-cell-derived epithelial progenitor cells at 12 hours after transplantation. *Arrows*: the basal or wing-cell-like transplanted cells. (I) Higher magnification of *n*-heptanol-injured cornea without transplantation 12 hours after the injury. No corneal epithelial cells were observed. (J) Limbus of *n*-heptanol-injured cornea without transplantation 24 hours after the injury. Migration of the host-originated progenitor cells onto the corneal surface was not observed. (K) Immunostaining for E-cadherin of the corneal epithelial cells 12 hours after transplantation of ES-cell-derived graft cells. E-cadherin-positive epithelial cells are stained *red*. Data shown are representatives of results in 10 independent experiments.

specifically expressed in the corneal epithelium. Whereas K4 and K13 are expressed in the conjunctival epithelium, at a lesser level, K4, has also been observed in the corneal epithelium.[18,50] The ES-cell-derived epithelial progenitor cells express a low level of K12, which is a specific corneal epithelium cell marker, suggesting the possibility that ES-cell-derived epithelial progenitor cells can differentiate further into mature corneal epithelial cells in appropriate microenvironments. Pax-6 expression was evident in the ES-cell-derived progenitor cells. Pax-6 is very much involved in controlling eye development, including forming the cornea.[51] It is necessary to stimulate K12 promoter activity.[52,53] Western blot analysis demonstrated that the expression of Pax-6 preceded K12 expression in the epithelial progenitor cells cultured in the presence of type IV collagen. This suggests that Pax-6 and other signals generated by interaction with type IV collagen were necessary for K12 promoter activity.

We successfully transplanted ES-cell-derived epithelial progenitor cells in conventional mice. Detection of the *Sry* gene specific to the male genotype is one of the most useful methods for identification of the donor cells that originate from ES cells (male mouse origin) in the female recipient cornea.[25,26] The evidence that PCR detected a male-specific sequence (*Sry* gene on the Y chromosome of ES cells) in genomic DNA extracted from the cornea of female recipient mice definitely confirmed histologically the survival of transplanted cells and reconstruction of the corneal epithelium. The ES-cell-derived epithelial progenitor cells expressed both E-cadherin and

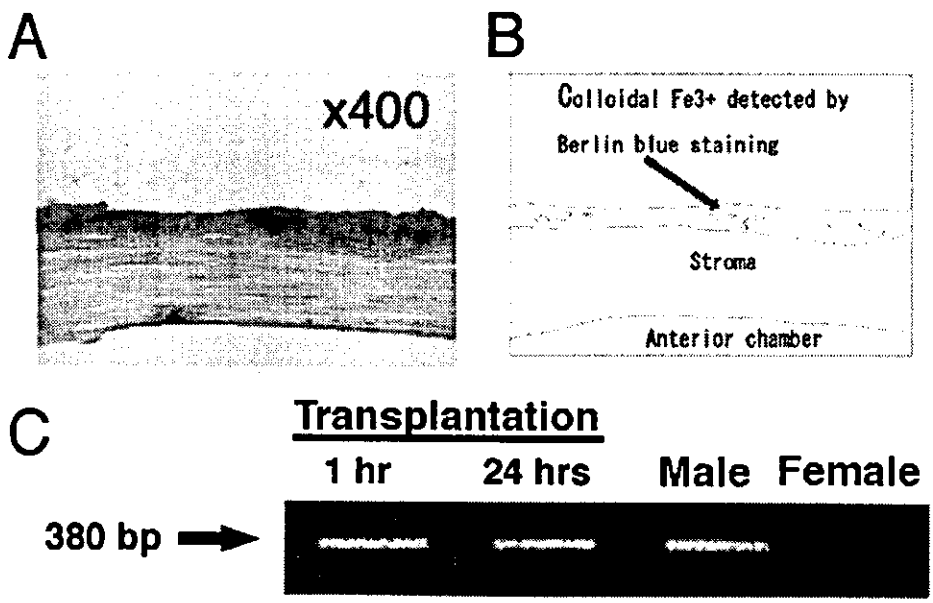

FIGURE 5. Expression of donor-cell-specific markers in epithelial cells covering the damaged cornea after transplantation of ES-cell-derived epithelial progenitor cells. (**A**) The ES-cell-derived epithelial progenitor cells were labeled with colloidal iron ($Fe3^-$) and transplanted to the *n*-heptanol-injured cornea of mice. At 24 hours after transplantation, cryostat sections were stained with Berlin blue solution, followed by counterstaining of nucleus with fast red solution. We detected the $Fe3^-$ positive ES-cell-derived cells as those with *blue* particles in their cytoplasm. (**B**) A schematic representation of (**A**). (**C**) At 1 hour and 24 hours after transplantation of the ES-cell-derived epithelial progenitor cells with a Y chromosome to *n*-heptanol-injured corneas of female recipient mice, genomic DNA was extracted from the corneal surface, and the *Sry* gene was detected by PCR. The *Sry* gene-specific PCR products from male and female mouse corneal DNA served as the positive and negative control, respectively.

CD44. The critical roles of E-cadherin during wound healing after corneal epithelial injury has been reported,[30] whereas CD44 has also been reported with its earlier increase of transcription during corneal epithelial wound healing.[31] Thus, the expression of adhesion molecules may contribute to successful transplantation by inducing tight cell-to-cell and/or cell-to-matrix interaction.

Taken together, both the location of cornea and the characteristics of ES-cell-derived progenitor cells may make it clinically possible to transplant ES-cell-derived cells to the cornea. This is one of the first successful trials toward using ES cells to reconstruct the corneal epithelium.

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Hyaluronic Acid–CD44 Interaction Mediates the Adhesion of Lymphocytes by Amniotic Membrane Stroma

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Purpose: To demonstrate the role of intrinsic hyaluronic acid (HA) in the entrapment of inflammatory cells by amniotic membrane (AM) in vitro.

Methods: HA concentration in AM was analyzed by the sandwich protein binding assay, and the approximate molecular weight was measured by gel filtration chromatography. To localize HA in AM tissue, toluidine blue staining with and without hyaluronidase treatment was performed. Adhesion of the lymphocyte cell lines, Jurkat and Molt 4, and peripheral blood mononuclear cells (PBMC) to AM and HA-coated glass slides was analyzed in an in vitro binding assay. Flow cytometry was performed to quantify the expression of the HA receptor, CD44, in Jurkat, Molt 4, and PBMC.

Results: HA was present in high levels in the stroma of AM, also demonstrated by intense staining with toluidine blue. Staining was inhibited by both hyaluronidase treatment and acidic pH. Molt 4, which constitutively expressed CD44, bound to AM and HA-coated slides significantly more than Jurkat cells ($CD44^-$). Adhesion of Molt 4 was inhibited by pretreatment with both soluble HA and anti-CD44 antibody. LPS- or TNF-α-treated PBMC also bound to AM and HA-coated slides and was inhibited by pretreatment with an anti-CD44 antibody.

Conclusion: HA in AM stroma may play an important role in the entrapment of inflammatory cells including lymphocytes when used as a patch in ocular surface disease.

Key Words: antiinflammatory, cornea wound healing, amniotic membrane, hyaluronic acid, CD44, extracellular matrix adhesion

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Amniotic membrane (AM) has a thick collagen layer and an overlying basement membrane supporting a single layer of epithelium.[1] The use of AM as a surgical material dates as far back as 1913, when Stern reported the use of AM in surgical procedures on the skin.[2] In regard to application of fetal membrane to ocular surface disorders, De Rotth first reported the treatment of conjunctival defects after symblepharon repair in 1940,[3] followed by several similar reports in the next decade.[4,5] More recently, Kim and Tseng reported the use of AM for ocular surface reconstruction in the rabbit,[6] followed by several groups who have reported clinical use of AM in the treatment of ocular surface disease.[7,8] Results of such studies show that AM has the ability to suppress both inflammation and fibrosis.[7,9,10] Kim et al reported that the patching of AM on epithelial defects of the cornea resulted in rapid epithelialization and decreased infiltration of inflammatory cells.[11] Data have also been presented showing that AM can suppress proteinase and matrix metalloproteinase activation.[11] However, the precise mechanisms involved in the antiinflammatory effects of AM still remain unclear.

We previously found inflammatory cells trapped in the amniotic stromal tissue after AM patching in patients with various ocular surface diseases.[12] To elucidate the mechanisms involved, we conducted an in vitro study to determine the ability of AM to bind inflammatory cells, with emphasis on hyaluronic acid (HA), a high-molecular-weight glycosaminoglycan distributed widely in the body. HA is a major carbohydrate component of the extracellular matrix that can be found in skin, joints, and Wharton jelly in the umbilical cord.[13] It is also involved in cell-to-matrix adhesion and plays a central role in scarless wound healing in the fetus.[14] HA has high water retention capacity and participates in the proliferation and differentiation of cells.[14–18] In this study, we hypothesized that HA found in AM acts as a ligand for CD44 expressed on inflammatory cells and thereby traps such cells that have infiltrated to the ocular surface. The entrapment of inflammatory cells may serve to reduce damage to ocular tissue.

MATERIAL AND METHODS

Amniotic membranes were donated by mothers seronegative for human immunodeficiency virus and hepatitis B and C

viruses at the time of cesarian sections. AM was stored with 15% dimethylsulfoxide (Sigma, St Louis, MO) with PBS at −80°C until use. Written informed consent was obtained from all donors before surgery.

Detection of HA in Human AM

HA was extracted from AM with 0.5 N sodium hydroxide at 4°C for 20 hours and neutralized with hydrochloric acid. HA concentration was measured using the sandwich binding protein method, using plates bound with immobilized HA and peroxidase-labeled HA binding protein (Chugai Pharmaceutical Co, Ltd, Tokyo, Japan). The molecular weight distribution of HA was obtained by gel filtration chromatography with an Asahipack GS-620 column (Asahi Kasei Co, Tokyo, Japan).

AM stroma was also stained with toluidine blue, which specifically stains glycosaminoglycans containing HA. AM tissue was fixed in 10% buffered formalin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and embedded in paraffin. The sections were stained with 0.05% toluidine blue (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at a pH of 7.0 (0.1 M citric acid, 0.2 M disodium hydrogen phosphate 12-water).[19] Hyaluronidase-digested sections were prepared by treating paraffin-fixed samples with bovine testis hyaluronidase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) in 0.1 M phosphate-buffered saline (pH 5.5) for 3 hours at 37°C.[20] To differentiate HA from other glycosaminoglycans, staining of toluidine blue was compared at 3 different pH values (7.0, 4.1, and 2.5) titrated with 0.1 M citric acid and 0.2 M disodium hydrogen phosphate 12-water.[19] Staining of HA by toluidine blue is weak under acidic conditions.

CD44 Expression by T-Lymphocyte Cell Lines and PBMC

T-cell lines used in the study (Jurkat and Molt 4) and human PBMC were analyzed by flow cytometry (Epics XL, Beckman Coulter, FL) to confirm the expression of CD44 on the cell surface. Jurkat, Molt 4, or human PBMC (1×10^6 cells) were incubated with either 33.3 μg/mL of FITC-labeled isotype IgG 1 (Immunotech, Marseilles, France) or a FITC-labeled anti-CD44 monoclonal antibody (Immunotech, Marseilles, France) containing 1.3% FCS and 0.1% sodium azide in PBS for 45 minutes at 4°C. Cells were washed twice with PBS before flow cytometry.

In Vitro Adhesion Assay

AM was fixed in Tissue-Tek OCT compound (Sakura Finetechnical Co, Ltd, Tokyo, Japan) and stored at −80°C until use. The cryostat sections were mounted on glass slides, air dried for 5 minutes, and fixed for 10 minutes at 4°C in 3% glutaraldehyde (TAAB, Berkshire, England) with PBS. The sections were washed in cold deionized water for 10 minutes. HA (0.1 mg/mL) (Biozyme Laboratories, CA) was applied to APS-coated (silanization) glass slides (Matsunami Glass Ind, Ltd, Osaka, Japan) and thoroughly air dried. Jurkat and Molt 4 (2.0×10^6 cells/mL) were suspended in RPMI 1640 (Gibco BRL, Grand Island, NY) containing 10% FCS.

PBMC from normal healthy donors were isolated by density centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). PBMC were cultured for 72 hours in RPMI 1640 medium supplemented with 10% vol/vol autologous serum at 37°C, 5% CO_2 in air. Supplements were used at the following concentrations: lipopolysaccharide (LPS) (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 1 μg/mL, TNF-α (Gibco BRL, Grand Island, NY) at 10 ng/mL. Cells were applied to HA-coated glass slides or fixed cryostat sections and then incubated for 10 minutes at 37°C. Samples were washed by dipping the glass slides in cold PBS and fixed for 10 minutes in 3% glutaraldehyde at 4°C. After washing with deionized water, slides were stained with hematoxylin and eosin stain (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

To inhibit the adhesion of cells, suspensions were preincubated with either 1 mg/mL HA for 30 minutes at 37°C or 20 μg/mL of anti-CD44 monoclonal antibody (Seikagaku Co, Tokyo, Japan) for 60 minutes at 37°C. The number of attached cells per square millimeter was analyzed by the NIH Image program (developed at the National Institutes of Health, Bethesda, MD). Statistical analysis was done by the Mann-Whitney *U* test using the StatView 5.0 program for the Macintosh (SAS Institute Inc, Cary, NC).

RESULTS

Detection of HA in Human AM

The HA content in AM was 140 ng/mg wet weight measured by the sandwich binding protein assay, with a high molecular weight in the range of 1670 kD. Figure 1 shows the distribution of glycosaminoglycans (GAGs), including HA, in AM when stained with toluidine blue. To differentiate staining of HA from other GAG members, treatment with 0.5% hyaluronidase markedly decreased toluidine blue staining of the stroma (Fig. 1B). This was also confirmed by toluidine blue stains of AM under various pH values: unlike other GAGs, HA does not stain at a pH of 2.5 (Fig. 1).

Detection of CD44 on Cell Lines of T-Lymphocytes

The T cell lines Jurkat and Molt 4 were examined for cell-surface CD44 expression by flow cytometry analysis. CD44, the receptor of HA, was expressed only in Molt 4 cells and not in Jurkat cells (Fig. 2A,B, solid peaks) when compared with the background values obtained with isotype IgG1 (Fig. 2A,B, open peaks).

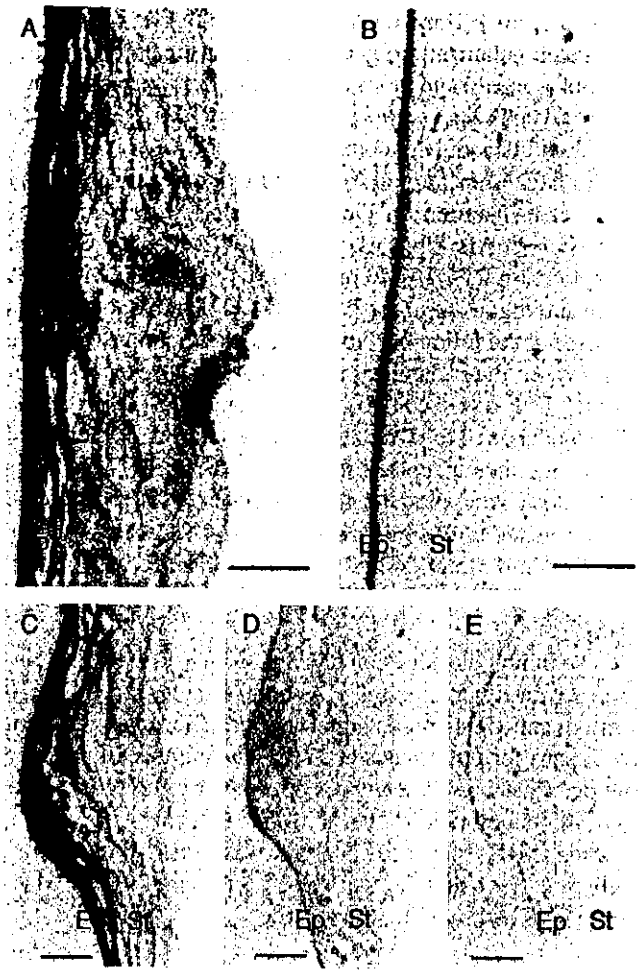

FIGURE 1. Paraffin sections of AM were treated with (B) or without (A) 0.05% bovine testicular hyaluronidase and stained with 0.05% toluidine blue (TB) (pH 7.0). Sections stained at pH 7.0 (C), 4.1 (D), and 2.5 (E) showed a gradual decrease in intensity, indicating that the glycosaminoglycan stained by TB was hyaluronic acid. Ep, amniotic epithelium; St, stroma of AM. The bar represents 50 μm.

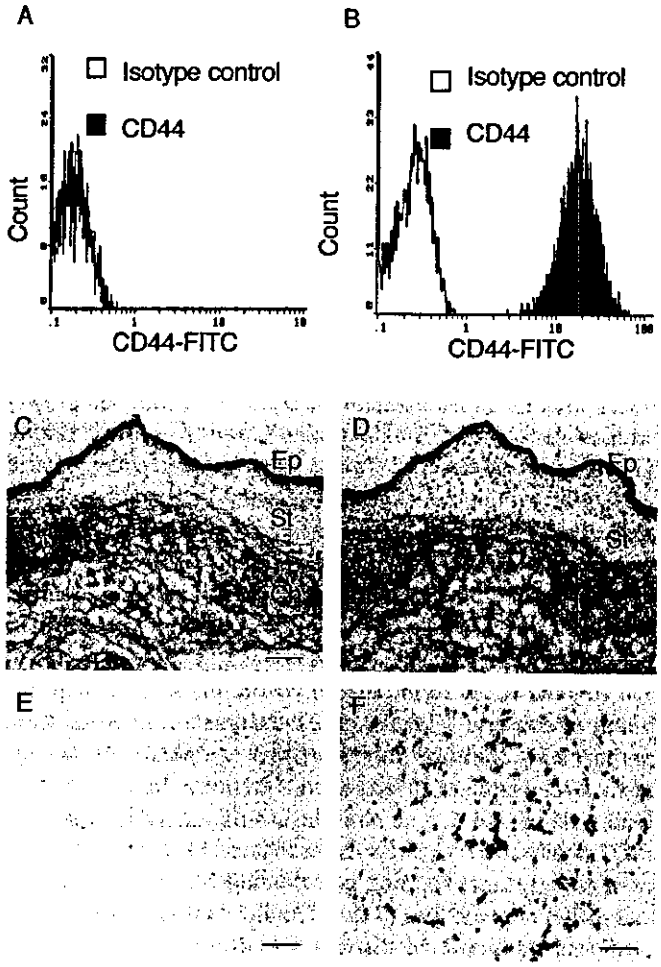

FIGURE 2. Flow cytometry of Jurkat (A) and Molt 4 (B) incubated with isotype control IgG1 (open peaks) or CD44 monoclonal antibody (solid peaks) confirmed the expression of CD44 on Molt 4. Adhesion assays of Jurkat (C, E) and Molt 4 (D, F) to cryostat sections of AM and immobilized HA both showed greater adhesion by Molt 4. E, amniotic epithelium; S, stroma of AM; C, chorion. The bar represents 100 μm.

Adhesion Assay of T-Lymphocytes Cell Lines

Adhesion assays of Molt 4 ($CD44^+$) and Jurkat ($CD44^-$) to AM revealed that only Molt 4 adhered to the AM stroma in areas with abundant HA and did not adhere to the epithelium and chorion (Fig. 2D). Adhesion of Jurkat cells was sparse (Fig. 2C), suggesting that HA-CD44 interaction is involved in lymphocyte adhesion to AM. Because AM contains other glycosaminoglycans and matrix components, Jurkat and Molt 4 binding assays were repeated using HA-coated glass slides. As with the AM adhesion assay, only Molt 4 bound to the immobilized HA (Fig. 2E,F). To confirm this, the same adhesion assay was done with Molt 4 preincubated with soluble HA or anti-CD44 mAb. Preincubation with soluble HA decreased the number of adhering Molt 4 cells to 6.5% of control (Fig. 3A–C, $P = 0.019$). Molt 4 adhesion was also blocked by anti-CD44 mAb to 11.7% of control (Fig. 3D–F, $P = 0.002$). Adhesion of Molt 4 to immobilized HA was blocked by anti-CD44 mAb (Fig. 3G,H).

Detection of CD44 on PBMC

PBMC were examined for cell-surface CD44 expression by flow cytometry analysis. To separate lymphocytes and monocytes in white blood cells, cells were analyzed by gating on forward and side scatter. CD44 was expressed on each cell type (Fig. 4A,B, solid peaks) when compared with the background values obtained with isotype IgG1 (Fig. 4A,B, open peaks).

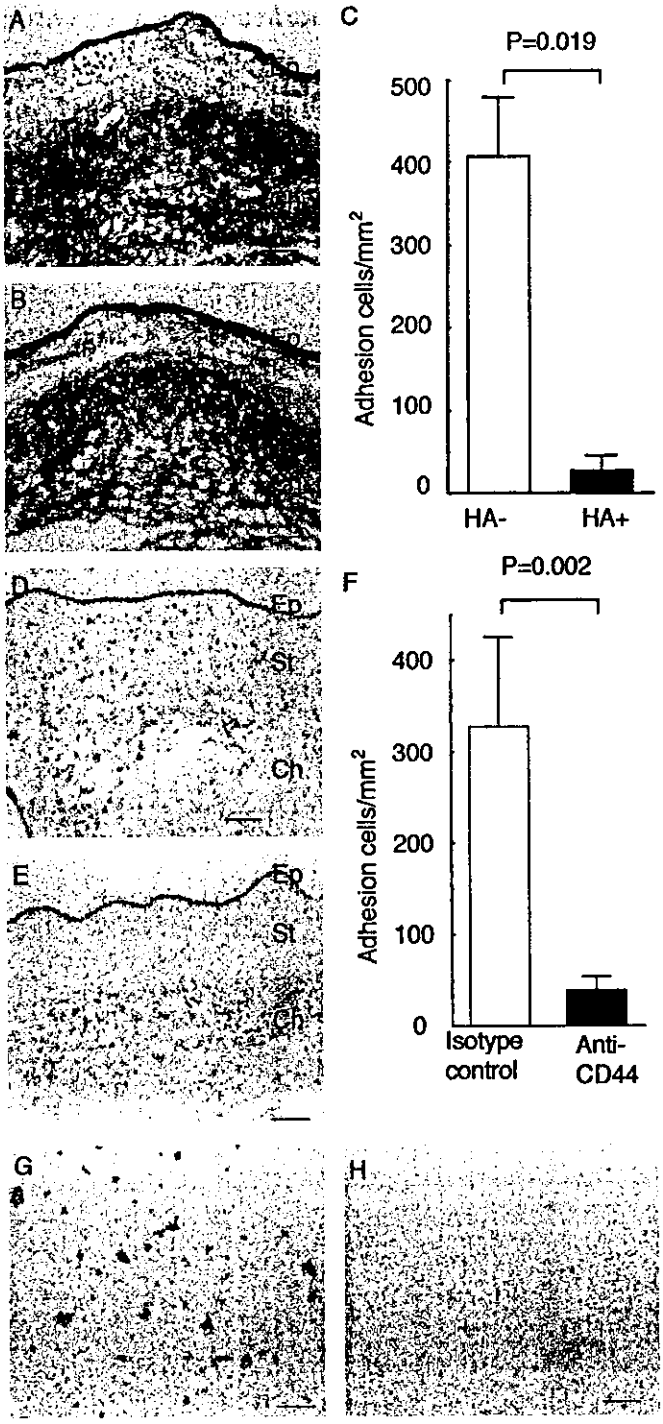

FIGURE 3. Molt 4 adhesion to AM sections was significantly inhibited by preincubation with soluble HA (1 mg/mL) (B) compared with control (A, C). (HA^-, n = 8; HA^+, n = 6). Adhesion of Molt 4 to AM was also inhibited by anti-CD44 mAb (E) but not by isotype control (D, F) (n = 10). Anti-CD44 mAb also inhibited Molt 4 adhesion to HA-immobilized glass slides (H) compared with control (G). Ep, amniotic epithelium; St, stroma of AM; Ch, chorion. The bar represents 100 μm.

Adhesion Assay of PBMC

Both LPS and TNF-α increased the adhesion of PBMC to the AM stroma but not to amniotic epithelium and chorion (Fig. 4D–F). PBMC binding assays were repeated using HA-coated glass slides. As with the AM adhesion assay, stimulation with LPS or TNF-α increased PBMC adhesion to immobilized HA (Fig. 4F–H). PBMC adhesion induced by LPS or TNF-α treatment was also blocked by anti-CD44 mAb to 11.5% and 7.7% of control, respectively (Fig. 5, $P = 0.003$, $P = 0.002$). To compare adhesion with other glycosaminoglycans, PBMC binding assays were repeated using chondroitin sulfate (CS)-coated glass slides. PBMC treated with LPS or TNF-α did not show an increase in adhesion to immobilized CS (Fig. 4I–K), suggesting that HA–CD44 interaction is the main mechanism involved in lymphocyte adhesion to AM.

DISCUSSION

High-molecular-weight HA (approximately 1.67×10^6) was detected in high levels within the stroma of AM but not in the amniotic epithelium. The pattern of HA distribution corresponded with the collagen-rich zones of AM[21] and consisted of typical fibrous connective tissue with a high concentration of type IV and V collagen.[1] Our results were consistent with a previous report measuring HA distribution in AM.[22] Various experimental studies have shown that the antiinflammatory effects of high-molecular-weight HA are associated with its scavenging of free radicals,[23] inhibition of cytokine production,[24] or suppression of elastase release from activated peritoneal leukocytes.[25] In a previous report, supplementation of dialysis fluid with high-molecular-weight HA reduced the intraperitoneal inflammatory reaction in rats maintained for 1 mouth on peritoneal dialysis.[26] The high-molecular-weight HA in AM may also exert such effects; however, physical sequestration alone, by trapping inflammatory cells, may also have antiinflammatory effects.

Pathology of clinical samples after AM patching to the ocular surface revealed the entrapment of inflammatory cells of monocyte/macrophage lineage and lympocytes throughout the thickness of the stroma.[12,22] An approximately equal ratio of $CD4^+$ and $CD8^+$ cells were found, indicating that both types of T lymphocytes were present. We found that CD44, expressed on Molt 4 and PBMC when stimulated with IL-2 and IFN-γ,[27] was required for adhesion to both HA-coated slides and fixed AM stroma sections. Although both CD44 and the β1 integrin heterodimers play a role in mediating the adhesion of ovarian carcinoma cells to mesothelial cells,[28] our previous study indicated that adhesion of Molt 4 was not inhibited by blocking the adhesion molecules β1 and β2 integrins.[12]

The abundance of the high-molecular-weight form of HA may be crucial to the physiological effects observed following AM patching of the inflamed ocular surface.