Table 3. Corneal Epithelialization after First LSCT in Each Group

LSCT Type	No. of Eyes	No PK	Simultaneous PK	2-Step PK	Total
Autograft	11	8/8 (100%)	1/2 (50.0%)	1/1 (100%)	10/11 (90.9%)
Allograft	21	2/3 (66.7%)	3/13 (23.1%)	2/5 (40.0%)	7/21 (33.3%)
Unilateral	10	2/2 (100%)	1/8 (12.5%)	0/0	3/10 (30.0%)
Bilateral	11	0/1 (0%)	2/5 (40.0%)	2/5 (40.0%)	4/11 (36.4%)

LSCT = limbal stem cell transplantation; PK = penetrating keratoplasty.

anesthesia, and adjacent superficial limbal tissue was also excised using a surgical knife (Micro-Feather Blade K-730, Feather Safety Razor Co., Tokyo, Japan). Typically, a tissue pair was obtained from the 6-o'clock and 12-o'clock positions, and the Tenon's capsule was left intact. The grafts were transferred to the injured eye and secured on the amniotic membrane at the 6-o'clock and 12-o'clock limbal area. They were secured using 10-0 nylon sutures and 8-0 vicryl sutures to the limbus and sclera, respectively. Sutures were placed through both amniotic membrane and sclera, trying to stretch the grafts. In KLAL, a ring-shaped limbal graft was prepared from the peripheral portion of an eye bank donor cornea. The scleral and deep stromal tissues were excised, trimmed, and then secured on the limbal portion of the host eye after AMT. In both CLAU and KLAL, amniotic membrane on the central cornea was excised unless underlying corneal stroma was severely degraded. At the end of surgery, either a therapeutic soft contact lens or an amniotic membrane patch was placed to protect the corneal surface. Subconjunctival injection of antibiotics (dibekacin sulfate [Panymicin, Meiji, Tokyo, Japan]) and corticosteroid (betamethasone [Rinderon, Shionogi Pharmaceutical Co., Osaka, Japan]) was performed.

After surgery, intensive management of epithelialization was performed. This included preservative-free artificial tears (Soft San-Tear, Santen Pharmaceutical Co., Osaka, Japan), hyaluronic acid eyedrops (Hyalein-Mini, Santen), and frequent use of 20% autologous serum eyedrops diluted in saline. Therapeutic soft contact lenses were placed until stable epithelialization was obtained. In eyes with decreased tear secretion, either punctal plug insertion or punctum occlusion was performed. Tarsorrhaphy was performed to protect the ocular surface in some cases.

Local and systemic immunosuppression was performed after KLAL. For immunosuppression, topical corticosteroids (0.1% dexamethasone [Sanbetasone, Santen] or 1% methylprednisolone) 5 times a day and systemic dexamethasone tapering from 8 mg/day were used for approximately 2 weeks. Cyclosporine A (0.05%) dissolved in α-cyclodextrin was applied topically 5 times a day, continued for at least 1 year after surgery. Systemic cyclosporine A starting from 3 mg/kg was used for at least 3 months. The blood trough level was maintained at approximately 100 ng/ml for at least 6 months, unless systemic adverse effects developed.

Efficacy Evaluation

The epithelium was evaluated by slit-lamp biomicroscopy with fluorescein dye staining and, in selected cases, impression cytology. Corneal epithelialization was considered a sign of failure when either a PED or a visually significant conjunctivalization occurred. A PED was defined as a delay in corneal epithelialization for more than 2 weeks. Corneal clarity was evaluated by slit-lamp examination. It was considered to be lost when visually significant corneal opacity developed after the first stem cell transplantation. In the visual acuity measurement, counting fingers, hand movements, and light perception were converted to 0.004, 0.002, and 0.001, respectively, for statistical analysis.

Statistical Analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed using the chi-square test and Fisher exact rank test to calculate the differences in incidence. Differences between groups were evaluated by the nonpaired *t* test. Wilcoxon rank analysis was used for nonparametric values. Rates of corneal epithelialization and clear grafts were analyzed using Kaplan–Meier analysis. For graft survival, trial time was calculated as duration between the date of PK and the date on which the graft lost its transparency. A level of $P<0.05$ was considered statistically significant.

Results

Mean follow-up for all patients in this study was 67 weeks. For all patients in this study, 17 of 32 eyes (53.1%) had corneal epithelialization after first ocular surface reconstruction surgery; 5 of the 17 had partial conjunctival invasion at the peripheral cornea. Upon last examination, 21 eyes (65.6%) succeeded in ocular surface reconstruction. During the observation period, 21 eyes had PK. At final examination, 18 eyes (56.3%) had clear corneas, including 8 eyes that had PK. There were no significant differences in either corneal epithelialization or clear corneas between eyes with alkali injury and those with other causes of chemical injuries.

Autograft Group

Of 11 eyes in the autograft group, 10 (90.9%) achieved corneal epithelialization after first surgery (Table 3). One eye that did not achieve corneal epithelialization in the autograft group had simultaneous LSCT and PK. The eye was complicated by a wound dehiscence in the nasal limbal graft on the first postoperative day, followed by a PED and conjunctivalization. Despite the subsequent surgical intervention, which included KLAL and PK, the cornea was conjunctivalized. Eight of 11 eyes in the autograft group did not have PK during the observation period, and all of them remained clear. Two eyes received PK and LSCT simultaneously, and one other eye had PK 10 months after LSCT. Two eyes that had simultaneous PK lost their clarity, giving rise to final clear corneas in 9 eyes (81.8%; Table 4).

As postoperative complications, one eye that had PK and limbal autograft simultaneously developed endothelial rejection in the central graft. One other eye developed a PED that required KLAL combined with PK, as described above. Two eyes showed increased intraocular pressure, and both eyes were successfully treated with antiglaucomatous eyedrops. Both limbal and conjunctival epithelium were healed promptly in the donor site of the opposite eye without any consequences.

Table 4. Clear Cornea after First LSCT in Each Group

LSCT Type	No. of Eyes	No PK	Simultaneous PK	2-Step PK	Total
Autograft	11	8/8 (100%)	0/2 (0%)	1/1 (100%)	9/11 (81.8%)
Allograft	21	2/3 (66.7%)	2/13 (15.4%)	1/5 (20.0%)	5/21 (23.8%)
Unilateral	10	2/2 (100%)	1/8 (12.5%)	0/0	3/10 (30.0%)
Bilateral	11	0/1 (0%)	1/5 (20.0%)	1/5 (20.0%)	2/11 (18.2%)

LSCT = limbal stem cell transplantation; PK = penetrating keratoplasty.

Allograft Group

In the allograft group, 7 of 21 eyes (33.3%) had corneal epithelialization after first LSCT (Table 3). There was no significant difference in the rate of corneal epithelialization between eyes with unilateral and bilateral injuries in the allograft group (30.0% vs. 36.4%, $P = 0.76$). All 14 eyes that did not achieve corneal epithelialization received further surgeries, including KLAL (3 eyes), PK (3 eyes), or both (8 eyes). At final examination, 11 of 21 eyes (52.4%) achieved corneal epithelialization. Eighteen eyes had PK either simultaneously with LSCT (13 eyes) or as a 2-step surgery (5 eyes) in the allograft group (Table 4). Clear grafts were observed in 3 (16.7%) and 6 (33.3%) eyes after first LSCT and at final examination, respectively. Both the rate of corneal epithelialization and the rate of clear corneas were worst in eyes that received PK and LCST simultaneously, followed by those that had PK as secondary surgery.

Endothelial rejection developed in 9 of 13 eyes (69.2%) that had simultaneous PK and KLAL (Table 5). Although 6 of the 9 eyes had repeated PK, only 4 grafts remained clear at final examination. A PED in the central cornea was observed in 15 eyes (71.4%). The development of PED was associated with a higher incidence of failed corneal epithelialization and opaque corneas (73.2%) compared with eyes that did not develop PED (60.0% vs. 16.7% and 73.2% vs. 33.3%, respectively). Repeated KLAL was more common in eyes having simultaneous PK (10 eyes [76.9%]) compared with those that did not receive simultaneous PK. Postoperative glaucoma was noted in 11 eyes (52.4%), of which 7 required surgical intervention such as trabeculectomy or laser cyclophotocoagulation.

Comparison of Autograft and Allograft

There were no significant differences in preoperative conditions such as age, male:female ratio, causative agents of injury, visual acuity, and the tear production rate between the autograft and allograft groups (Table 2). Kaplan–Meier analysis revealed that the differences in survival of corneal epithelialization between the 2 groups were statistically significant (Fig 1, $P = 0.003$). Survival of clear cornea was also significantly higher in the autograft group (Fig 2, $P = 0.01$). Although many of the eyes in the allograft group received repeated surgery, the rates of either corneal epithelialization or clear cornea at final examination were still significantly better in the autograft group than in the allograft group ($P = 0.049$ and $P = 0.0075$, respectively).

Visual prognosis was also better in the autograft group. The number of eyes obtaining ≥20/200 corrected visual acuity was 9 (81.8%) in the autograft group, whereas there were only 6 such eyes (28.6%) in the allograft group ($P = 0.008$) at final examination. Development of a postoperative PED was significantly more common in the allograft group (15 eyes vs. 1 eye, $P = 0.0021$). Postoperative glaucoma was also more common in the allograft group, although the difference did not reach statistical significance ($P = 0.13$, Table 5).

Simultaneous versus 2-Step Approach

In the present study, 21 of 32 eyes received PK during the observation period. Fifteen eyes had PK simultaneously with LSCT, and 6 had PK as a secondary surgery. The duration between limbal transplantation and PK in the latter group ranged from 1 to 13 months (mean = 7.7). Although both corneal epithelialization and clear corneas were more common in eyes with the 2-step approach than in those with simultaneous PK, the differences were not statistically significant (Tables 3, 4).

As postoperative complications, the number of eyes that required repeated LSCT was higher in the simultaneous group than in the 2-step group (66.7% vs. 16.7%, $P = 0.06$). The incidence of endothelial rejection was significantly higher in eyes that had

Table 5. Postoperative Complications in Each Group

	Rejection (eyes)*	PED (eyes)	Re-LSCT (eyes)	Glaucoma (eyes)
Autograft				
No PK (n = 8)	—	0	0	1
Simultaneous PK (n = 2)	1	1	1	0
2-step PK (n = 1)	0	0	0	1
Subtotal (n = 11)	1 (33.3%)	1 (9.1%)	1 (9.1%)	2 (18.2%)
Allograft				
No PK (n = 3)	—	2	1	1
Simultaneous PK (n = 13)	9	10	10	7
2-step PK (n = 5)	0	3	0	3
Subtotal (n = 18)	9 (50.0%)	15 (71.4%)†	11 (52.4%)†	11 (52.4%)

LSCT = limbal stem cell transplantation; PED = persistent epithelial defect of the cornea; PK = penetrating keratoplasty.
*Endothelial rejection in the central graft after PK.
†$P<0.05$.

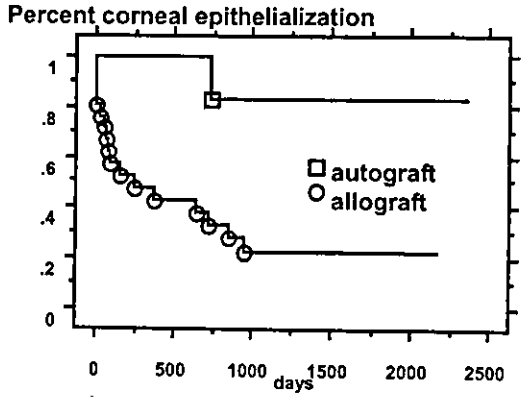

Figure 1. Kaplan–Meier survival analysis for corneal epithelialization (P = 0.003, log rank test).

simultaneous PK than in those with PK as a secondary procedure (53.3% vs. 0%, P = 0.019).

Discussion

The survival of the stem cells of the corneal epithelium is a key factor that determines the prognosis of chemical or thermal injury in the cornea. If functional limbal stem cells are intact even partially, medical treatment alone can restore corneal epithelialization in most cases. In contrast, transplantation of limbal tissue is needed when stem cells are totally depleted.[29,30] In such cases, limbal stem cells can be transferred from the opposite eye of the patient, from cadaver eyes, or from living relatives.[1–14,16–18] Although these approaches have shown favorable results, there have been few reports comparing the clinical outcomes among these approaches.

In the present study, we found that LSCT using an autograft produced significantly better results than allografts in both long-term corneal epithelialization and clear corneas. Although we used relatively intensive epithelial management, eyes that had KLAL often developed epithelial problems. Only one third of eyes achieved corneal epithelialization after the first surgery, and more than half of the eyes required repeated KLAL. The results indicate that the corneal epithelium is more fragile after KLAL than CLAU.

There are theoretical advantages and disadvantages in both autografting and allografting. The most obvious merit in autografting is the absence of immunologic rejection. One of the potential risks of autograft is the effect on the donor eyes. Because limbal stem cells are partially excised, the donor eye may reduce its reserved potential for epithelialization. Although we did not encounter any epithelial problems in donor eyes, it is conceivable that these eyes have a higher risk of future problems, especially after injury or surgery. To minimize this potential risk, we excised 2 pieces of autografts of ≈5×5 mm. Therefore, the amount of limbal tissue transplanted in CLAU was less than that in KLAL, in which the entire limbal area was covered. The difference may also produce better limbal barrier function in KLAL than in CLAU. However, from the results in the present study, we can conclude that the autograft is superior to the allograft as a source of corneal epithelium. As differences in the clinical outcomes were so striking, we recommend using an autograft instead of an allograft in unilateral ocular surface chemical/thermal burns. Although it is difficult to distinguish epithelial breakdown caused by mechanical injury from immunologic rejection, some form of immunologic response may be involved in the susceptibility of epithelial damage in the KLAL group. We do not perform autografts when the potential donor eye has the slightest possibility of injury, even if there is no clinical abnormality. There have been reports of epithelial problems in the donor eye with subclinical injury after limbal grafts were excised.[6,7]

Another frequently encountered issue that surgeons must decide upon is the timing of keratoplasty when treating ocular burns associated with corneal opacity. Although it appears that simultaneous keratoplasty will result in faster visual rehabilitation, it has been unclear whether this simultaneous approach has a higher risk of complications. It is well recognized that keratoplasty to inflamed and vascularized corneas has a higher risk of immunologic rejection and epithelial problems. In the present study, we found that the eyes receiving PK several months after LSCT showed fewer complications than those with simultaneous PK. More than half of the eyes in the simultaneous group developed immunologic rejection in the central graft, whereas the complication was not observed in the 2-step group. The result was in good accordance with our recent report that endothelial rejection was common after combined LSCT and PK.[31,32] The event is probably attributed to the difference in the immunologic response between the limbal and central corneal transplants. Also, the rate of corneal epithelialization and clear graft tended to be superior in the 2-step groups compared with the simultaneous group, although the differences did not reach statistical significance. This is probably due to the small number of eyes that had 2-step approaches.

It should be noted, however, that there was a potential bias in the present study. This is a retrospective study, and therefore the surgical procedure was not randomly selected. In addition, the relatively small number of subjects studied may make it difficult to draw conclusions. It is conceivable

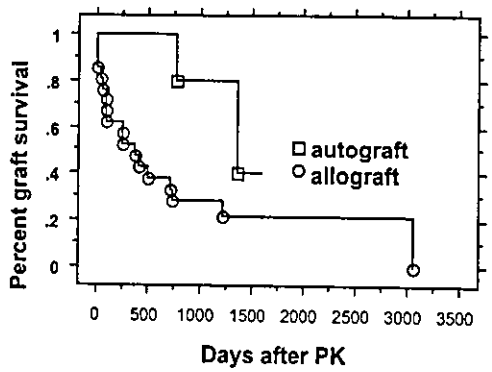

Figure 2. Kaplan–Meier survival analysis for clear cornea (P = 0.01, log rank test). PK = penetrating keratoplasty.

that eyes undergoing KLAL had more severe injury than those undergoing CLAU, because many in the former group had bilateral injury. However, there were no significant differences in both corneal epithelialization and clear corneas between eyes undergoing CLAU and those undergoing KLAL with unilateral involvement (Tables 3, 4). This finding indicated that the differences in surgical prognosis were likely attributable to the surgical methods used. Also, there was a potential bias in the analysis of the simultaneous and 2-step groups. Eyes that had combined limbal and central graft transplantation might have had more severe involvement in the corneal stroma compared with those with the 2-step approach. Although we did not find any differences in the preoperative conditions, such as patient profile and ocular surface parameters, between the groups analyzed (Table 2), a prospective study is necessary to draw a final conclusion. Also, new surgical approaches such as transplantation of cultivated limbal epithelium onto amniotic membrane may be worth investigating.[33–36]

In summary, we found that surgical approaches influence the clinical outcome in ocular surface reconstruction for chemical and thermal burns of the cornea. We recommend performing limbal autograft transplantation combined with AMT in patients with unilateral injury. In cases of opaque corneal stroma, it seems to be safer to perform ocular surface reconstruction first, and then keratoplasty several months later, to reduce postoperative complications.

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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 gtacgacagaggcatacagg; pax-6 (206 bp), sense aacaacctgcctatgcaacc and antisense cttggacgggaactgacact; K12 (437 bp), sense cgagagtggtatgaaaca and antisense tgggctctcatttcattg; 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.[50] Similarly, an earlier increase of CD44 transcription is observed during corneal epithelial wound healing.[51] 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-

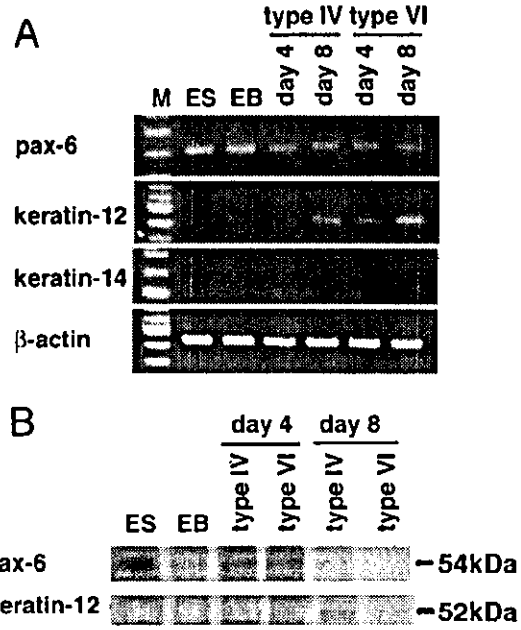

FIGURE 2. Expressions of keratins and *Pax-6* in ES-cell-derived cells. (A) ES cells were induced to differentiate into epithelial progenitor cells. Total RNA was extracted at each differential stage, reverse transcribed, and PCR amplified. The product was electrophoresed on 1.5% agarose gel and visualized 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.

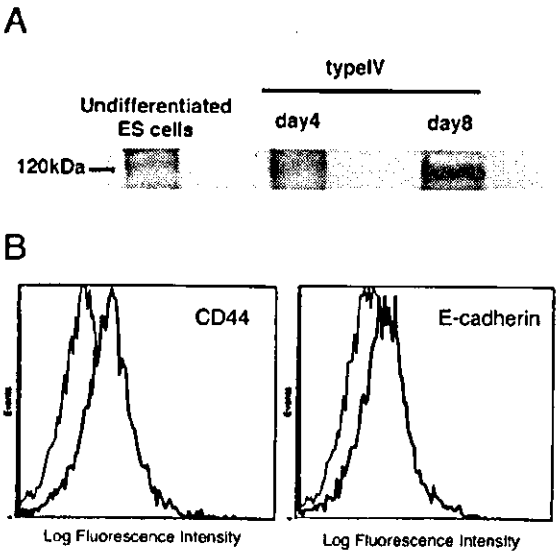

FIGURE 3. Expressions of E-cadherin in ES-cell-derived epithelial progenitor cells. (A) ES cells were induced to differentiate into epithelial progenitor cells by culture on type-IV collagen-coated plates. At each stage of differentiation, cell lysates were prepared, and expression of E-cadherin was 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

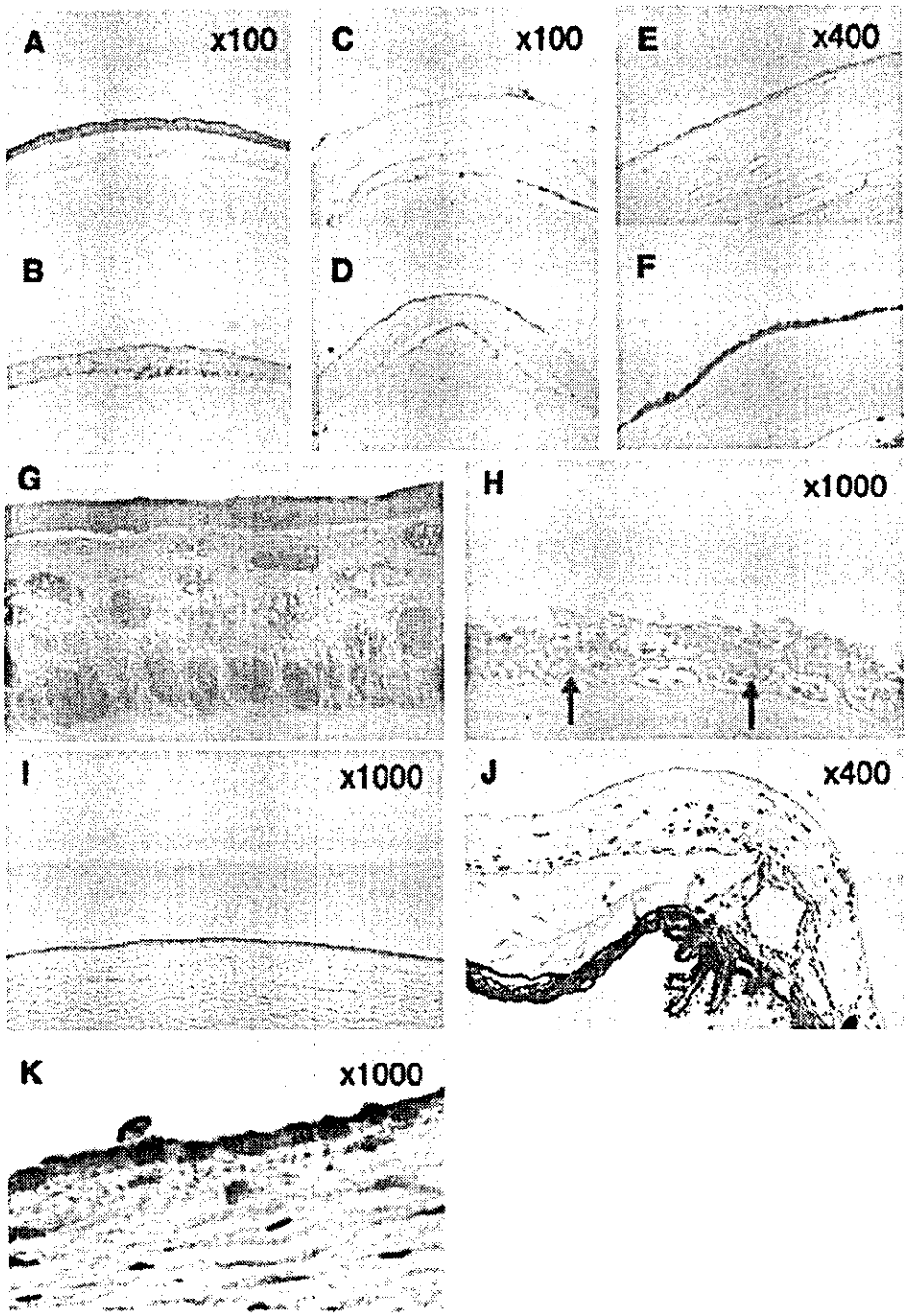

FIGURE 4. Histologic analysis of injured cornea, with or without transplantation of the ES-cell-derived epithelial progenitor cells. The ES-cell-derived epithelial progenitor cells (day 8 culture) were transplanted to *n*-heptanol-injured cornea of mice. (A) Normal mouse cornea. (B). *n*-Heptanol-injured 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

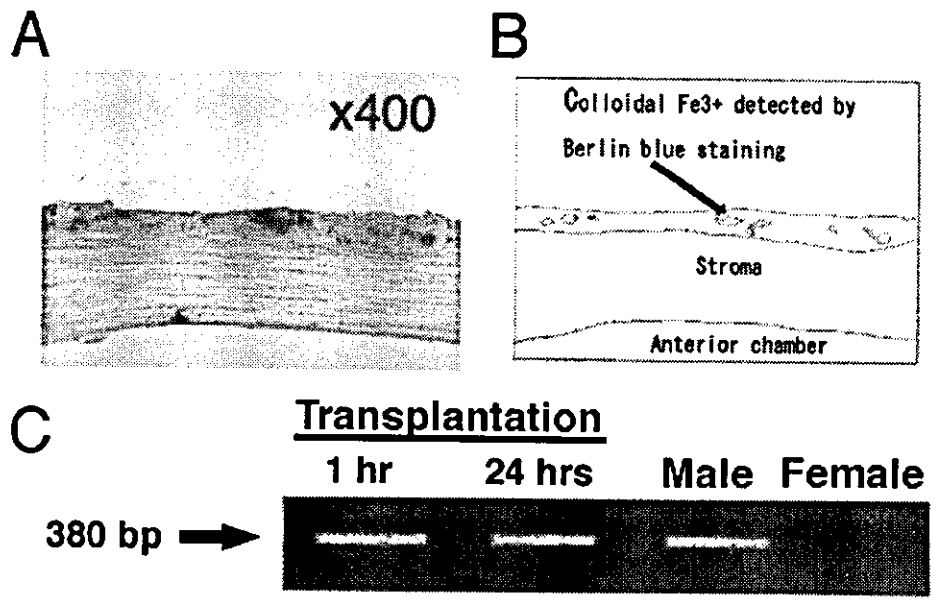

FIGURE 5. Expression of donor-cell-specific markers in epithelial cells covering the damaged cornea after transplantation of ES-cell-derived epithelial progenitor cells. (A) The ES-cell-derived epithelial progenitor cells were labeled with colloidal iron ($Fe3^-$) and transplanted to the *n*-heptanol-injured 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).

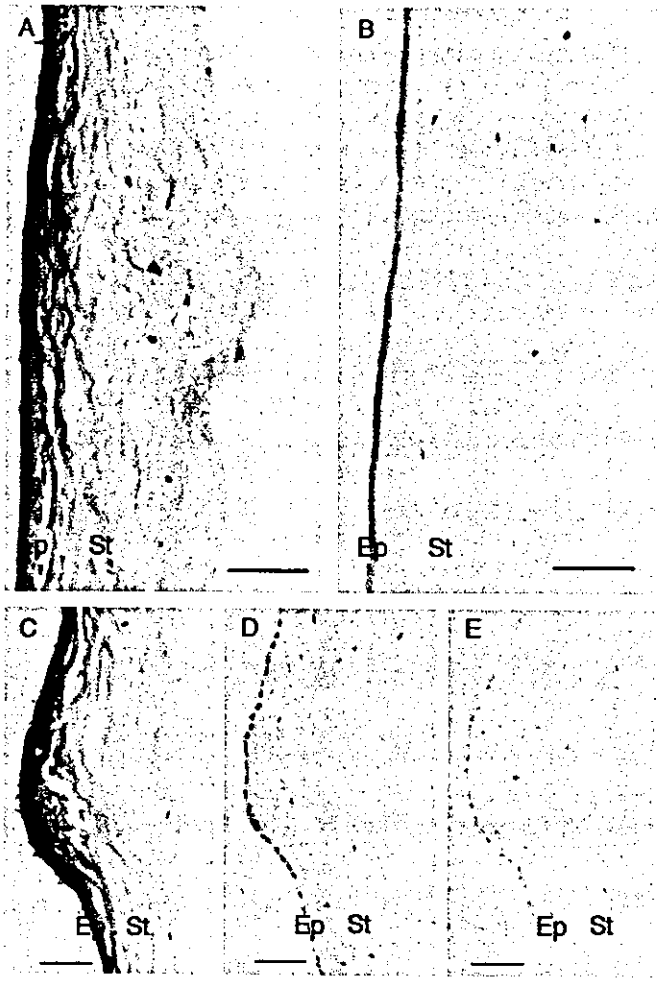

FIGURE 1. Paraffin sections of AM were treated with (B) or without (A) 0.05% bovine testicular hyaluronidase and stained with 0.05% toluidine blue (TB) (pH 7.0). Sections stained at pH 7.0 (C), 4.1 (D), and 2.5 (E) showed a gradual decrease in intensity, indicating that the glycosaminoglycan stained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

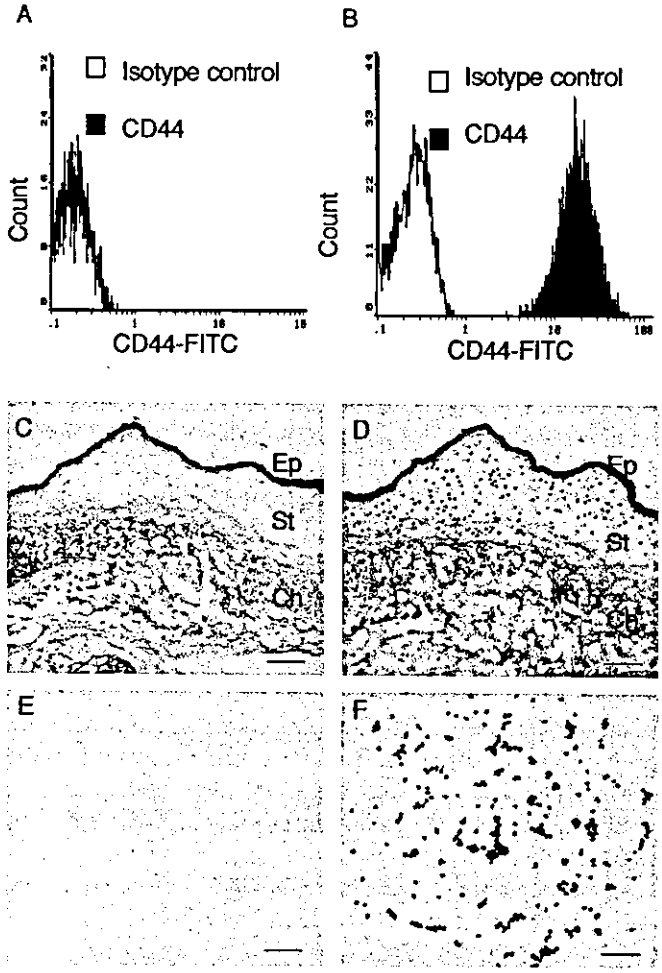

FIGURE 2. Flow cytometry of Jurkat (A) and Molt 4 (B) incubated with isotype control IgG1 (open peaks) or CD44 monoclonal antibody (solid peaks) confirmed the expression of CD44 on Molt 4. Adhesion assays of Jurkat (C, E) and Molt 4 (D, F) to cryostat sections of AM and immobilized HA both showed greater 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).

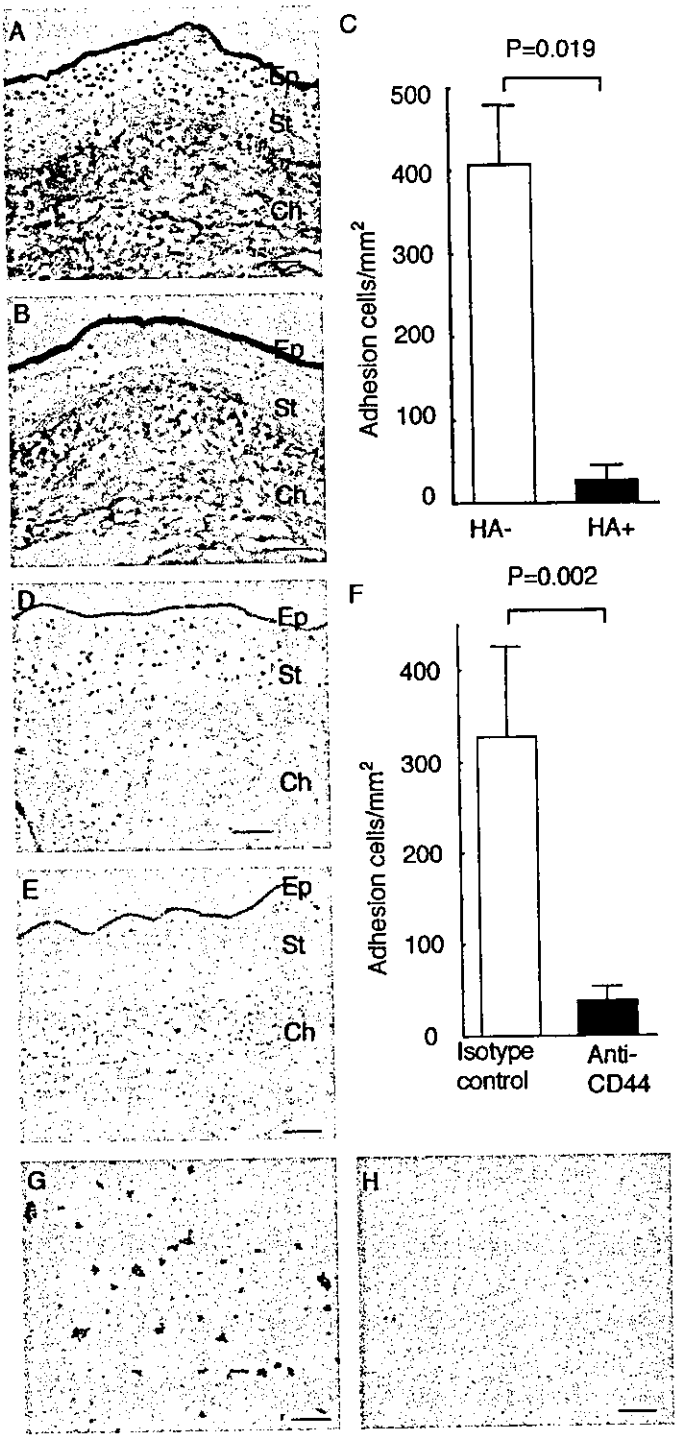

FIGURE 3. Molt 4 adhesion to AM sections was significantly inhibited by preincubation with soluble HA (1 mg/mL) (B) compared with control (A, C). (HA^-, n = 8; HA^+, n = 6). Adhesion of Molt 4 to AM was also inhibited by anti-CD44 mAb (E) but not by isotype control (D, F) (n = 10). Anti-CD44 mAb also 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.

The entrapment of inflammatory cells may explain the antiinflammatory effects often observed following AM patches. As presented in our previous study, in a clinical situation, infiltrating cells are found throughout the AM stroma and not only at the junction of AM and cornea/sclera.[12] Most of the infiltrating lymphocytes and/or monocytes were TUNEL positive in the AM obtained from clinical samples.[12] Fetal membranes express FasL, by which the fetus is afforded protection against cytolytic actions of lymphocytes from the mother.[29–32] Whether the apoptosis process is an active effect exerted by AM remains to be clarified because there is the possibility that lymphocytes simply underwent a physiologic course of apoptosis while being trapped within the AM. Because none of the histologic samples showed infiltrating cells invading the basement membrane, most of the cells seem to have been sequestered from the ocular surface lying directly beneath the AM.[12] There is also the possibility that the AM also acts as a physical barrier, as with contact lenses, to protect the ocular surface from inflammatory cells in the tear film.

AM has the ability to suppress allo-reactive T cells in vitro,[33] an effect that may be mediated by secretory factors such as PGE_2,[34] HLA-G,[35] and FasL.[29–32] Because high-molecular-weight HA is also associated with inhibition of cytokine production,[24] HA in AM stroma may regulate both T_H1(IL-2 and IFN-γ) and T_H2 (IL-6 and IL-10) types of cytokine production.[33]

In summary, HA was present in high levels in the stroma of AM. Our data demonstrate that HA–CD44 interaction plays an important role in the adhesion of inflammatory cells, including lymphocytes, to AM stroma. Although further studies are required to elucidate the molecular events involved, entrapment of inflammatory cells may explain some of the clinical effects observed in the use of AM in ocular surface reconstruction.

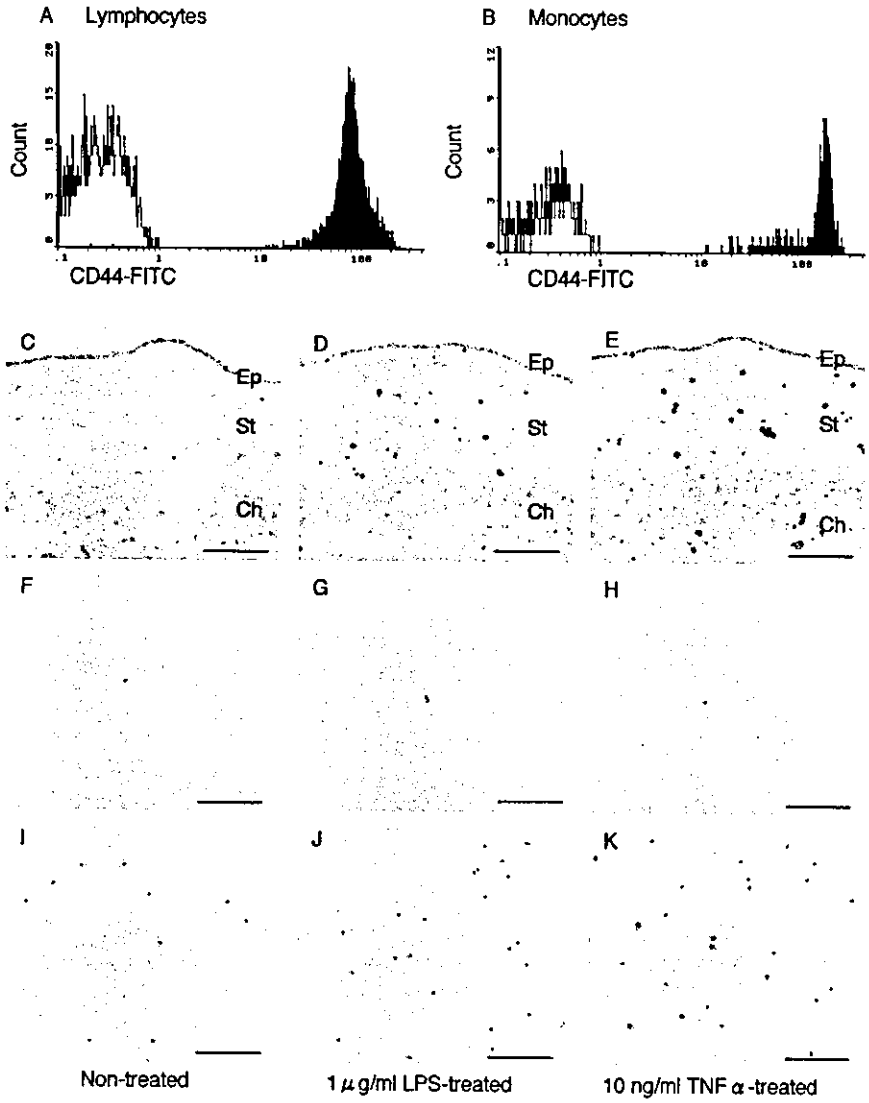

FIGURE 4. PBMC expressing CD44 (A, B) were used in an adhesion assay to AM (C, E), immobilized chondroitin sulfate (CS) (F, H), and immobilized HA (J, K). Activation of PBMC by preincubation with 1 μg/mL LPS or 10 ng/mL TNF-α for 72 hours caused increased adhesion in AM and HA-coated slides but not in CS-coated slides. Ep, amniotic epithelium; St, stroma of AM; Ch, chorion. The bar represents 100 μm.

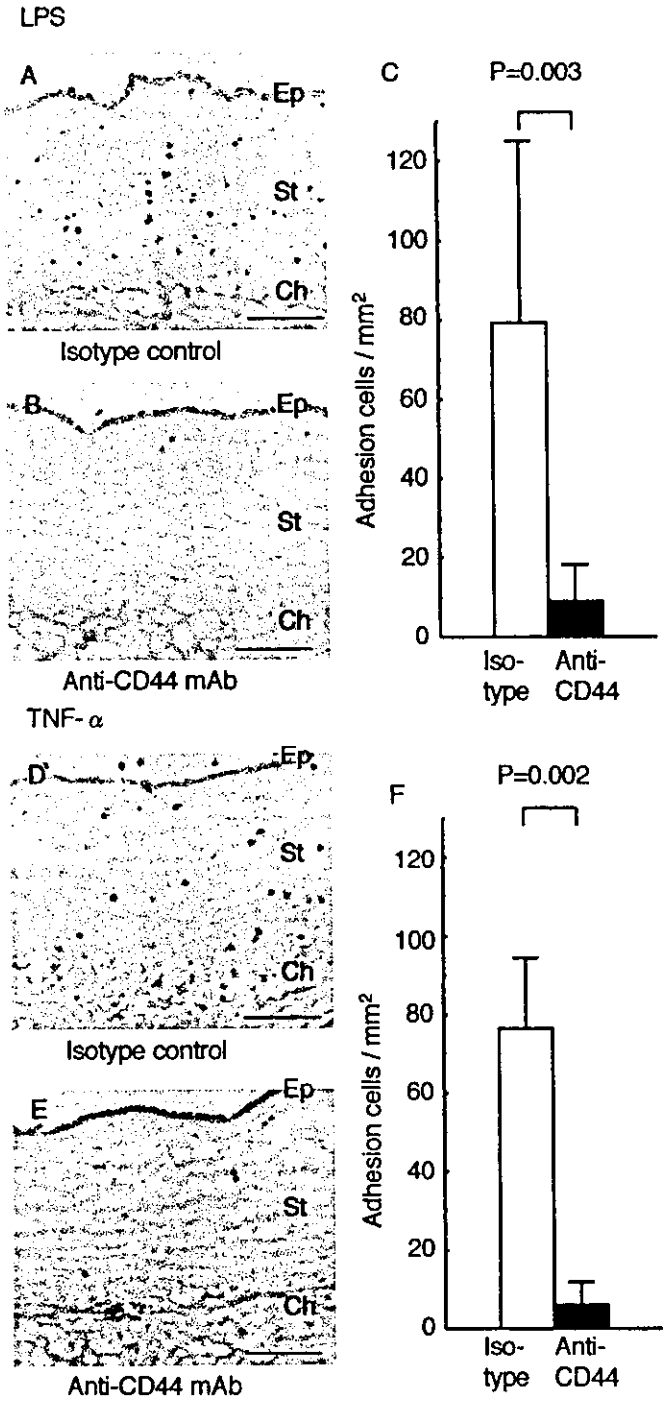

FIGURE 5. Adhesion of LPS-treated (A) and TNF-α–treated (D) PBMC to AM was inhibited by the anti-CD44 mAb (B, E). Anti-CD44 mAb significantly inhibited adhesion of PBMC activated by both LPS (C) and TNF-α (F) (n = 10 each). Ep, amniotic epithelium; St, stroma of AM; Ch, chorion. The bar represents 100 μm.

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Characterization and Distribution of Bone Marrow–Derived Cells in Mouse Cornea

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Purpose. Bone marrow (BM)–derived stem cells are thought to possess extensive differentiation capacity. The present study was conducted to investigate the characteristics and distribution of these cells in the normal mouse cornea.

Methods. BM cells and BM-derived hematopoietic stem/progenitor cells (HSCs) from enhanced GFP (eGFP) transgenic mice (lin^-, $Sca\text{-}1^+$) were intravenously transplanted into irradiated wild-type C57BL/6 mice. At 4 to 6 months after transplantation, the mice were killed, and their whole corneas examined by histologic and immunohistochemical methods (CD11c, CD11b, and CD45).

Results. At 2 weeks after BM cell transplantation, GFP^+ cells gradually migrated into the cornea from the limbal area. At 2 to 6 months, they were distributed over the entire cornea. In cross sections of whole cornea, GFP^+ cells comprised 27.3% ± 11.1% (BM) and 24.0% ± 8.01% (HSC) of total cells in the peripheral corneal stroma. In the center of the corneal stroma, GFP^+ cells were 7.58% ± 2.63% (BM) and 8.06% ± 1.76% (HSC) of total cells. Immunohistochemistry showed that GFP^+ $CD11c^+$, $CD11b^+$, $CD11c^-$, and $CD11b^-$ cells occupied the entire corneal stroma.

Conclusions. The present study provides direct evidence of the distribution of BM-derived cells in the mouse cornea. Immunohistochemical study showed that some of these cells are BM-derived antigen-presenting cells such as dendritic cells and macrophages. Some elements of BM-derived cells may continue to exist in the corneal stroma. (*Invest Ophthalmol Vis Sci.* 2005;46:497–503) DOI:10.1167/iovs.04-1154

Adult somatic stem cells have been isolated from several tissue sources including neurons,[1,2] retina,[3] corneal limbal epithelium,[4,5] and bone marrow (BM).[6–8] It had been thought that somatic stem cells preferentially generate differentiated cells of the same lineage as their tissue of origin. However, recent studies suggest that tissue-specific stem cells can differentiate into lineages other than their tissue of origin and that, with respect to the developmental potential of different adult cell types, there is far more plasticity than previously thought. Particular attention has been focused on the plasticity of BM-derived stem cells. They are reported to possess extensive differentiation capacities and can differentiate into several epithelial types such as liver, lung, and skin.[9] Furthermore, BM-derived mesenchymal stem cells can differentiate in vitro not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endoderm characteristics.[10] These findings suggest that BM-derived stem cells may have the ability to transdifferentiate into a variety of tissues, including those of the eye.

Normal corneal tissue is located in the anterior segment of the eye, and it participates in several major functions. It is the gateway into the eye of visual images and plays a critical role in maintaining corneal transparency and avascularity. It is composed of three layers: the corneal epithelium, stroma, and endothelium. Corneal epithelial stem cells exist in the basal cell layer of the limbal region[4,5] and in the transitional zone between the cornea and conjunctiva. They are supported by the limbal vascular arcade. Little is known about stem cells of the corneal stroma and endothelium, and the origin of these cells is not well understood.

From an immunologic point of view, the normal avascular cornea was thought to be an immune-privileged site without functional antigen-presenting cells (APCs) and largely devoid of BM-derived cells. Therefore, higher success rates would be expected with corneal than other organ transplants. This notion has lost favor since the demonstration of large numbers of resident BM-derived cells of different lineages—for example, macrophages and dendritic cells—in both the epithelium and stroma of the normal cornea.[11–15] Until now, indirect evidence obtained by immunohistochemical studies has shown these cells to be present and important questions, such as the original cell type and the physiological and functional significance of these progenitors, remain unanswered.

We are the first to attempt the characterization and clarification of the distribution of BM-derived cells in the normal mouse cornea. In the current study, we sought to acquire a direct demonstration by transplanting BM cells from enhanced green fluorescence protein (eGFP) transgenic mice using our unique protocol.[11–16] We transplanted GFP-labeled BM cells and hematopoietic stem/progenitor cells (HSCs) into syngeneic C57BL/6 (wild-type) mice and found BM-derived cells distributed in the mouse cornea. We then evaluated the characteristics of these BM-derived cells by immunohistochemical studies.

Materials and Methods

Experimental Animals

The mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental

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