Immunogenicity and Antigenicity of Allogeneic Amniotic Epithelial Transplants Grafted to the Cornea, Conjunctiva, and Anterior Chamber

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PURPOSE. To determine the immunogenic characterization of amniotic epithelium (AE), by examining the fate of allogeneic AE grafts heterotopically transplanted in the eye.

METHODS. Intact AE from enhanced green fluorescence protein (EGFP) transgenic mice (C57BL/6 background) and wild-type C57BL/6 mice were transplanted onto cornea or conjunctiva, or inserted into the anterior chamber (AC) of normal BALB/c mice, C57BL/6 mice, or BALB/c mice presensitized to donor antigens. For repeated AE transplantation experiments, AE was grafted in the other eye 7 days after the first grafting. Graft fate was assessed clinically and histologically at selected intervals after grafting. Infiltrating inflammatory cells were examined immunohistochemically. Sensitization to alloantigens by AE was assessed by the delayed hypersensitivity (DH) response.

RESULTS. In normal recipients, GFP+ cells were absent in EGFP donor-derived AE grafts by day 21 on cornea and by day 7 on conjunctiva. AE grafts implanted in the AC survived for >8 weeks. In presensitized recipients and recipients that underwent repeated AE implantation, graft survival was markedly shorter than in normal recipients. DH was induced at 2 weeks, but failed to be induced at 4 weeks after grafting on cornea or at 8 weeks after grafting on conjunctiva and in the AC of normal recipients.

CONCLUSIONS. Fresh allogeneic AE expressed immunogenicity when placed on the ocular surface, although no memory of allospecific DH was acquired. Allogeneic AE is clearly vulnerable to immune rejection in specifically sensitized recipients. (*Invest Ophthalmol Vis Sci.* 2006;47:1522–1532) DOI: 10.1167/iovs.05-0787

Transplantation of human amniotic membrane (AM) in ocular disorders was introduced into ophthalmology more than 60 years ago.[1,2] Since 1995, when Kim and Tseng[3] reported the use of preserved human AM to cover rabbit cornea that had been damaged to produce extensive neovascularization, AM transplantation has been successfully applied for ocular surface reconstruction in patients with severe ocular diseases.[4-8]

A variety of characteristics make AM ideally suited for use in ocular surface reconstruction. It has an anti-inflammatory effect by inhibiting proteinase activity and infiltration of polymorphonuclear leukocytes[9] and by suppressing interleukin (IL)-1α and -1β.[10] Li et al.[11] reported that supernatant from human amniotic epithelial cells (HAECs) significantly inhibits the chemotactic activity of neutrophils and macrophages and reduces the proliferation of both T and B cells after mitogenic stimulation. In a prior study, we found that conditioned medium from HAECs suppresses development of corneal neovascularization, migration of major histocompatibility complex (MHC) class II+ antigen-presenting cells (APCs), and expression of mRNA for proinflammatory cytokines in the inflamed cornea.[12] In addition, AM displays antifibroblast activity in suppressing transforming growth factor (TGF)-β and subsequent fibroblast differentiation.[13] Moreover, AM has antimicrobial properties, reducing bacterial counts and promoting healing in infected wounds.[14] Hao et al.[15] also confirmed that various antiangiogenic and anti-inflammatory proteins are expressed in amniotic epithelial and mesenchymal cells. Aside from these characteristics, AM has been thought to display very low immunogenicity. Amniotic epithelial cells reportedly do not express human leukocyte antigen (HLA)-A, -B, -D, or -DR antigens on the cell surface,[16] suggesting that acute rejection would not occur after transplantation. Akle et al.[17] performed amniotic tissue transplantation in seven volunteers, none of whom displayed clinical signs of acute rejection.

Although experimental and clinical studies implanting AM as a graft or a patch have demonstrated that AM promotes re-epithelialization, decreases inflammation and fibrosis, and inhibits angiogenesis, uncertainties remain regarding the fate of grafted AM and thus also the mechanisms through which long-term effects are exerted. In fact, slit-lamp examination has demonstrated that AM gradually disappears after transplantation, and the period over which this disappearance occurs depends on the underlying disease and whether the AM is implanted as a graft or patch.[18,19] Notwithstanding AM dissolution, the ocular surface remains stable, and stromal corneal thickness is maintained.[20] Although low, immunogenicity is inherent in AM and remains unclarified and controversial. Reports have described AE cells frequently expressing MHC class I molecules, but that expression may be modulated in situ by extrinsic factors.[21,22] In 1940, De Roth[1] reported that the success rate is low when live AM and chorion are used together for plastic repair of conjunctival defects, implying that the live fetal membrane is immunogenic. Akle et al.[17] reported that low-grade inflammatory responses are observed under conditions in which viable amniotic epithelial cells are present. At present, most AM tissue used clinically has been cryopreserved. However, Gabler and Lohmann[23] reported a patient who underwent AM transplantation on three occasions and developed hypopyon after both the second and third transplantations. Because these AMs were all taken from the same donor, this finding suggests that immunologic responses of the recipient to donor tissue may have been involved.

FIGURE 1. Fresh AE grafts (approximately 2.0 × 2.0 mm) from EGFP mice or wild-type C57BL/6 mice transplanted on cornea (**A**), on conjunctiva (**B**), and in the AC (**C**) of BALB/c mice, shown just after surgery. *Arrows:* AE grafts.

As components of solid-tissue allografts, epithelial cells have long been known to be potently immunogenic. Determining the fate and immunogenicity of amniotic epithelial (AE) cells as allogeneic grafts represents an important step in understanding the potential use of AM transplantation to reconstruct the ocular surface. To approach the question, we transplanted freshly isolated, intact sheets of murine allogeneic AE onto cornea or conjunctiva, or into the anterior chamber (AC) of normal eyes. AE from enhanced green fluorescence protein (EGFP) transgenic mice[24] was transplanted into the eyes of GFP$^-$ recipients. We demonstrate that GFP$^+$ allogeneic AE cells gradually disappeared from the ocular surface and sensitized the recipient. Moreover, AE cells became a target of acute rejection reactions in the eyes of presensitized recipients.

MATERIALS AND METHODS

Mice and Anesthesia

Male BALB/c (H-2^d) and C57BL/6 (H-2^b) mice and female 18-day pregnant C57BL/6 mice were purchased from Sankyo Laboratory Service Corp. (Tokyo, Japan). Breeding pairs of EGFP transgenic mice (C57BL/6-TgN (AcTbEGFP); Jackson Laboratories, Bar Harbor, ME) were purchased and bred in our animal colony. All mouse recipients were used at 8- to 10-weeks-old and were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Each mouse was anesthetized by intramuscular injection with a mixture of 3.75 mg ketamine and 0.75 mg xylazine before all surgical procedures. Tissues of EGFP mice are green under excitation light, with the exception of hair and erythrocytes. In these mice, EGFP is expressed in the cytosol. As the optimal excitation wavelength for EGFP is close to 488

nm, cells from EGFP transgenic mice are suitable for analysis under fluorescence microscopy.

Preparation of AE

Pregnant uteri were obtained by cesarean section from 18-day pregnant EGFP and wild-type C57BL/6 mice, and AE was then peeled as an intact sheet from the uterus and fetus and washed in ice-cold RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan). The AE sheet was cut into fragments of approximately 2.0 × 2.0 mm each, which were then used for grafting.

Transplantation of AE Grafts on Cornea and Conjunctiva and in AC

Fragments of AE were heterotopically transplanted onto cornea or conjunctiva or into the AC of recipient mouse eyes. Briefly, a freshly prepared AE graft was placed on the corneal or conjunctival surface, then fixed with two interrupted sutures (11-0, nylon; Figs. 1A, 1B). Through a lateral incision in the recipient cornea, the AE graft was implanted into the AC, and the corneal wound was closed with an interrupted 11-0 nylon suture (Fig. 1C) that was removed 7 days later.

Clinical and Histologic Evaluation of Heterotopic AE Grafts

The fate of each heterotopic AE graft was assessed clinically under an operative microscopy by a single observer (MCW) at selected time points after transplantation. At each time point, graft-bearing mice were anesthetized and a clinical inspection was made, evaluating both the presence of AE grafts at the graft sites and neovascularization of cornea. After clinical examination, the graft-bearing eye was removed, fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and
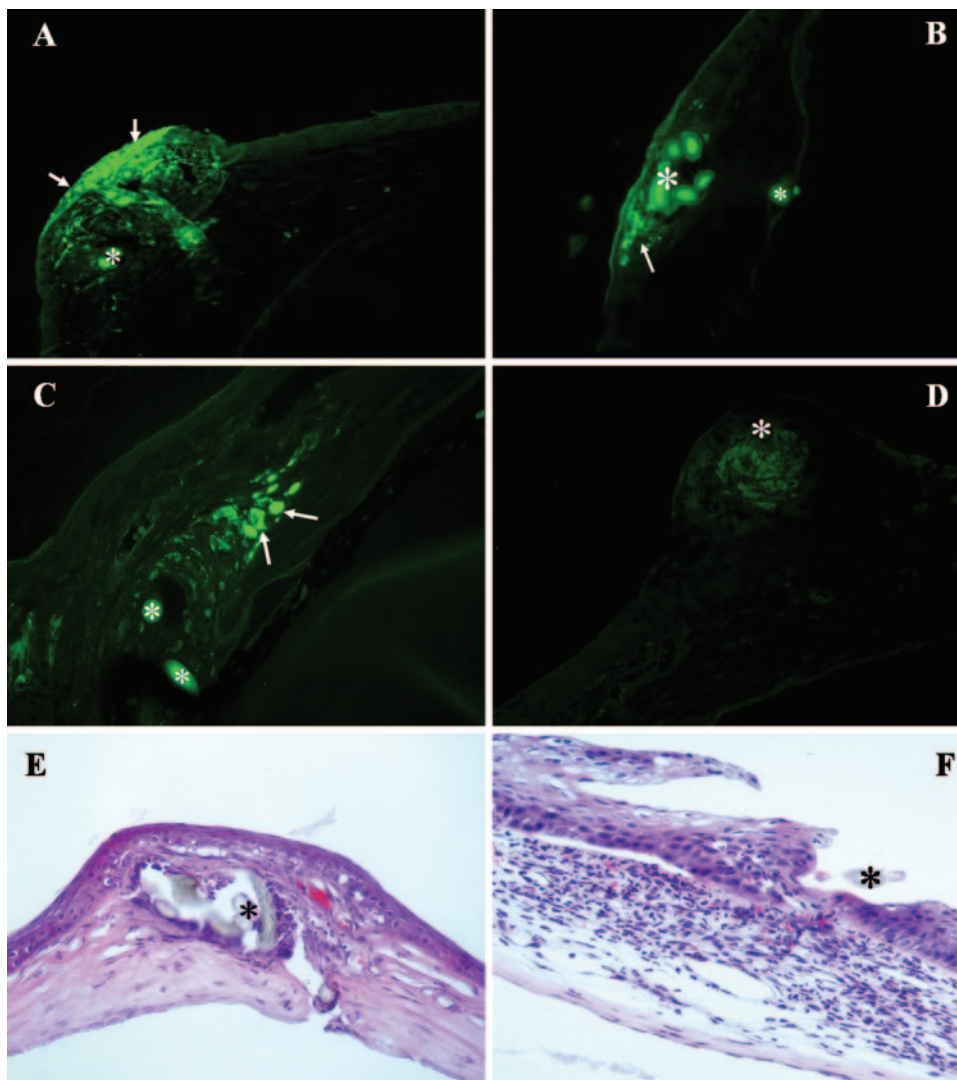
FIGURE 2. Histologic appearances and fluorescence microcopy of syn- and allogeneic GFP+ AE cells transplanted on corneas of normal recipients. At day 14, syngeneic GFP+ AE cells were detected in AE graft (**A**); whereas allogeneic GFP+ AE cells were detected in corneal stromal (**B**). At day 28, syngeneic GFP+ AE cells were detected (**C**), whereas all allogeneic GFP+ AE cells had disappeared (**D**). Histologic aspects of syngeneic (**E**) and allogeneic (**F**) AE transplants at day 14. *Arrows*: GFP+ AE cells; (✱) sutures. Magnification: (**A–D**) ×20; (**E**, **F**) ×40.

stained with hematoxylin and eosin (HE) for histologic examination. Approximately 20 sections were prepared from each graft-bearing eye.

Immunohistochemical Assessment of Heterotopic AE Grafts

Immunohistochemical studies for CD4, CD8, B220, and Gr-1 expression were performed on frozen sections of AE grafts on the cornea or conjunctiva or in the AC. Purified rat anti-mouse CD4, CD8, B220, or Gr-1 monoclonal antibodies (eBioscience, Tokyo, Japan) were used as primary antibodies. Cy-3 conjugated goat anti-rat IgG (Jackson ImmunoResearch, Tokyo, Japan) was used as secondary antibody. Graft-bearing eyes were removed on day 4, 7, 10, 14, 21, 28, or 56, fixed in 4% paraformaldehyde, frozen in optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan) in acetone-dry ice and stored at $-80°C$. Frozen specimens were sectioned at 5 μm with a cryostat and air dried. Approximately 20 sections were prepared from each graft-bearing eye. After they were washed with phosphate-buffered saline (PBS), sections were incubated in each primary antibody diluted to 2.5 μg/mL for 2 hours and in secondary antibody diluted to 7.5 μg/mL for 1 hour at room temperature. After a wash with PBS, sections were mounted and observed under fluorescence microscopy or confocal microscopy. Immunohistochemical studies for mouse MHC class I and II expression on AE cells were performed with frozen sections of BALB/c eyes bearing C57BL/6 AE allografts. PE-conjugated

mouse anti-mouse H-2K^b and I-A^b monoclonal antibodies (PharMingen Technical, Tokyo, Japan) were diluted to 4 μg/mL. After incubation in antibodies for 2 hours at room temperature and a wash with PBS, sections were mounted and observed under confocal microscopy.

Delayed Hypersensitivity Assessment after AE Transplantation

At selected time-points after allogeneic AE grafting in the eye, 1×10^6 irradiated (2000 rad) splenocytes from C57BL/6 donors were injected into the right ear pinnae of recipient mice for an ear-swelling assay. As the positive control, a similar number of irradiated splenocytes were injected into the ear pinnae of normal BALB/c mice that had been immunized 1 week previously by subcutaneous injection of 10×10^6 donor splenocytes. As the negative control, 1×10^6 splenocytes were injected into the ear pinnae of naïve mice. At 24 hours after injection, ear thickness was measured with a low-pressure engineering micrometer (Mitsutoyo, Kanagawa, Japan). Ear swelling was expressed as follows: specific swelling = (24-hour measurement of right ear − 0-hour measurement of right ear) − (24-hour measurement of left ear − 0-hour measurement of left ear) × 10^{-3} mm. Ear swelling responses at 24 hours after injection are presented as individual measurements (10^{-3} mm) for each tested animal and as a group mean ± SEM.
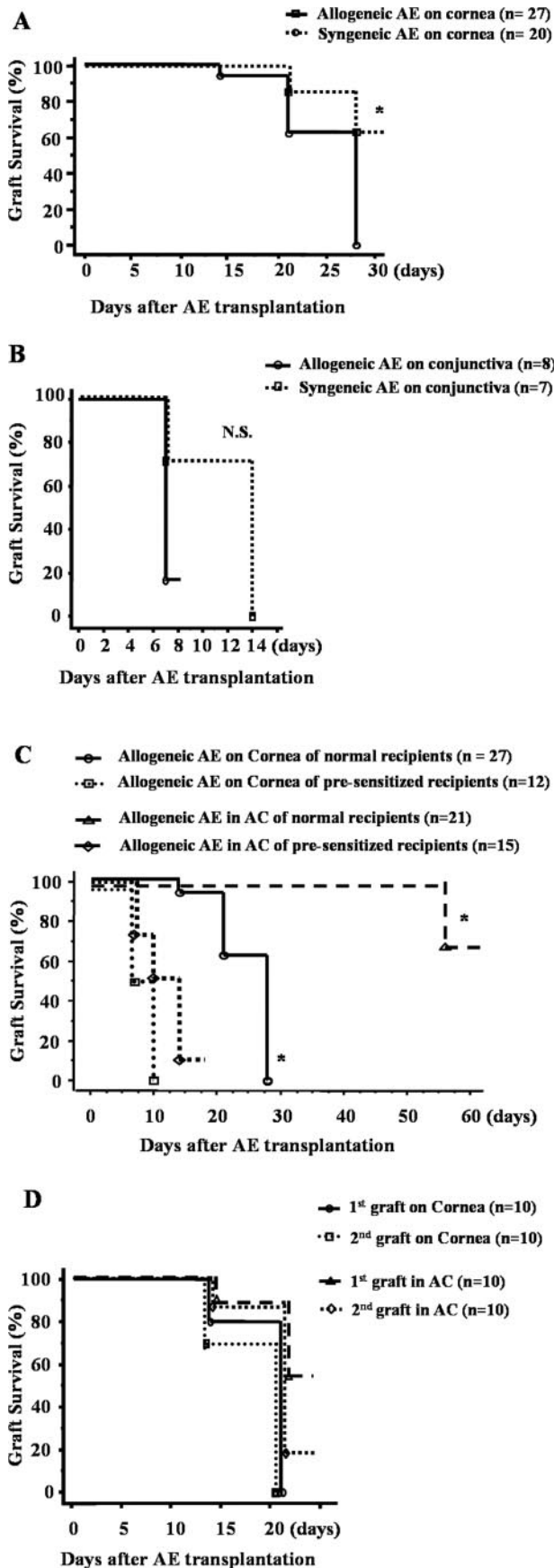
A

Graft Survival (%)

— Allogeneic AE on cornea (n= 27)
·○· Syngeneic AE on cornea (n= 20)

Days after AE transplantation

B

Graft Survival (%)

— Allogeneic AE on conjunctiva (n=8)
·□· Syngeneic AE on conjunctiva (n=7)

N.S.

Days after AE transplantation

C

○ Allogeneic AE on Cornea of normal recipients (n = 27)
·□· Allogeneic AE on Cornea of pre-sensitized recipients (n=12)

▲ Allogeneic AE in AC of normal recipients (n=21)
·◇· Allogeneic AE in AC of pre-sensitized recipients (n=15)

Graft Survival (%)

Days after AE transplantation

D

● 1st graft on Cornea (n=10)
·□· 2nd graft on Cornea (n=10)

▲ 1st graft in AC (n=10)
·◇· 2nd graft in AC (n=10)

Graft Survival (%)

Days after AE transplantation

FIGURE 3. Fate of heterotopic C57BL/6 AE graft on corneas of normal BALB/c (allogeneic) and C57BL/6 (syngeneic) recipients (**A**), on con-

Transplantation of AE Allografts in Presensitized Recipients

GFP[+] AE grafts were transplanted onto the cornea or into the AC of BALB/c mice that had been subcutaneously injected with 10×10^6 C57BL/6 splenocytes 1 week earlier. After clinical inspection under operative microscopy at day 4, 7, 10, or 14 after transplantation, AE graft-bearing eyes were removed for histologic or immunohistochemical examination.

Second Transplantation of AE Allografts in Normal Recipients

The first transplantation of AE was performed on the cornea or in the AC of the right eye of normal BALB/c mice. A second transplantation was performed 7 days later on the same site in the left eye. After clinical observation by operative microscopy at day 7, 14 or 21 after second implantation, both eyes were removed for histologic or immunohistochemical examination.

Statistical Analyses

Grafts with GFP^{+} cells detected in the graft area by fluorescence microscopy were considered surviving grafts. Graft survival in panels of recipient mice was compared by Kaplan-Meier survival curves and the Breslow-Gegan Wilcoxon test. Ear-swelling measurements were evaluated statistically with a two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Fate of Allogeneic AE Grafts Transplanted Heterotopically on Corneas of Normal Mouse Eyes

AE grafts from EGFP mice were placed on the cornea of syngeneic C57BL/6 and allogeneic BALB/c recipients and examined at day 4, 7, 10, 14, 21, or 28 after grafting by visual inspection through an operation microscope. GFP[+] AE was grafted onto the cornea of 60 BALB/c mice and 30 C57BL/6 mice, and all graft-bearing eyes were removed for histologic or immunohistochemical examination.

Under the operation microscope, heterotopic allogeneic AE grafts were observed as membranes on the corneal surfaces until day 10 after implantation, and the most severe blood vessel invasion was observed visually at day 14, subsequently declining as the AE grafts gradually faded. Syngeneic AE grafts remained on the cornea as a membrane until day 14, and GFP^{+} cells were easily detected in retained AE grafts under examination by fluorescence microscopy (Fig. 2A). At day 14, allogeneic AE grafts were not visualized as a membrane by operation microscopy, but donor-derived GFP^{+} cells were still detected in corneal epithelium and stroma (Fig. 2B) under fluorescence microscopy. As shown in Figure 3A, approximately 60% of allogeneic AE grafts had lost GFP^{+} cells by day 21, increasing to 100% by day 28 (Fig. 2D). Conversely, GFP^{+} cells from syngeneic AE grafts were detected even at day 28 (Fig. 2C). Survival was significantly longer for syngeneic grafts than for allografts ($P < 0.05$, Fig. 3A). HE staining revealed

junctiva of normal BALB/c and C57BL/6 mice (**B**), on the corneas or in the ACs of presensitized BALB/c mice (**C**), on the corneas or in the ACs of normal BALB/c nice in a second transplantation (**D**). Grafts with GFP^{+} cells detected in the graft area by fluorescence microscopy were considered surviving grafts. (**A**, *asterisk*) Longer survival on corneas of syngeneic AE grafts than of allogeneic graft ($P < 0.05$). (**C**, *asterisk*) Longer survival of allogeneic AE grafts in the normal recipient than in presensitized recipients, irrespective of site ($P < 0.001$).
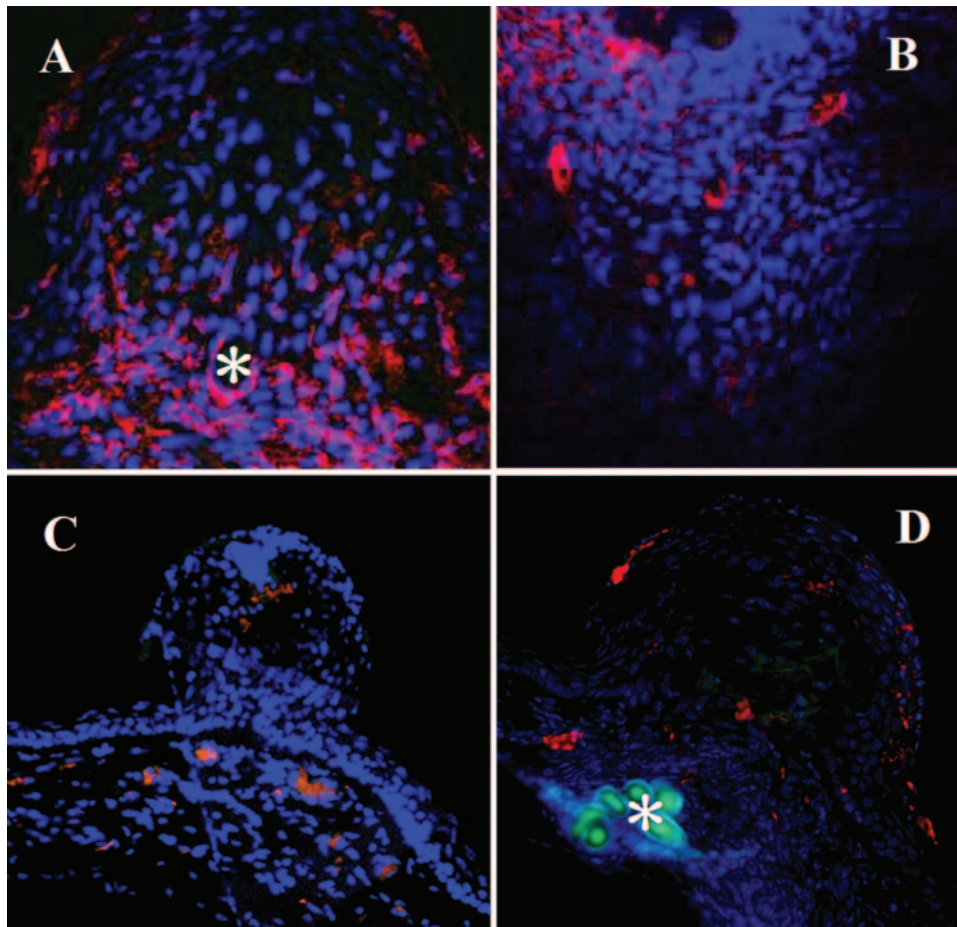
FIGURE 4. Immunohistochemical analysis of allogeneic GFP$^+$ AE grafts on the cornea at day 21. (A) Gr-1-, (B) B220-, (C) CD4-, or (D) CD8-positive cells (*red*) were present at the corneal graft site at day 21, but GFP$^+$ cells had disappeared by this time point. Purified anti-Gr-1, anti-B220, anti-CD4 or anti-CD8 antibodies were used as primary antibodies, and Cy-3-conjugated goat anti-rat IgG was used as the secondary antibody. Nuclei were stained with DAPI (*blue*). (✱) Sutures. Magnification: (A, B) ×40; (C, D) ×20.

numerous inflammatory cells infiltrating around the suture in the corneal epithelium and stroma after both allogeneic and syngeneic transplantations, but inflammatory response and neovascularization were more severe after allogeneic than after syngeneic AE grafting (Figs. 2E, 2F).

Immunohistochemical studies were performed in frozen tissue sections. CD4-, CD8-, or B220-positive cells were not present in cornea bearing allogeneic AE grafts at days 7 and 14, but many Gr-1$^+$ cells were present at these early observation points. At day 21, CD4-, CD8-, and B220-positive cells started to appear at the corneal graft site (Fig. 4). Conversely, except for Gr-1$^+$ cells, CD4-, CD8-, and B220-positive cells were not detected in corneas bearing syngeneic AE grafts (data not

shown). These results confirm that an innate immune reaction was involved in allogeneic AE grafting on the cornea, since Gr-1 was expressed throughout the period of examination. Moreover, acquired immune response by T and B cells was included in allogeneic AE grafting, but not in syngeneic AE grafting.

Fate of Allogeneic AE Grafts Heterotopically Transplanted on Conjunctiva of Normal Mouse Eye

Allogeneic GFP$^+$ AE grafts placed on the conjunctival surface disappeared in the early period after transplantation. GFP$^+$
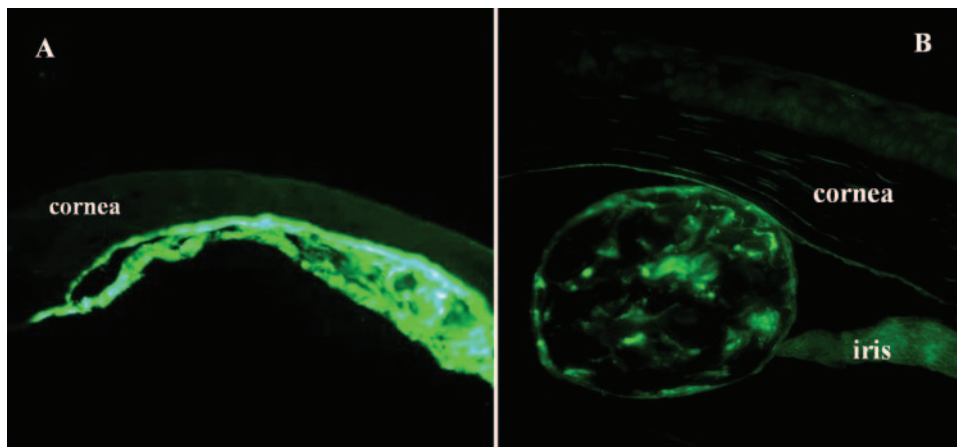
FIGURE 5. Fluorescence microscopy of allogeneic GFP$^+$ AE graft in the anterior chamber at days 28 (A) and 56 (B) after transplantation. Magnification: (A) ×20; (B) ×40.
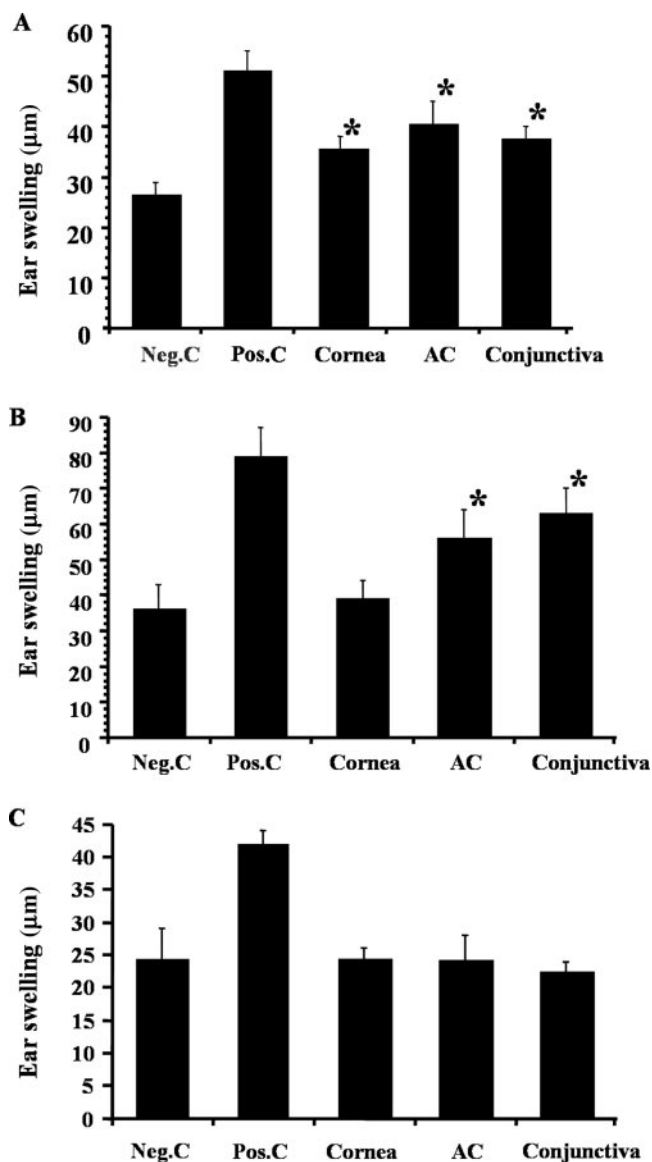
FIGURE 6. Induction of donor-specific delayed hypersensitivity (DH) assessment after transplantation of allogeneic AE grafts on cornea or conjunctiva or in AC at 2 (**A**), 4 (**B**), or 8 (**C**) weeks. AE grafts from 18-day pregnant wild-type C57BL/6 were transplanted on cornea or conjunctiva or into the AC of BALB/c eyes. Positive control mice received subcutaneous injection of 10×10^6 C57BL/6 splenocytes 1 week before assay. At 2 (**A**), 4 (**B**) or 8 (**C**) weeks after grafting, right ear pinnae received injection of 1×10^6 x-ray-irradiated C57BL/6 splenocytes, and swelling responses were assessed 24 and 48 hours later. Negative control mice received right ear pinnae challenge only. Mean 24-hour ear swelling responses were compared with the negative control. *$P < 0.05$ vs. negative control.

cells were undetectable as early as day 7 after transplantation. As shown in Figure 3B, no significant difference was identified between syngeneic and allogeneic grafting on the conjunctiva ($P > 0.05$). With HE staining, infiltrating inflammatory cells were observed around the suture (data not shown). Immunohistochemical staining showed that numerous Gr-1$^+$ cells had infiltrated around the suture site in both allo- and syngeneic implantation (data not shown). This suggests that the conjunctival surface is unsuitable for survival of syngeneic or allogeneic AE cells. AE cells grafted to this site presumably lost viability because of a nonimmunologic process.

Fate of Allogeneic AE Grafts Heterotopically Transplanted into the AC of Normal Mouse Eye

In next experiments, freshly isolated allogeneic GFP$^+$ AE grafts were implanted into the AC of normal BALB/c mouse eyes. A total of 35 BALB/c mice were used as recipients. After clinical observation under an operative microscope at day 7, 14, 28, or 56, all recipient eyes were removed for histologic or immunohistochemical analysis.

AE grafts were easily visualized in the AC by operating microscopy up to day 28. When AE grafts were examined under fluorescence microscopy, GFP$^+$ cells were apparent in the membrane covering the posterior surface of the cornea (Fig. 5A). At day 56, all the six AE grafts were visualized as a membrane in the AC by operative microscopy, and GFP$^+$ AE cells were still detectable in four of these six allogeneic AE grafts by fluorescence microscopy (Figs. 3C, 5B). Including GFP$^-$ AE-bearing eyes, HE staining showed no evidence of any inflammation in cornea, in AE grafts, or in the AC. CD4$^+$ or CD8$^+$ cells were not detected in either GFP$^+$ or GFP$^-$ AE grafts on immunohistochemical examination. Failure of long-term survival of two allografts was attributed to nonimmunologic responses.

Delayed Hypersensitivity Assessment

Given the presence of CD4$^+$ and CD8$^+$ T cells after allogeneic AE transplantation on the cornea, we sought to determine whether allogeneic AE grafts transplanted on the ocular surface induce donor-specific delayed hypersensitivity (DH). Panels of BALB/c recipients received AE allografts on the cornea or conjunctiva or in the AC. As a positive immunizing control, additional BALB/c mice received a subcutaneous injection of 10×10^6 C57BL/6 splenocytes, instead of AE allografts. At 2, 4, or 8 weeks after AE grafting, 1×10^6 x-ray-irradiated (2000 rad) C57BL/6 splenocytes were injected into the ear pinnae. Each panel comprised five to six mice. AE allografts induced donor-specific DH 2 weeks after transplantation on the cornea or conjunctiva or in AC ($P < 0.05$, Fig. 6A). At 4 weeks, DH was not induced after grafting on the cornea ($P > 0.05$), but was induced after grafting on conjunctiva or in the AC ($P < 0.05$, Fig. 6B). Of interest, at 8 weeks, DH was not induced in any recipients bearing allogeneic AE grafts ($P > 0.05$, Fig. 6C). These findings imply that fresh allogeneic AE graft is relatively immunogenic and able to sensitize recipients, but long-term memory is not acquired.
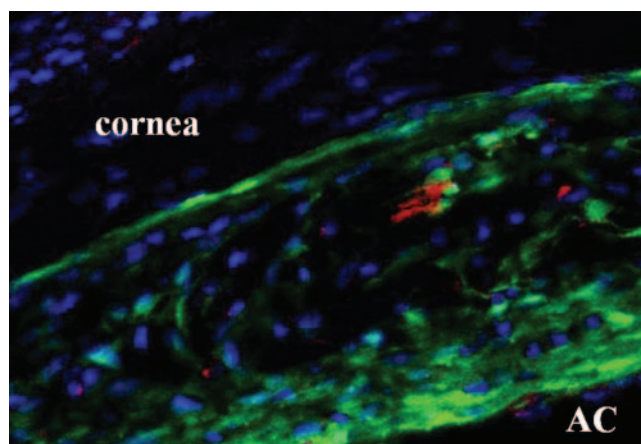
FIGURE 7. Expression of MHC class I on AE cells. A PE-mouse anti-H2K^b antibody was used. H-2K^{b+} (*red*) and GFP$^+$ (*green*) AE cells were apparent on the section for 4 days after EGFP-C57BL/6 AE grafting in the AC of BALB/c eye. Magnification, ×40.
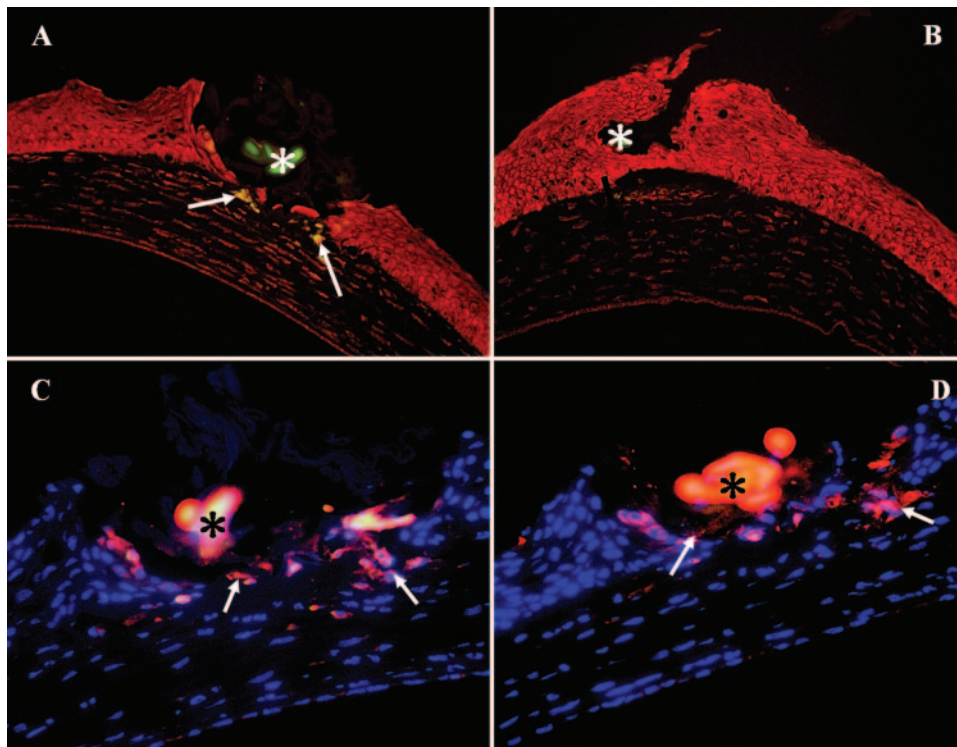
FIGURE 8. Immunohistochemical analysis of allogeneic GFP[+] AE grafts on cornea of presensitized recipients. Allogeneic GFP^{+} AE graft at days 4 (**A**) and 7 (**B**). Propidium iodide (PI) indicates cell nuclei (*red*). Fluorescence microscopy of CD4^{+} (**C**) and CD8^{+} (**D**) cells in the grafted area at day 4 after transplantation. Nuclei were stained with DAPI (*blue*). *Arrows*: GFP^{+} AE cells (**A**, **B**), CD4^{+} cells (*red*, **C**), CD8^{+} cells (*red*, **D**). (✱) sutures. Magnification: (**A**, **B**) ×20; (**C**, **D**) ×40.

Expression of MHC on Mouse Amniotic Epithelium

Because allospecific DTH induction and T-cell infiltration at the graft site was observed after allogeneic AE transplantation in the eyes, we hypothesized that allosensitization was probably due to expression of MHC antigens on AE cells. To test the expression, we used anti-mouse class I and II antibodies (H-2K[b] and I-A[b]) to detect mouse class I and II antigen on AE of C57BL/6 mice. AE cells were found to express MHC class I after grafting in the eye. Low expression of MHC class I was detected on allogeneic AE cells (Fig. 7), whereas MHC class II–expressing AE cells were not detected (data not shown).

Fate of Allogeneic AE Grafts in Eyes of Presensitized Recipients

To determine whether AE cells can be a target of effector cells in alloimmune rejection, we transplanted GFP[+] AE grafts onto corneas or into the ACs of BALB/c mice that had been subcutaneously injected with 10×10^6 C57BL/6 splenocytes 1 week earlier. A total of 40 BALB/c mice underwent sensitization and AM transplantation.

Survival of AE grafts in presensitized recipients was markedly curtailed compared with that in normal recipients (Fig. 3C, $P < 0.001$). When transplanted on cornea, a small number of GFP^{+} cells were detected in the corneal stroma, but these were not detected at day 7 in either AE grafts or corneal stroma (Figs. 8A, 8B). Under fluorescence microscopy, CD4^{+} and CD8^{+} cells were present at the graft area (Figs. 8C, 8D). When transplanted in AC of presensitized recipients, a few GFP^{+} cells were detected in AE grafts at day 7, but had disappeared from the remaining AE membrane by day 10 (Figs. 9A, 9B). With HE staining, numerous inflammatory cells were seen to have infiltrated AE grafts (Figs. 9C, 9D). Immunohistochemical examination revealed CD4^{+} and CD8^{+} cells present in the iris and ciliary body at day 7 (Figs. 9E–G), and, at day 10, these cells began to appear in the AE graft (Fig. 9H). Allogeneic AE cells

are thus not only able to sensitize recipients but also to become a target of rejection in specifically sensitized recipients.

Fate of Allogeneic AE Grafts after the Second Transplantation

The next experiment was designed to determine the fate of allogeneic AE grafts after the second transplantation. The first transplantation of AE was performed on corneas or in the ACs of the right eyes of normal BALB/c mice. A second transplantation was performed 7 days later on the same site in the left eye. A total of 30 BALB/c mice were used as recipients.

Whereas 60% of first grafts on the right cornea survived to day 14 after the second transplantation, 0% survived to day 21 (Fig. 3D). Likewise, 40% of second grafts on the left cornea survived to day 14 after grafting, whereas 0% survived to day 21. Survival of the grafts after the first grafting on cornea was thus similar to that in single transplantations, whereas survival of second grafts was markedly shorter and AE cells in these grafts disappeared by the same time the first grafts disappeared. CD4^{+} and CD8^{+} cells were present both in first and second grafts at day 14 after second grafting (Figs. 10A, 10B). Conversely, only 60% of first allogeneic AE graft in the AC and only 20% of second grafts survived to day 21 after second transplantation (Fig. 3D). These results show that survival of first and second grafts in the AC was markedly curtailed for repeated transplantation. CD4^{+} and CD8^{+} cells were present in the iris, ciliary body, and AE grafts by day 14 after the second transplantation (Figs. 10C–10F).

DISCUSSION

AM transplantation has been used successfully in patients for ocular surface reconstruction.[4–8] The satisfactory clinical results mostly benefit from the re-epithelialization and decreased inflammation and fibrosis promoted by AM. Various mechanisms of action have been proposed to explain the anti-inflammatory effects. Li et al.[11] recently reported that HAECs secrete
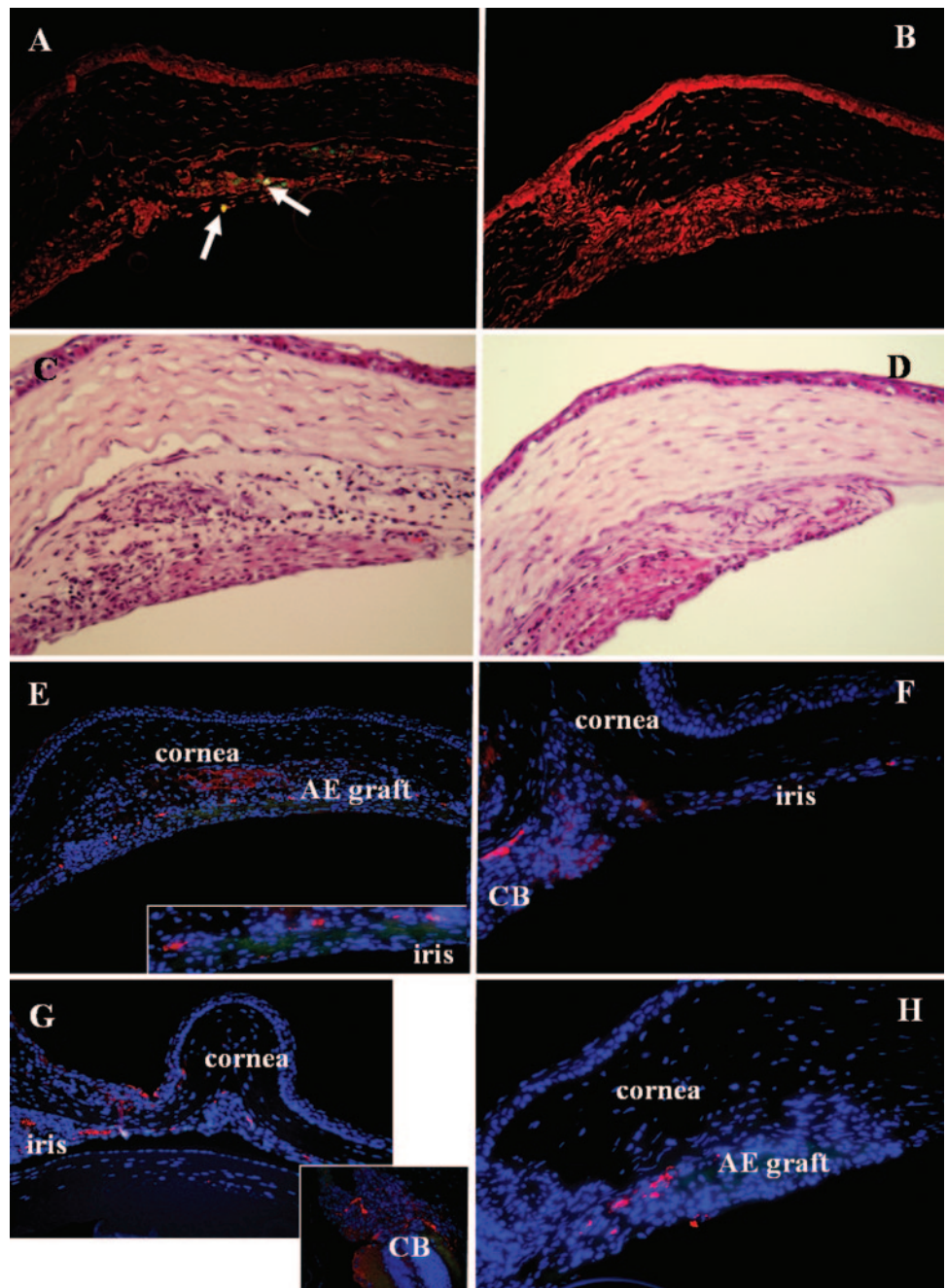
FIGURE 9. Histologic appearances and immunohistochemical analysis of allogeneic GFP⁺ AE grafts in the anterior chamber of presensitized recipients. Allogeneic AE graft at days 7 (**A**) and 10 (**B**) after transplantation. PI indicates cell nuclei (*red*). Histologic aspects of allogeneic AE grafts at days 7 (**C**) and 10 (**D**). Fluorescence microscopy of CD4⁺ and CD8⁺ cells (*red*) at days 7 (**E-G**) and 10 (**H**). Nuclei were stained with DAPI (*blue*). CB: ciliary body. *Arrows*: GFP⁺ AE cells (**A**). Magnification (**A**, **B**, **E**, **G**) ×20; (**C**, **D**, **F**, **H**, *inset* **E**, *inset* **G**) ×40.

factors that inhibit both the innate and adaptive immune systems. According to their study, HAEC-derived factors inhibit migration of neutrophils and macrophages. HAECs also secrete factors inhibiting T- and B-cell proliferative responses to mitogens and induce apoptosis of activated T cells. In addition, we recently reported that topical application of HAEC supernatant leads to profound suppression of corneal neovascularization, migration of MHC class II⁺ antigen presenting cells (APCs) in the epithelium, and expression of inflammatory cytokines mRNA in cornea. We have proposed that HAECs represent a source of soluble anti-inflammatory factors, which can suppress corneal inflammation.[12] This progress in identifying the anti-inflammatory properties of AM could suggest that immune rejection does not arise after AM transplantation. AM transplantation has been applied more and more widely to the treatment of ocular disorders. However, repeated transplantation of AM derived from the same donor to a single patient has recently

been reported to result in serious inflammation, with hypopyon developing after both the second and third transplantations.[23] The immunogenicity and antigenicity of AE as an allograft thus demands a fresh evaluation.

The present results demonstrate for the first time that AE has the capacity to sensitize recipients and can be the target of alloimmune effector cells in rejection. Transplantation of freshly isolated allogeneic AE grafts onto cornea or conjunctiva or into the AC sensitizes the recipient, and all recipients in this study acquired allospecific DH within 2 weeks after transplantation. However, none of these recipients acquired long-lasting memory of sensitization. Survival of allogeneic AE cells differed among graft sites, with AE cells losing viability within 1 week on conjunctiva and disappearing within 21 days in cornea. Conversely, these cells remained viable for >8 weeks when implanted into the AC. This indicates that the fate of allogeneic AE cells depends on the degree of immunogenicity and privi-
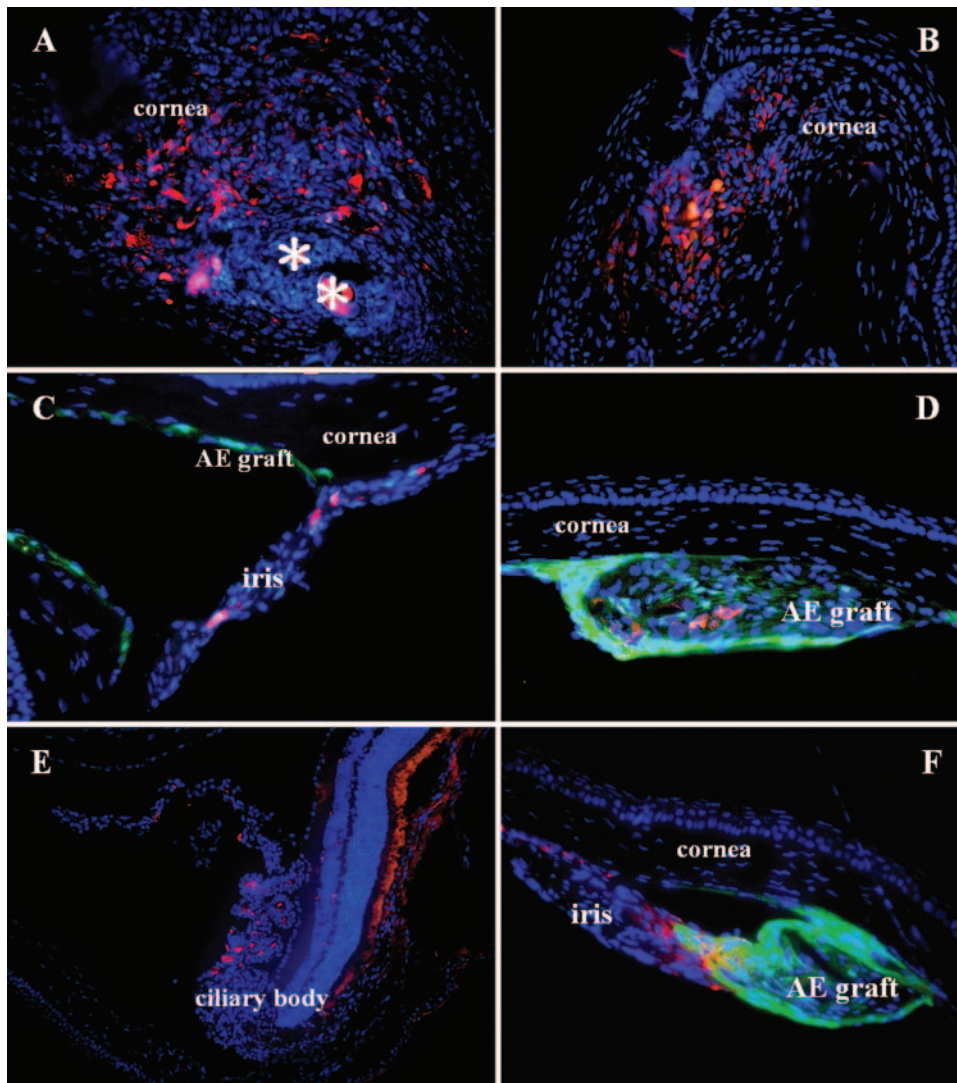
FIGURE 10. Immunohistochemical analysis of allogeneic GFP⁺ AE grafts on the cornea or in the AC with repeated transplantation. Presence of CD4⁺ T cells (*red*) in the first (**A**) and second (**B**) corneal grafts at day 14 after the second transplantation. The presence of CD4⁺ T cells (*red*) in first graft (**C**) and second graft (**D**) in AC at day 21 after the second transplantation. The presence of CD8⁺ T cells (*red*) in the first (**E**) and second (**F**) grafts in the AC at day 21 after the second transplantation. Nuclei were stained with DAPI (*blue*). (1002) Sutures. Magnification: (**A–D**, **F**) ×40; (**E**) ×20.

lege of the graft site. In addition, AE cells disappeared not only because of the immunogenic response, but also because of the nonimmunogenic response after transplant to the ocular surface, since even syngeneic AE cells were not able to remain viable for long on cornea or conjunctiva in normal recipients. HLA-A, -B, -C, and -DR and β_2-microglobulin have been reported to be undetectable in cultured human amniotic epithelium,[16] but MHC class I antigen manifestations in AE have subsequently been reported in several studies.[21,22,25] To the best of our knowledge, no reports have described MHC antigens expressed on mouse AE cells. The present study demonstrated for the first time that MHC class I⁺ antigens are weakly expressed on mouse AE cells after grafting into the eye. In our experiments, both allospecific DH induction and infiltration of CD4 and CD8 T cells were observed at the graft site after allogeneic AE transplantation in normal mice. These results led the hypothesis that weak allosensitization is acquired, due to low MHC expression on AE cells. Thereafter, cell-mediated immune responses were induced, and T cells infiltrated the graft site. Because of the short period of viability of AE cells grafted on the ocular surface, by the time effector CD4⁺ and CD8⁺ T cells reached the graft site at day 21, most donor-derived AE cells had already lost viability and were unable to display enough antigens to represent a target for these effector cells. As a result, no long-term memory of sensitization was acquired. At 8 weeks, DH was not induced in AC transplant-

recipient mice, such as those receiving transplants to the cornea or conjunctiva. However, AE allografts survived only in the AC at this time point. These results suggest that anterior chamber-associated immune deviation (ACAID) may be induced in AC transplant recipients. ACAID is a well-known antigen-specific deviant systemic immune response induced after antigen injection into the AC. CD4⁺ helper 1 (Th1), Th2, and B cells that secrete complement-fixing antibodies are reportedly suppressed, but CD8⁺ cytotoxic T cells and generation of non-complement-fixing antibodies remain induced or even enhanced in ACAID.[26] Although our findings of B cells in the graft site indicate the possibility of inducing antibody-mediated immune responses, whether these B cells play any role as effectors of rejection or other responses remains unclear. Further studies are necessary to address these possibilities.

In presensitized recipients, survival of allogeneic AE cells was markedly reduced. CD4⁺ and CD8⁺ T cells infiltrated the graft area rapidly, as early as day 4 after transplantation on the cornea and day 7 after transplantation into the AC, resulting in destruction of AE cells. Because recipients had been allosensitized, donor antigen-specific CD4 and CD8 T cells infiltrated more rapidly and in greater quantities in the graft site. Allogeneic AE cells that induced donor-specific DH in normal recipients were rejected in recipients sensitized systemically to donor alloantigens, implying that AE cells transplanted in the eye display both immunogenicity and antigenicity. The terms

"immunogenic" and "antigenic," when applied to tissue transplantation, indicate the ability of an allograft to sensitize the recipient and the vulnerability of the graft to specific immune effectors of rejection, respectively.[27] We have therefore demonstrated for the first time that AE has sufficient antigenicity to represent a target of alloimmune effector cells in rejection. Although cases of presensitization do not occur in clinical scenarios, many eyes need repeated transplantation, and rejection after repeated transplantation has been reported.[23] The present results clearly show that rejection arises after repeated AE allografting.

In our experiments, syngeneic (EGFP-C57BL/6 W/t) and allogeneic (EGFP-C57BL/6 to BALB/c W/t) AE cells were transplanted into mouse eyes. Although proteins expressed as selectable makers have been described as potentially immunogenic,[28] Skelton et al.[29] reported that the enhanced GFP is minimally immunogenic. We have also reported that syngeneic corneal grafts from EGFP C57BL/6 donors survive indefinitely in W/t C57BL/6 recipients.[30] Moreover, we have found that W/t recipients after receiving subcutaneous injection of EGFP[+] syngeneic spleen cells did not undergo EGFP-specific DH (Hori J, unpublished data, 2000). We can thus conclude that immune rejection of EGFP[+] AE allografts is induced by alloantigens, not by the GFP protein.

Evidence from murine experiments suggests that MHC class I and II molecules are relatively unimportant in promoting graft rejection, whereas minor histocompatibility (H) antigens represent more formidable barriers to corneal allograft acceptance.[31–33] However, expression and roles of minor H antigens on AE allografts have not been studied in detail. In our present experiments, BALB/c mice were used as recipients that recognize both MHC and minor H antigens on donor C57BL/6 mice tissue. Minor H antigen-only disparate mice strains should be helpful in determining the role of minor H antigen in rejection reactions of the AE. Experiments to examine this are now under way.

The gradual disappearance of AE cells after transplantation is interesting, and has recently attracted much attention from researchers. Our experiments also found that when implanted in the AC, some allogeneic AE grafts disappeared by day 56 without any evidence of inflammation. Runic et al.[34] reported that human fetal membranes undergo apoptosis. Kubo et al.[25] speculated that some amniotic cells are apoptotic and readily disappear under particular conditions such as transplantation. The disappearance of allogeneic AE grafts transplanted heterotopically in the eye may thus be due to the process of apoptosis, although further studies are needed to support this notion.

In clinical situations, cryopreserved AM has been used widely. Immunogenicity of cryopreserved tissues is generally thought to be less than that of fresh tissues. Cryopreserved AM is thus expected to have a lower risk of rejection than fresh AM. However, ≥50% of AE cells cryopreserved for 2 months reportedly remain viable and able to grow in culture.[25] Conversely, secretion of anti-inflammatory factors by AE cells does not mean that AE is nonimmunogenic or nonantigenic as an allograft. Under some exceptional conditions, such as transplantation of AE cells with high viability or after repeated transplantation of tissues from the same donor, rejection can arise after transplantation. The present study is the first to demonstrate the fate of freshly isolated allogeneic AE cells transplanted to the ocular surface and AC. Our results indicate that AE is not a completely immune-privileged tissue, displaying partial immunogenicity after transplantation into the normal mouse eye and acting as a target of rejection in the eyes of presensitized recipients. We therefore suggest that the partial immunogenicity of AM should not be ignored and the use of AM from different donor placentas should be emphasized when repeated AM transplantation is necessary in patients clinically.

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