

Immunogenicity of Human Amniotic Membrane in Experimental Xenotransplantation

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PURPOSE. The immunogenic characterization of amniotic membrane is still unknown. This study was designed to examine the immunogenicity of human amniotic membrane, by using experimental xenotransplantation models.

METHODS. Anti-human class I, class II, and Fas ligand monoclonal antibodies were used against cryopreserved amniotic membrane and cell viability tested for cryopreserved amniotic membrane. Amniotic membranes were then transplanted to the limbal area, intracorneal space, and under the kidney capsule. The scores of transparency and neovascularization after transplantation were recorded by slit lamp microscopy. Host cell infiltration was examined by hematoxylin-eosin or immunohistochemical staining. Control grafts were transplanted human cryopreserved skin grafts.

RESULTS. Strong class I expression was observed in amniotic epithelium, mesenchymal cells, and fibroblasts in cryopreserved amniotic membrane. Some fibroblast cells unexpectedly expressed class II antigen. Fas ligand-positive cells were also detected in mesenchymal cells of amniotic stroma. Approximately 50% of epithelial cells of amniotic membrane cryopreserved for several months were still viable. In limbal transplantation, although some CD4⁺ and CD8⁺ T cells surrounded the amniotic graft, the response was mild. In intracorneal transplantation, all grafted amniotic membranes were accepted and clear, without host cell infiltration. In contrast, all skin grafts were rejected within 3 weeks after intracorneal transplantation. In amniotic membrane transplantation under the kidney capsule, extremely few host vessels and cells infiltrated the amniotic membrane; however, more host cells infiltrated the skin tissues under the kidney capsule.

CONCLUSIONS. Amniotic membrane seems to be immune-privileged tissue and to contain some immunoregulatory factors, including HLA-G and Fas ligand. The amniotic membrane may be useful to supplement corneal collagen, and it may be applied not only to the ocular surface but also intracorneally. (*Invest Ophthalmol Vis Sci.* 2001;42:1539-1546)

The amniotic membrane has been used as a biological membrane to treat burn injury and skin ulcer.¹ Recently, remarkable therapeutic effects by amniotic membrane have been reported for serious conjunctival disease such as chemical burn, ocular pemphigoid, and Stevens-Johnson syndrome.^{2,3} One of the reasons for using amniotic membrane is that it rarely causes immunologic rejection. Although no HLA was

initially detected in cultured amniotic membrane,⁴ the manifestation of class I antigen in the amniotic membrane has been reported since then. Although the fetus has semiallogeneic antigens recognized by the mother, pregnancy is established normally in many cases, except in some abortions. Furthermore, kidney allograft avoided allograft rejection in one case when immunosuppression was discontinued during and after pregnancy.⁵ It is implied that an active immunoregulatory mechanism may be generated, although it is not clear whether the mechanisms of unresponsiveness are related to the fetal antigens. So far, it is thought that a special immunologic mechanism may protect the fetus from maternal aggression. By the hypothesis of Medawar in 1953,⁶ (1) because fetal antigen is immature, the maternal body cannot recognize the fetus, (2) the fetus is isolated from maternal immunity, (3) the maternal immunologic activity decreases during pregnancy, and (4) the placenta is a barrier between the fetus and the maternal body. However, recently, special manifestations of fetal antigen and its recognition during pregnancy have attracted attention.

The amniotic membrane is of embryonic origin, and the intrauterine fetus is located in the amniotic cavity surrounded by the amniotic membrane. The amniotic cavity is filled with amnion fluid secreted from the amniotic membrane and fetus. Because type IV collagen is abundant in the amniotic membrane, recently, the main concept of amniotic membrane transplantation in ophthalmology is "substrate transplantation"—that is, basement membrane transplantation to develop normal corneal or conjunctival epithelium in ocular surface disorders. Most amniotic membrane grafts, however, are placed on the amniotic cavity side of the sponge layer, and various nucleated cells are contained in the amniotic membrane, including not only collagen but also amniotic epithelium, mesenchymal cells, and fibroblasts. In 1940 De Roth⁷ reported that the success rate is low when live amniotic membrane and chorion are used together for plastic repair of conjunctival defect, implying that the live fetal membrane is immunogenic. At present, most amniotic membrane tissues used in clinical cases has been cryopreserved. However, the immunogenicity of cryopreserved human amniotic membrane is not fully understood and is still controversial. We therefore examined the immunogenicity of the amniotic membrane after amniotic membrane transplantation, by using xenotransplantation models.

MATERIALS AND METHODS

Animals

This study was carefully performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Experimental Committee of Tokyo Medical University. Lewis rats (aged 8-10 weeks) were prepared, anesthetized by intramuscular injection of ketamine (7 mg/kg) and xylazine (0.7 mg/kg), and subjected to general anesthesia.

Human Tissues

Informed consent was received from pregnant donors in accordance with the Declaration of Helsinki. Fetal membrane obtained on Caesar

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ian section was washed in isotonic sodium chloride solution. Amniotic membrane was separated from the chorion gently and was cut in 5-cm² sections. These membrane specimens were washed in 0.5 M, 1.0 M, and 1.5 M dimethyl sulfoxide (DMSO), in that order, and finally were stored in 1.5 M DMSO at -80°C. When used after spontaneous thawing, amniotic membrane was prepared in balanced salt solution (BSS). Adult human skin was obtained during surgery for dermatochalasis from a patient who gave informed consent. Skin grafts were cryopreserved similar to the amniotic membrane for 2 months in 1.5 M DMSO at -80°C.

Cell Viability and Growth in Cryopreserved Amniotic Membrane

To examine cellular viability of amniotic membrane after cryopreservation, cells in amniotic membrane after 2 months or 18 months cryopreservation at -80°C were counted after 5 minutes of mixing with 0.4% trypan blue followed by washing with phosphate-buffered saline (PBS). For cell culture, amniotic membranes were cultured at 37°C in a 5% CO₂ atmosphere for 4 weeks with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) after spontaneous thawing. Each piece of amniotic membrane was cultured in plastic wells. Culture medium was exchanged every 3 days, and each week the cultures were examined for appearance of new amniotic cells, by light microscopy. At that time amniotic membranes were stained by 5 minutes of mixing with 0.4% trypan blue.

Transplantation Designs

Limbal Transplantation. Lewis rats (aged 8-10 weeks) were anesthetized by intramuscular injection of ketamine and xylazine. First, 3.0 × 4.0-mm sections of upper limbal conjunctiva were removed, and then human amniotic membrane (2-3 months' cryopreservation) was placed on the sclera and sutured with 10-0 nylon. After transplantation, tarsorrhaphy was performed, and eyelid sutures were removed at 3 days.

Intracorneal Transplantation. After rat corneal marking by 3.0-mm trephine, a semilayer incision of the corneal stroma was performed with Vannas scissors, and amniotic membrane (2-3 months' cryopreservation) was inserted into the intrastromal layer. Three sutures of 10-0 nylon were placed around the corneal wound. All sutures were removed on day 7. Antibiotic eyedrops (ofloxacin) were used for 14 days after transplantation; however, no immunosuppressive drug was used in these experiments. As a positive control in intracorneal transplantation, human skin (2 months' cryopreservation) was grafted inside corneal stroma by a similar technique. The corneal transparency and neovascularization were analyzed and scored by slit lamp microscopy. The graft-bearing corneas were stained histopathologically with hematoxylin and eosin (HE) at 2 to 3 weeks.

Heterotopic Transplantation under the Kidney Capsule. Lewis rats were used as recipients, and human amniotic membranes were used as donor tissues. Cryopreserved amniotic membranes were transplanted under the left kidney capsule. Cryopreserved human skin grafts were transplanted as positive controls. Five rats were used in each group. The transplantation procedure under the kidney capsule was based on the method of Hori et al.⁸ A skin incision was made in the left side of the Lewis rats. A subcapsular pocket was created under the kidney, and 4.0 × 5.0-mm donor tissues were placed in the pocket. Amniotic membranes were either transplanted under the kidney capsule so that the amniotic epithelial side faced down toward the kidney, or they were folded over with the amniotic epithelial side inside and then transplanted under the kidney capsule. The kidney was replaced in the abdominal cavity, and skin was closed with a 4-0 Dacron suture. Recipients were killed at 1 week, and the graft-bearing kidneys were stained with HE histopathologically.

Evaluation and Scoring of Limbal and Intracorneal Transplantation

In limbal and intracorneal transplantation, the transparency and neovascularization after transplantation were analyzed and scored by slit lamp microscopy. A 0 to 3+ scoring system was devised to describe semiquantitatively the extent of opacity, as follows: 0, clear graft without edema; 1+, minimal opacity with slight edema; 2+, moderate opacity with edema; and 3+, intense opacity with marked edema. A similar scoring system was developed to semiquantitatively describe the extent of neovascularization as follows: 0, no vessels extending toward the graft; 1+, vessels reaching the graft margin; 2+, vessels invading the graft; and 3+, many vessels traversing the graft.

Monoclonal Antibodies

Two anti-human class I antibodies against class I heavy chain, B9.12.1 (diluted 1:50; Immunotech, Inc., Marseilles, France) and W6/32 (diluted 1:50; Leinco Technologies, Inc., St. Louis, MO), were used against human class I antigen. Anti-human class II antibody BL-IA/6 (diluted 1:50; Monosan, Inc., Uden, The Netherlands) was used against human class II antigen, and anti-human Fas-ligand antibody G247-4, diluted 1:25; Pharmingen, San Diego, CA) was used against human Fas ligand. As monoclonal antibodies against the grafting models, anti-rat CD4⁺ T cell W3/25 (diluted 1:20; Chemicon International, Inc., Temecula, CA), anti-rat CD8⁺ T cell OX8 (diluted 1:20; Chemicon International, Inc.), anti-rat macrophages ED2 (diluted 1:20; Cosmobio, Tokyo, Japan), anti-human class I W6/32 (diluted 1:50), and anti-human class II antibody BL-IA/6 (diluted 1:50) were used.

Immunohistochemical Examination

We performed immunohistochemical analysis (ABC method) using monoclonal antibodies. Human amniotic membranes or experimental rat's eyes were capsulated by OCT compound, and frozen sections of 5-μm thickness were cut by a microtome cryostat. With the ABC method, sections were fixed in acetone, and sections were treated with horse serum as the blocking serum. The sections were incubated with horse serum (diluted 1:70) for 20 minutes in 37°C in a 5% CO₂ atmosphere. All sections were washed 3 × 10 minutes with PBS and incubated with each primary mouse monoclonal antibody for 45 minutes in 37°C in a 5% CO₂ atmosphere. As controls, sections without primary antibodies were used. After incubation with primary antibodies, sections were incubated with horse anti-mouse antibody-peroxidase conjugate. Color development in peroxidase reaction was performed using the diaminobenzidine supplied with an ABC staining kit (Vectastain; Vector Laboratories, Inc., Burlingame, CA). Finally, each section was counterstained with hematoxylin.

RESULTS

Detection of Tissue Antigens on Amniotic Membrane

Two different anti-human class I monoclonal antibodies (B9.12.1 and W6/32), which combined different epitopes, were used to detect human class I antigen on amniotic membrane. Generally, it is thought that B9.12.1 reacts against human HLA-A, -B, and -C. W6/32 is not an exclusive monoclonal antibody against HLA-G; however, it is known that it is able to capture the HLA-G molecule well.^{9,10} Normal cryopreserved amniotic membranes expressed strong class I antigen in epithelium, mesenchymal cells, and fibroblasts (Figs. 1A, 1B). β₂-microglobulin was stained in the same way (data not shown). Some fibroblasts unexpectedly expressed class II antigen (Fig. 1C). No significant difference was observed between amniotic membrane stored at -80°C for 1 month and 6 months. Fas ligand-positive cells were also detected in some mesenchymal cells of amniotic stroma (Fig. 1D).

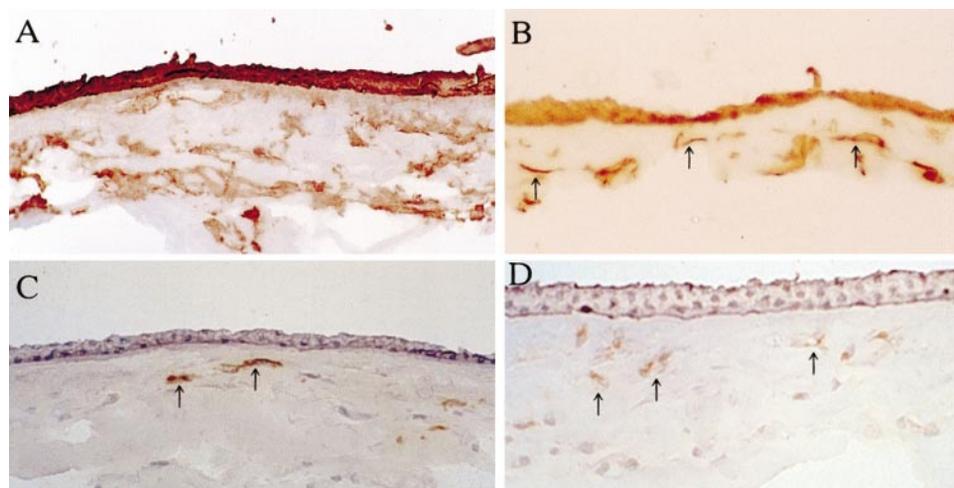


FIGURE 1. Detection of tissue antigens on amniotic membrane. Two different anti-human class I monoclonal antibodies, B9.12.1 (A) and W6/32 (B). Arrows: class I-positive fibroblasts in (B). (C) Some fibroblasts expressed class II antigen (arrows). (D, arrows) Fas ligand-positive amniotic mesenchymal cells. Magnification, (A, B, C) $\times 200$; (D) $\times 400$.

Cell Viability on Amniotic Membrane after Cryopreservation

Most amniotic epithelial cells preserved for 18 months at -80°C were stained with 0.4% trypan blue, indicating that most cells were not alive (Fig. 2A). However, more than 50% of amniotic epithelial cells preserved for 2 months at -80°C were not stained with 0.4% trypan blue (Fig. 2B). This result indicates that at least 50% of amniotic epithelial cells might be still alive, if the period of preservation lasts a few months. Next, we wanted to determine whether these amniotic epithelial cells grow in culture. New amniotic epithelial cells that were not stained by trypan blue appeared overlying the original epithelial cells at 2 weeks (Fig. 2C). Gradually, the number of new amniotic cells increased. These cells combined at 3 weeks, and new amniotic epithelial cells covered more than 50% of the total area (Fig. 2D). These results clearly indicate that some proportion of amniotic epithelial cells were viable and had the capacity to grow in culture after preservation for a few months.

Limbal Transplantation

Two weeks after grafting, some new vessels appeared on the amniotic membrane; however, no severe rejection, including

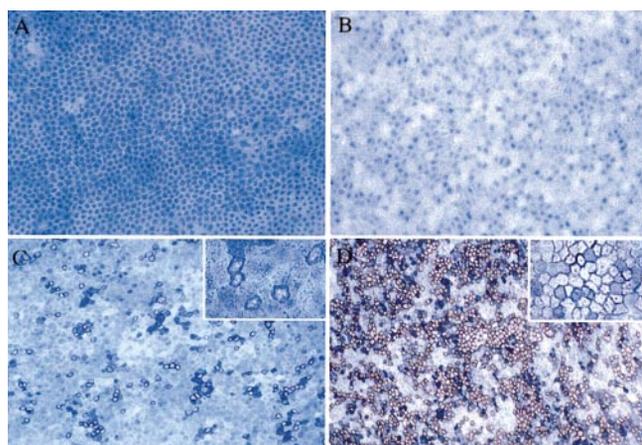


FIGURE 2. Amniotic membrane cell viability after cryopreservation. (A) Amniotic epithelial cells preserved for 18 months at -80°C stained with 0.4% trypan blue. (B) Amniotic epithelial cells preserved for 2 months at -80°C . (C) In culture new amniotic epithelial cells grew, overlying the original epithelial cells at 2 weeks after culturing. Inset: higher power image of the new amniotic epithelial cells. (D) The number of new amniotic cells increased, and these cells combined at 3 weeks. Inset: higher power image of the combined new amniotic epithelial cells. Magnification, $\times 100$; insets, $\times 400$.

strong edema or necrosis, was seen by slit lamp microscopy, and collagen tissue was still present (Fig. 3A). Neovascularization on the amniotic membrane diminished gradually, but the collagen tissue of amniotic membrane remained and was clear at 4 weeks (Fig. 3B). On immunohistochemical observations at 1 week after grafting, CD4^{+} T cells had surrounded the amniotic membrane, predominantly under it (Fig. 3C, arrows). CD8^{+} T cells also had surrounded the amniotic membrane; however, the number of positive cells was smaller than that of the accumulating CD4^{+} T cells and CD8^{+} T cells, particularly at the suture area (Fig. 3D, arrows). In a preliminary study, rat macrophages also infiltrated at a site similar to where T cells infiltrated (data not shown). A summary of the clinical scores is shown in Figure 4A. In limbal transplantation, grafted amniotic membrane had moderate opacity and edema at 1 week, and some new vessels invaded the graft. The mean scores of transparency and neovascularization were both 2 at 1 week; however, vessels diminished gradually, and the transparency improved.

Intracorneal Transplantation

At day 3 after amniotic membrane transplantation, only slight graft edema was seen, and the grafts were clear (Fig. 5A). No rejection reaction with edema was observed, and all grafts ($n = 10$) were completely clear at 4 weeks (Fig. 5B). HE staining at day 3 showed no inflammatory infiltration into the amniotic membrane (Fig. 5C) and showed that transplanted collagen tissues remained at 4 weeks. Also of note, some host keratocytes appeared inside the matrix of the transplanted amniotic membrane (Fig. 5D). A summary of clinical scores for intracorneal transplantation is shown in Figure 4B. In intracorneal transplantation, the mean scores of transparency and neovascularization were 1 at 1 week, with no new vessels invading the amniotic membrane.

Generally, the mean scores of intracorneal transplantation were lower than those of limbal transplantation. Both scores reached approximately 0 at 4 weeks, staying the same for 6 months. Immunohistochemical observations at 1 week after grafting showed that rat CD4^{+} and CD8^{+} T cells did not surround or infiltrate the amniotic membrane (Figs. 6A, 6B). Rat macrophages also did not infiltrate the transplanted amniotic membrane even when some host macrophages were present in limbal areas (Fig. 6C). Furthermore, although amniotic epithelium still expressed human class I antigen at 1 week after grafting, no host cells infiltrated human class I-positive cells (Fig. 6D), and human class II-positive fibroblasts had disappeared (Fig. 6E). In intracorneal skin transplantation, all cryopreserved skin grafts ($n = 4$) were clearly rejected by 3

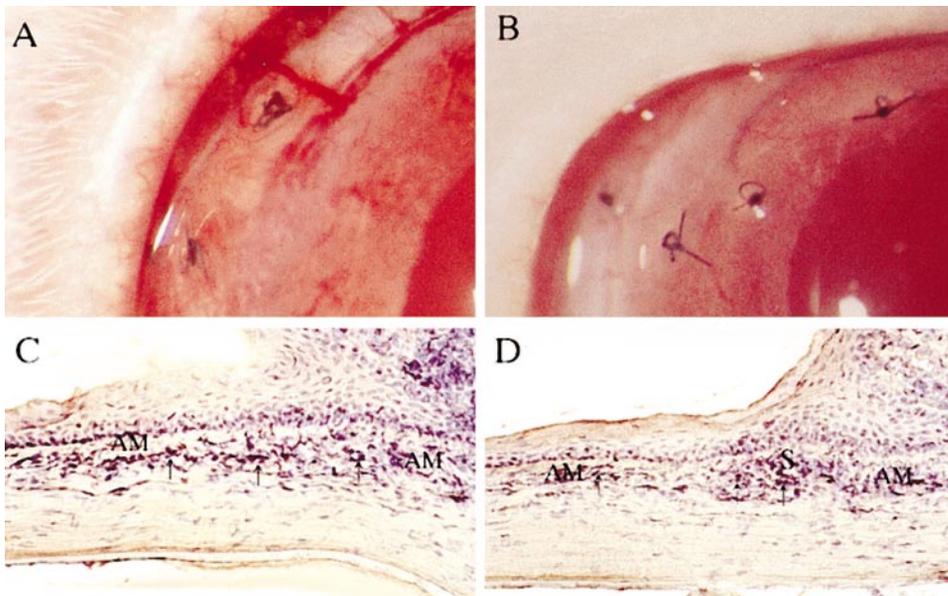


FIGURE 3. (A) Two weeks after limbal transplantation. (B) Four weeks after grafting, the collagen tissue of the amniotic membrane remained. (C) CD4⁺ T cell infiltration (arrows). (D) CD8⁺ T cells also surrounded the amniotic membrane; however, the number of positive cells was smaller than that of CD4⁺ T cells. Arrows: CD8⁺ T cells. Magnification, (C, D) ×100. AM, amniotic membrane; S, suture area.

weeks after transplantation. Neovascularization against corneal sutures decreased once the sutures were removed; however, new vessels subsequently approached the skin graft (Fig. 7A), and finally intense corneal edema and neovascularization were seen in all models (Fig. 7B). With HE staining, intense lymphocyte infiltration was seen of the host cornea and donor skin grafts, indicating immunologic rejection (Fig. 7C). The mean score of transparency and neovascularization in the host cornea gradually increased beyond 2 to 3 weeks (Fig. 4C).

Heterotopic Transplantation under the Kidney Capsule

To determine the immunogenicity of amniotic membrane more clearly, we performed another experimental transplantation under the kidney capsule using the cryopreserved amniotic membrane. This site of transplantation is thought to be an immunologically unprivileged site. As a control, human cryopreserved skin grafts were transplanted. At 1 week after amni-

otic membrane transplantation under the kidney capsule, grafted amniotic membrane was smooth with no edema. Some host vessels crossed over the amniotic membrane but did not invade it. In HE staining, the amniotic membrane clearly maintained its general structure, and extremely few cells infiltrated the amniotic stroma. A few amniotic epithelial cells remained on the kidney side, although host cells infiltrated between the kidney and the amniotic membrane. A few amniotic fibroblasts were present inside the amniotic membrane without infiltration of host cells (Fig. 8A). Similar results were seen in other rats. When the amniotic membrane was folded over and transplanted under the kidney capsule, only a few host cells infiltrated the amniotic membrane. An interesting observation was that most of the amniotic epithelial cells were protected from host aggression and remained, even in immunologically unprivileged sites (Fig. 8B). However, more vessels were seen reaching the skin graft in subcapsular transplantation. HE staining showed that more host cells infiltrated the skin tissue than the

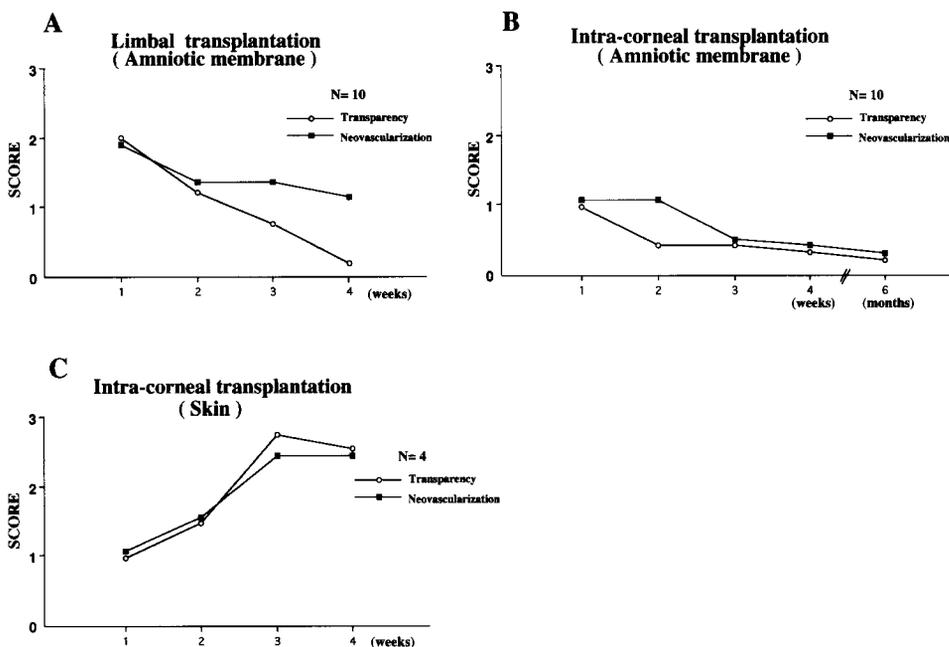


FIGURE 4. Summary of clinical scores for limbal transplantation (A) and intra-corneal (B) transplantation of the amniotic membrane and intracorneal transplantation of the skin tissue (C).

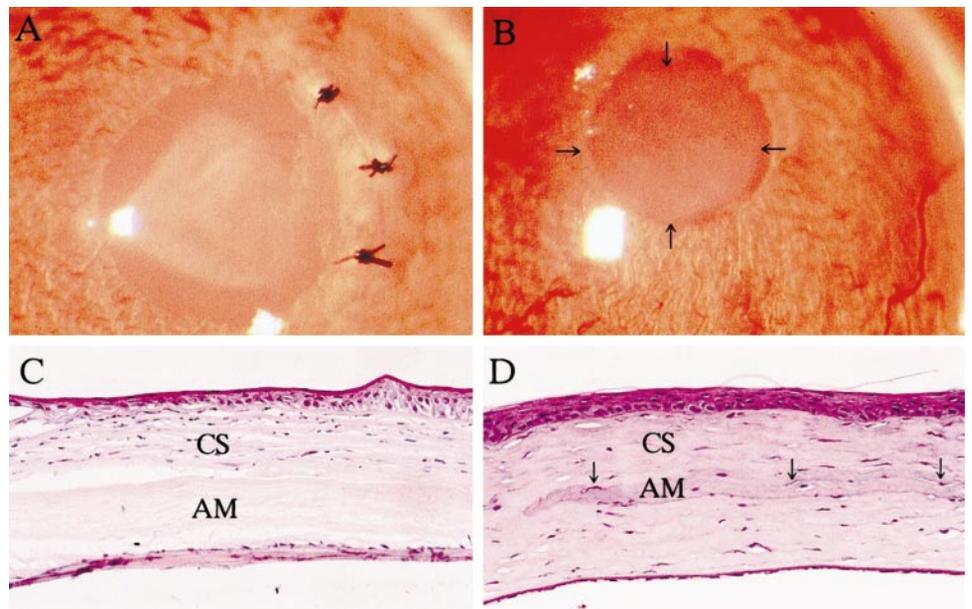


FIGURE 5. (A) Day 3 after intracorneal transplantation. *Black markings* represent sutures. (B) No rejection reaction with edema was observed and the graft was completely clear at 4 weeks. *Arrows:* intracorneal amniotic membrane. (C) HE staining at 3 days showed no inflammatory infiltration into amniotic membrane. (D) HE staining at 4 weeks. Some host keratocytes appeared inside the matrix of the transplanted amniotic membrane. *Arrows:* host keratocytes. Magnification, (C, D) $\times 200$. AM, amniotic membrane; CS, corneal stroma.

amniotic membrane, and some vessels invaded the peripheral site of the skin graft (Fig. 8C).

DISCUSSION

Because the amniotic membrane is a part of the fetal membrane and is composed of amniotic epithelium with basement membrane, mesenchymal cells, fibroblasts, and collagen tissues, not only collagen but also nucleated cells are transplanted in amniotic membrane transplantation. It is generally thought that the immunogenicity of cryopreserved tissues is less than

that of fresh tissues and that cryopreserved cells are expected to be nonviable. However, the amniotic membrane is originally fetal tissue, and some proportion of amniotic cells may still be viable, even after cryopreservation. Recently, it was reported that cryopreservation of amniotic membrane impairs cell viability and proliferative capacity.¹¹ However, our results indicate that at least 50% of amniotic epithelial cells cryopreserved for 2 months in DMSO at -80°C were still alive and had growth capacity in culture. Therefore, the immunologic characterization of these cells could not be ignored. Usually, amniotic membrane cryopreserved for less than 3 months at -80°C is

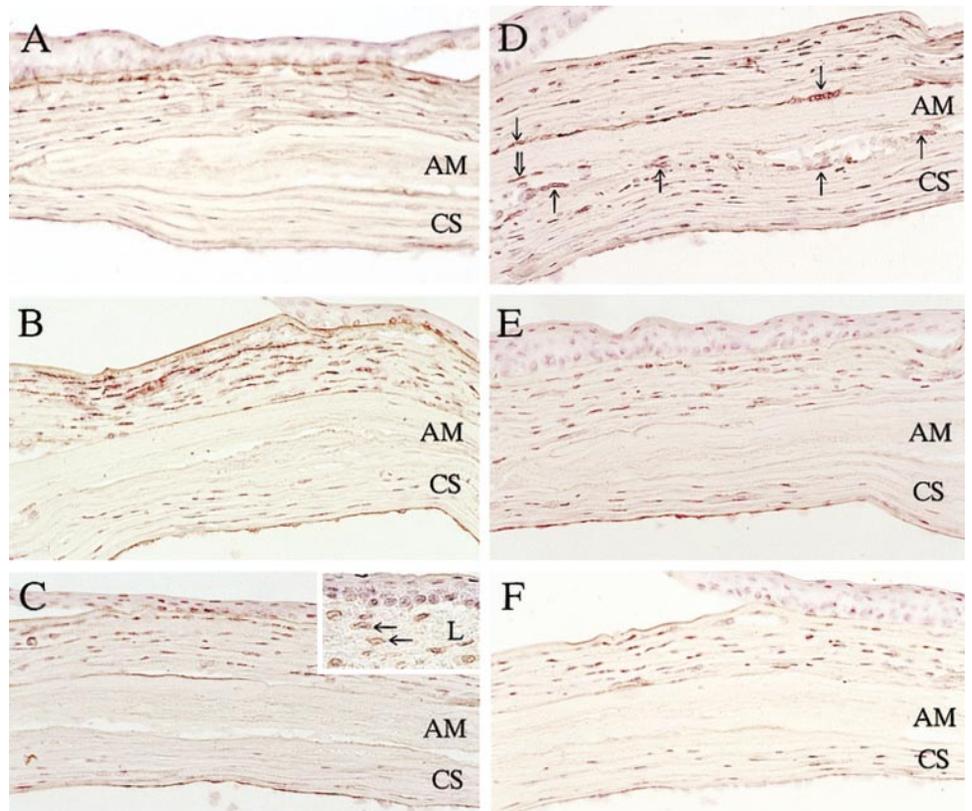


FIGURE 6. Immunohistochemical observation in intracorneal transplantation at 1 week after grafting. No rat CD4⁺ (A) or CD8⁺ (B) T cells infiltrated. (C) Rat macrophages also did not infiltrate, even if some host macrophages were present in the limbal area. (C, *inset, arrows*) Limbal macrophages in the same group. (D) Human class I-positive cells. Some positive amniotic epithelial cells were separated inside the host stroma. *Thin arrows:* human class I-positive cells. *Thick arrow:* amniotic fibroblast. (E) No human class II-positive cells were seen. (F) Control section without primary antibodies. Magnification, $\times 200$. AM, amniotic membrane; CS, corneal stroma; L, limbal area.

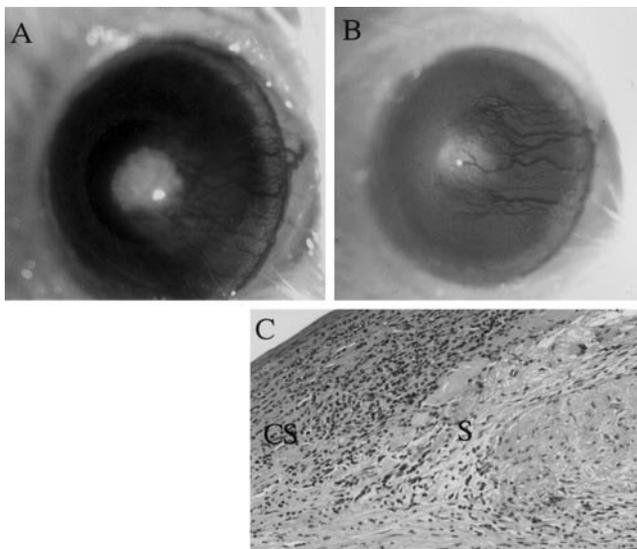


FIGURE 7. Skin graft in intracorneal transplantation. (A) New vessels approached the skin graft from the wound side at 2 weeks after grafting. (B) An intense rejection reaction was seen at 3 weeks. (C) HE staining revealed intense lymphocyte infiltration inside the host cornea and donor skin. Magnification, (C) $\times 100$. CS, corneal stroma; S, skin graft.

used for clinical cases in our institution, and in this study we used similar amniotic membrane for our experimental transplantation.

In the 1980s, it was reported that HLA-A, -B, -C, and -DR or β_2 -microglobulin were not detected in cultured amniotic epithelium⁴; however, class I antigen manifestation in amniotic epithelium was subsequently reported by several groups.^{12,13} In particular, Hammer et al.⁹ reported the comanifestation of class Ia and class Ib antigens in amniotic epithelium. Our results showed strong positive staining against anti-heavy and -light chains of class I antigen against all nucleated cells in epithelium, mesenchymal cells, and fibroblasts, indicating that cryopreserved amniotic membrane strongly expresses class I antigens, even after 6 months' cryopreservation. Based on our results concerning cell viability and immunohistochemistry, it is suggested that some proportion of cryopreserved amniotic

membrane may contain viable cells expressing especially class I antigen, if storage is less than a few months.

Next, we examined amniotic membrane immunogenicity, using limbal (presumably an immunologically unprivileged site) and intracorneal (presumably an immune-privileged site) transplantation models. Our results suggest that mild cellular infiltration was present in limbal transplantation; however, the amniotic membrane was not totally destroyed or lysed. Immunohistochemical analysis at 1 week after grafting showed that T cells (especially $CD4^+$ T cells) surrounded the transplanted amniotic membrane, indicating a mild cell-mediated reaction. A similar result was reported in murine xenogeneic responses to human testis, supporting a $CD4^+$ T-cell-mediated reaction.¹⁴ Our result may indicate that cryopreserved amniotic membrane is relatively immunogenic after discordant xenotransplantation in an immunologically unprivileged site. Because amniotic membrane expresses HLA antigens, including class Ia antigen, these antigens may be indirectly presented to T cells by host antigen-presenting cells (APCs) of the limbal area. However, all intracorneal transplanted grafts showed absolutely no immune reaction and retained complete transparency on long-term observation.

Because there are no blood or lymphatic flows inside the cornea, the intracorneal area has been considered to be an immune-privileged site. However, all cryopreserved skin tissues were rejected, even when transplanted into intracorneal spaces. Therefore, the difference in immunogenicity between amniotic membrane and skin tissue is of interest. To further determine the immunogenicity of amniotic membrane, we performed another experimental transplantation under the kidney capsule, which is an extraocular non-immune-privileged site. Only a very mild reaction was observed around the amniotic membrane, and a few host cells infiltrated the amniotic stroma. However, when cryopreserved human skin tissues were transplanted under the kidney capsule as control grafts, many host cells infiltrated the skin tissues. These results indicate that amniotic membrane may be immune-privileged tissue, clearly different from skin tissue. Skin grafts contain many donor-derived class II-positive dendritic cells (passenger leukocyte) including Langerhans' cells, but amniotic membrane has a lower number of class II-positive fibroblasts. Thus, the skin tissue may be rejected easily even when transplanted into intracorneal spaces. Recently, Hao et al.¹⁵ reported that several anti-angiogenic and inflammatory proteins were expressed in

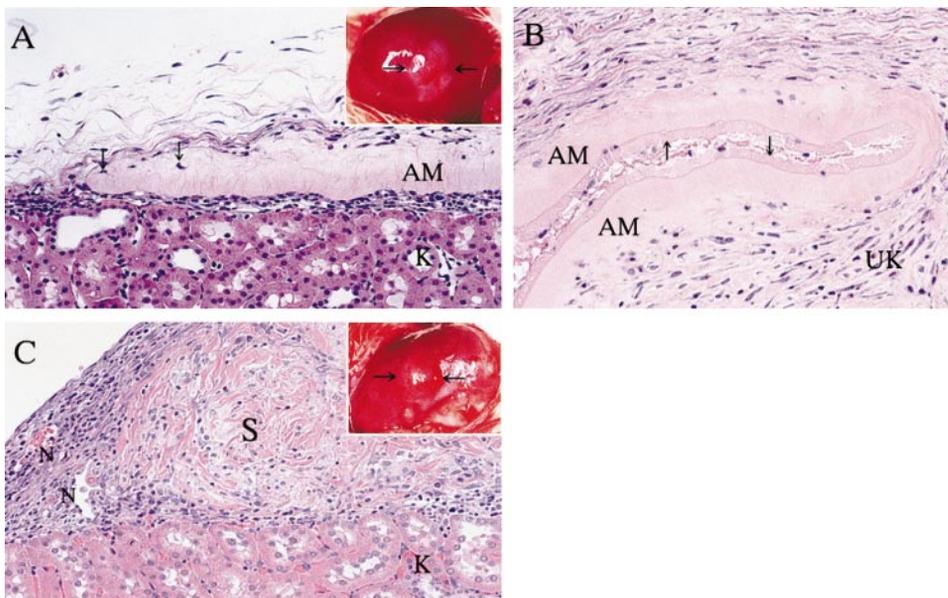


FIGURE 8. Heterotopic transplantation under the kidney capsule. (A) In HE staining, the amniotic membrane clearly maintained its general structure, and extremely few cells infiltrated the amniotic membrane. Arrows: amniotic fibroblasts. Inset, arrows: grafted amniotic membrane. (B) Folded amniotic membrane under the kidney capsule. Only a few host cells infiltrated the amniotic membrane. Arrows: amniotic epithelial cells. (C) Skin graft under the kidney capsule. Many host cells infiltrated the skin graft and some vessels invaded the peripheral site of the graft. Inset, arrows: grafted skin. Magnification, (A, C) $\times 200$; (B) $\times 400$. AM, amniotic membrane; K, kidney; UK, under the kidney capsule; S, skin graft; N, neovessel.

amniotic epithelial and mesenchymal cells, including IL-1 receptor antagonist and IL-10. Several proteins released from amniotic membrane may contribute to suppression of angiogenic or inflammatory factors in intracorneal transplantation.

It has been reported that the apoptosis of maternal lymphocytes may be mediated by Fas ligand expression in placenta.^{16,17} Generally, immune-privileged sites such as the cornea, testis, and uterus express Fas ligand, and apoptosis of host lymphocytes may be induced by Fas ligand binding.¹⁸ Recently, Fas ligand was detected in amniotic epithelial cells by immunohistochemistry.¹⁹ We detected Fas ligand-positive cells in mesenchymal cells of amniotic stroma in this study. The manifestation of the Fas ligand in mesenchymal cells may prevent lymphocyte infiltration of amniotic stroma. During pregnancy, fetus nonclassical HLA-G (class Ib antigen) are expressed exclusively in extravillous trophoblasts, interstitial trophoblasts, and endovascular trophoblasts in placenta.^{20,21} Because the HLA-G molecule has low polymorphism compared with class Ia antigen, aggression against the fetus is not easily initiated by HLA-G expression in the fetal-maternal interface. The expression of class Ib antigen (HLA-G and HLA-E) in the amniotic membrane has been reported.^{9,22} An antigen (MamuAG) similar to human HLA-G also has been detected in the amniotic membrane and testis of the monkey.²³ Moreover, soluble HLA-G is produced from amniotic epithelial cells and is contained in amniotic fluid, and soluble HLA-G molecule is thought to be an important immunosuppressive factor during pregnancy.^{10,24} HLA-G also controls lymphocyte proliferation in a mixed lymphocyte reaction.²⁵ Expression of HLA-G in amniotic membrane implies two possibilities for the host immune system. First, HLA-G may play the role of tolerogenic peptide, and the host lymphocyte or dendritic cell may be inactivated by HLA-G's binding to inhibitory receptors. Secondly, HLA-G may be recognized by certain T cells. Then, it may serve as an activator of CD8⁺ T cells, because CD8 can bind to HLA-G, and these cells may have a suppressor function.

It is interesting that in intracorneal transplantation amniotic epithelial cells, mesenchymal cells, and fibroblasts gradually disappeared, even if no rejection reaction was seen. It is not clear why amniotic cells disappeared after intracorneal transplantation without any rejection reaction. A possibility is that some amniotic cells are apoptotic cells and easily disappear under particular conditions such as transplantation. Recently, Fas-positive cells were detected in amniotic epithelial cells by immunohistochemistry, and apoptotic cells in amniotic epithelial cells were shown by the TUNEL method.²⁶ Moreover, apoptosis of amniotic cells by matrix metalloproteinase (MMP) has been reported.²⁷ It was thought that Fas signaling or MMP may play an important role in remodeling of amniotic membrane during pregnancy or in amniotic membrane rupture at birth. By such factors, the membrane-binding HLA and Fas ligand may be easily released from apoptotic amniotic cells as soluble forms. It is known that some soluble forms of HLA or Fas ligand show biologically immunosuppressive activity in vitro.²⁸ We speculate that the soluble form of amniotic antigens may protect the immunologic reaction after transplantation, even if amniotic cells disappear spontaneously.

In ophthalmology, the amniotic membrane is currently applied for reconstruction of the ocular surface environment. Several investigators have reported the intrastromal implantation of amniotic membrane.^{29,30} Our findings indicate the absence of immune response in the intracorneal model, even in discordant xenotransplantation, suggesting that amniotic membrane may be applied, not only for the ocular surface but also intracorneally, because of the similarity of the tissue. For example, amniotic membrane may be useful as a human collagen matrix for corneal stroma reconstruction after excimer laser keratectomy, including laser in situ keratomileusis

(LASIK). Recently, Park and Tseng³¹ reported that polymorphonuclear cell (PMN) infiltration and corneal keratocytes death after photorefractive keratectomy (PRK) is prevented by amniotic membrane transplantation. We observed that newly appearing host keratocytes were maintained inside grafted amniotic membrane after intracorneal transplantation and that the amniotic membrane may protect against host cell infiltration similar to a barrier membrane under the kidney capsule. Therefore, the amniotic membrane may play a role, not only as immune-privileged tissue but also as a tissue-engineered barrier.

Recently, we have used amniotic membrane transplantation for patients with Mooren ulcer. The inflammatory symptoms reduced in most cases and no recurrent ulcer was observed. Several advantages of amniotic membrane may contribute to our result in Mooren ulcer. Moreover, amniotic membrane is an interesting therapeutic material from the viewpoint of transplantation immunity. Because amniotic membrane expresses immunosuppressive factors, the cotransplantation of allogeneic or xenogeneic organs with amniotic membrane may improve the survival rate of these organs, creating a new immunosuppressive environment at the transplantation site. From the same point of view, coencapsulated islet xenografts with testis cells (Sertoli cells) have already been transplanted in diabetic mice,³² in that Sertoli cells also may produce several immunosuppressive factors, such as TGF- β or Fas ligand. The immunologic character of the amniotic membrane could be useful for new therapeutic approaches in the prevention of immunologic rejection.

In this study our first approach was to examine the immunogenicity of human cryopreserved amniotic membrane, because it is widely applied for ocular surface reconstruction in clinical ophthalmology. It is interesting how amniotic epithelial cells affect the immunoregulation after amniotic membrane transplantation; therefore, the transplantation using denuded amniotic membrane may be another approach. Moreover, analysis of alloreaction may be helpful in understanding the immunologic characterization in clinical transplantation. The establishment of an allogeneic transplantation model is under way.

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