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Heterologous Corneal Endothelial Cell Transplantation

— Human Corneal Endothelial Cell Transplantation in Lewis Rats —

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A heterologous corneal endothelial transplantation was attempted using human endothelial cells and a Lewis rat penetrating keratoplasty model. Cultured human endothelial cells were seeded to a Lewis rat cornea, which was denuded of its endothelium. When grafted into the syngeneic Lewis rat, the graft remained clear for at least five days, and then became opaque and edematous because of immune rejection reaction. In contrast, corneas denuded of their endothelium became opaque and edematous immediately after transplantation. These results demonstrate that transplanted endothelial cells have enough antigens to induce rejection reaction even though they have the functional capacity to deturge the cornea.

Key Words: *heterologous transplantation, corneal endothelium, immune rejection reaction*

INTRODUCTION

Corneal transplantation is the only means of restoring vision to eyes in which the cornea has became opacified as a result of injury, infection, or hereditary diseases. The success rate in obtaining clear transplants has been increased because of understanding of the endothelial pump function, new immunosuppressive agent, improved surgical skill and instruments, and so on. However, 6-40% of corneal transplants, still, undergo immune rejection reaction (allograft rejection). Currently, rejection reaction is a leading cause of graft failure (Alldredge and Kramer, 1981; Arentsen, 1983; Chandler and Kaufman, 1974). The immune rejection reaction is regulated by molecules of the major histocompatibility complex (MHC) (Braude and Chandler, 1983; Elliott, 1976; Foulks and Sanfilippo, 1982; Stark et al., 1978). This complex is cluster of genes coding for cell surface proteins, which are the major antigens involved in the transplantation responses. There are two main types of MHC antigens: class I antigens, which are present in most cells including corneal endothelium, keratocytes and epithelium; and class II antigens, which are present predominantly in lymphoid cells (Fujikawa et al., 1982; Mayer et al., 1983; Pels and Van der Gaag, 1984/1985; Tchah et al., 1989, Treseler et al., 1984; Whitsett and Stulting, 1984). The only corneal cell that normally expresses class II antigens is the Langerhans cell of the epithelium (Kelly et al., 1985; Mayer et al., 1983; Treseler et al., 1984; Vantrappen et al., 1985; Williams et al., 1985).

Recent evidence demonstrates that a stimulus from both class I and class II is required for the complete allograft rejection reaction. Class I corneal antigens are the major target antigens for rejection (Braude and Chandler, 1983; Elliott, 1976; Foulks and Sanfilippo, 1982; Stark et al., 1978; Treseler and Sanfilippo, 1985). The majority of these class I antigens are on the endothelial and epithelial layers. Studies have shown that rejection of each of the individual corneal layers can occur (Khodadoust and Silverstein, 1969). In addition, graft survival is much better in lamellar transplants in which just the stroma and epithelium are transplanted (Khodadoust and Silverstein, 1972). Class II antigens are identified in rejected corneal endothelium (Pepose et al., 1985) and are induced by γ-interferon (Donelly et al., 1985; Young et al., 1985) and in preservation media (Tchah et al., 1989). These indicate that the endothelial layer may be the most important layer in rejection. If the transplantation of the endothelial layer without stroma and epithelium is possible, the role of

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the endothelial layer in corneal graft rejection can be evaluated further.

In this experiment, human corneal endothelium was transplanted in rats to evaluate whether the antigens of endothelium can elicit the rejection reaction. Heterologous corneal transplantation was used to maximize MHC antigen disparity. An inbred Lewis rat penetration keratoplasty model was used to eliminate the affect of MHC antigens of the corneal epithelium and stroma on the immune rejection reaction.

MATERIALS AND METHODS

Human endothelial cell culture

Primary human endothelial cell cultures were established from the corneas of a neonate (3.5 month old). Donor eye globes were immersed in 10% povidone iodide in normal saline for three (3) minutes, followed by a one (1) minute immersion in normal saline. The globes were rinsed with 12cc of normal saline in a syringe with an 18G needle. Corneal scleral rims were removed and placed in sterile 35×15mm tissue-culture dishes (Corning Glass Works) and covered with complete culture medium. All tissue culture media were from Gibco (Life Technologies Inc.) and Sigma (Sigma Co.). The complete media contained modified Eagle's minimum essential medium with Earle's salt (Gibco), supplemented with 1.35% chondroitin sulfate (Sigma), 10% fetal bovine serum (Gibco), 1% sodium pyruvate (Sigma), 25mM HEPES (Gibco), 100 μ/ml penicillin/streptomycin (Gibco), sodium bicarbonate (Sigma), and antioxidant. Corneas were incubated for one (1) week at 37°C in 95% air-5% CO_2. Using sterile technique, the Descement's membrane and endothelium were gently microdisected, intact, from the cornea and cut into three or four pieces. Four to six pieces were then placed in one 25cm^2 culture flask (Corning) containing the culture media. Confluent endothelial culture was then subcultured with trypsinization. For trypsinization, cells in culture flasks were washed with Eagle's MEM and incubated with 1.5ml of 0.5% trypsin-EDTA solution (Gibco) at 37°C and then monitored closely with a phase contrast microscope. When they became rounded and refractile, 2-3ml of complete media were added to the flasks to stop the action of the trypsin. A one to two minutes exposure to trypsin-EDTA solution was the standard time used for most cells. The cell suspension was then placed into 15ml sterile, polystrene centrifuge tubes and spun down for ten (10) minutes at 1100 rpm. After centrifugation, the medium was aspirated off and the cells were resuspended in 10ml of complete culture media to initiate two to four subcultures per primary culture. The subcultures typically reached confluence in two to four weeks. These cells were used in the next step.

Seeding of human corneal endothelial cells to rat corneas denuded of endothelium

Female rats of the Lewis inbred strains (100-150gm) were used. The rats were killed by ether inhalation. Eyes were enucleated and decontaminated following the procedures of human cornea decontamination. Under a dissection microscope, the recipient cornea with 1 mm scleral rim was isolated and incubated for 24 hours in complete culture medium. Donor rat corneal endothelium was denuded by gentle scraping with a cotton-tipped applicator (10-15 times). Complete endothelial removal was verified by staining control corneas with Alizarin red. The cornea were rinsed in complete culture medium and placed into small polystyrene holders. These holders facilitated the seeding of donor endothelial cells by maintaining the normal shape of the rat corneas. Cultured human endothelial cells were seeded onto the denuded corneas at a cell density of 9.0×10^4 cells per ml (endothelial seeded group) The seeding procedure consisted of pippetting 50 μl of cells directly onto the denuded surface. The corneas were left undisturbed for 3-4 hours until the cells were attached. At this time, the corneas were incubated in 1ml of complete culture medium at 37°C. The medium was changed daily. Five to seven days after seeding, the corneas were transplanted.

Endothelial denuded rat corneas without human endothelial seeding were prepared (endothelial denuded group). All procedures were identical as mentioned above except for the seeding of endothelial cells. These corneas were used as a control to evaluate the function of the transplanted endothelium.

Penetrating keratoplasty procedure

Penetrating keratoplasty technique has been described previously (Tchah et al., 1991). The Lewis rats were anesthetized with intramuscular injections of ketamine (25 mg/kg) and xylazine (3 mg/kg). After anesthesia, 10% phenylnephrine and 2.5% mydriacyl were instilled in one eye of the recipient animal for maximal dilation of the pupil. The endothelial seeded donor cornea (or endothelial denuded donor cornea) was then placed on the operating tables trephined with a 3.5-mm diameter trephine on a Tefron block with the endothelium facing upward under an operating microscope. The donor tissues were covered with balanced salt solution until suture onto the recipient cornea.

With the aid of an operating microscope, a 3.0 mm

diameter trephine was used to cut the central corneal button from the recipient eye. After entering the anterior chamber, the excision was then completed with corneal scissors. The donor button was then transferred and sutured to the recipient wound with eight (8) interrupted 10-0 nylon sutures (Fig. 1). The loose ends were cut as short as possible. The cornea and lens were moistened throughout the procedure with balanced salt solution. Subconjunctival and topical gentamycin were administered at the end of the procedure. No steroids were given. The sutured were not removed postoperatively. The whole procedure is illustrated in Figure 2.

Clinical and histologic evaluation

Each animal was inspected twice a week under the operating microscope. Quantitative evaluation was based on grades of 0-4 for each of the following categories: corneal opacity (opacity grade, OG), edema (edema grade EG), and vascularization (neovascularization, NV) (Table).

The recipient animals were killed by ether inhalation three (3) weeks after the operation. The corneas of the enucleated eyes were stained using hematoxyline-eosin, as well as monoclonal antibody against hyman endothelial cells (PHM-5). The avidin-biotin-peroxidase complex method (Hsu et al., 1981) was used for this immunohistochemical staining.

Table 1. Items and Scales of Rejection Score

Grade	Opacity	Edema	Neovascularization
0	none	none	none
1	slight	slight	present but not to the suture
2	moderate (iris vessel obscured)	moderate	to the suture
3	marked (hardly visible iris)	marked	passed the suture
4	extreme (whitish opaque)	extreme	to the center

RESULTS

Endothelial seeded group (Heterologous endothelial transplantation).

Eleven (11) corneal transplantations were performed. Two (2) rats were killed during observation because of wound disruption. In nine (9) rats, mild postoperative edema (EG=1-2) was seen on the transplanted cornea immediately (Fig. 3). The transplanted cornea remained clear until day 5 (OG≤2, EG≤1, NV=0). Starting at the second week, peripheral vascularization was seen surrounding the sutures in many of the transplants (NV=1-2). At day 9, seven (7) transplants (77.8%) showed rejection reaction characterized by corneal opacity (OG=2-3) with moderate edema (EG=2) (Fig. 4). Rejection reaction became more severe at day 12-the iris vessels were not visible be-

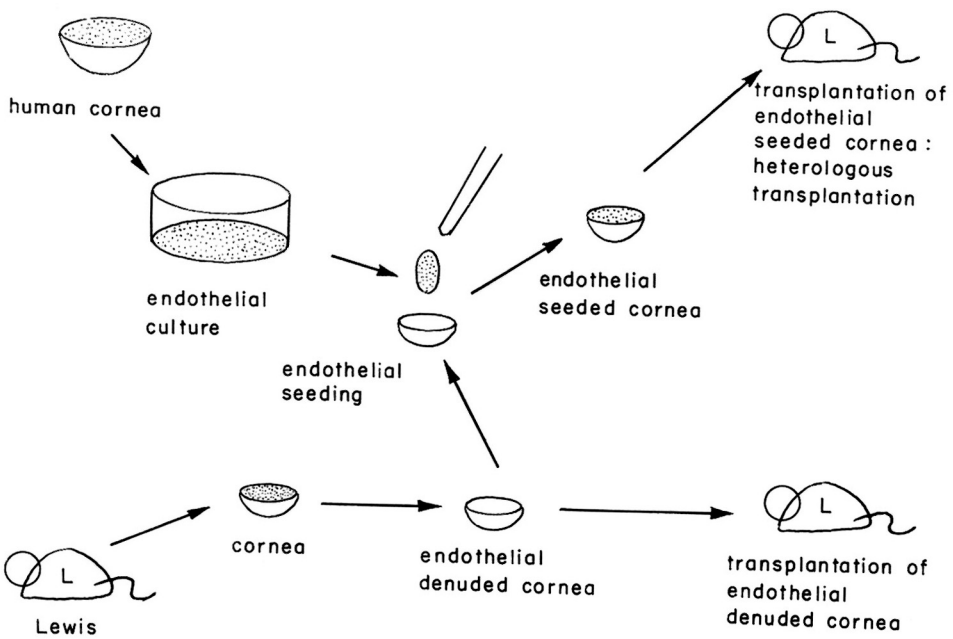

Fig. 2. Schematic diagram illustrating the procedure used for heterologous transplantation of human corneal endothelial cells.

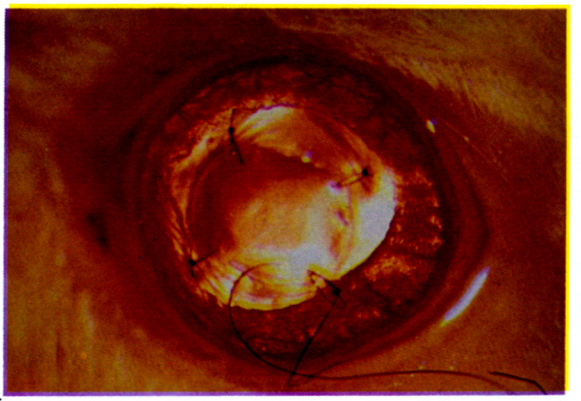

Fig. 1. Rat penetrating keratoplasty model. Good wound apposition between donor button and recipient cornea was obtained after four (4) cardinal sutures.

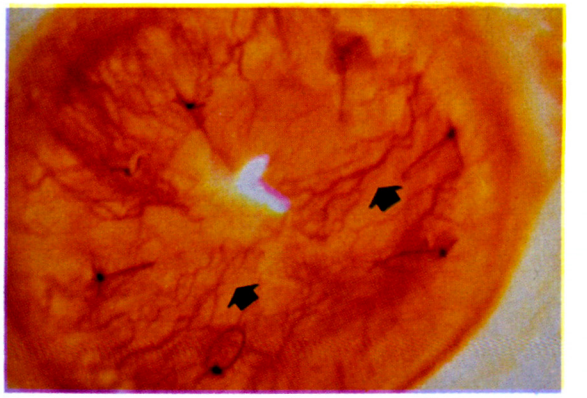

Fig. 5. Rejected corneal graft in the rat with human endothelium, 12 days after transplantation. The iris vessel was hardly visible through the opaque graft and new vessels (arrow) grew into the graft. Corneal edema was also present.

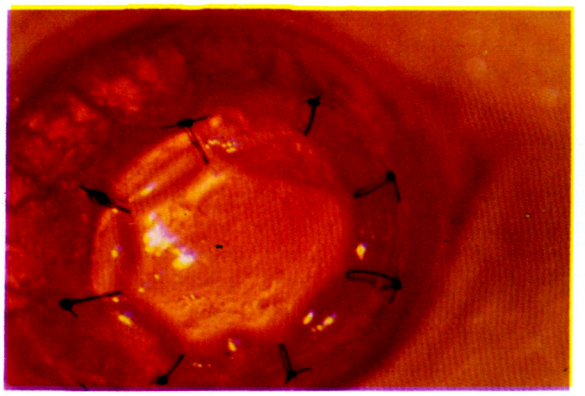

Fig. 3. Clear corneal graft in the rat with human endothelium, one (1) day after transplantation. There was only mild edema around the transplantation wound.

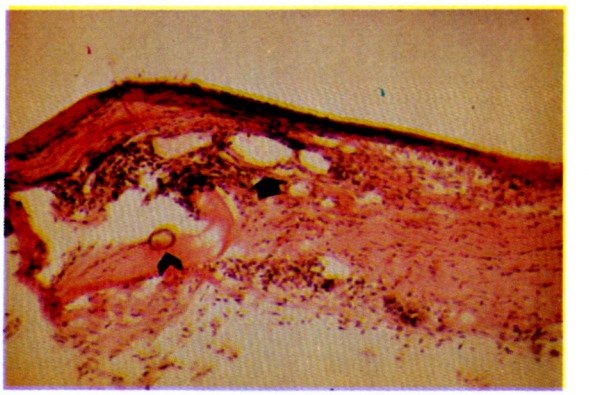

Fig. 6. Hematoxylin eosin staining of rejected corneal graft. There was diffuse infiltration of inflammatory cells and some new vessels (arrow) especially around the sutured (arrow head). Endothelial layer was not found.

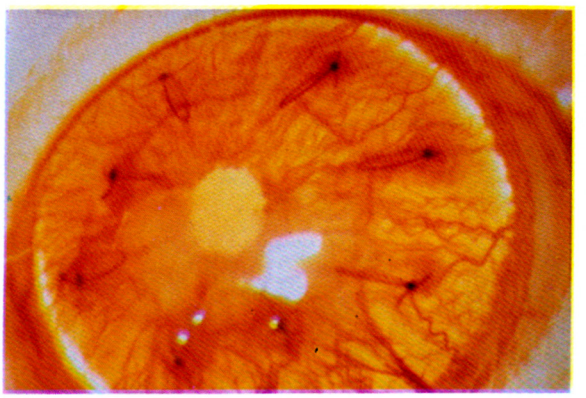

Fig. 4. Partially rejected corneal graft in the rat with human endothelium, nine (9) days after transplantation. The superior half of the graft was transparent so that the underlying iris was clearly visible, but the inferior half was opaque and edematous because of the rejection reaction.

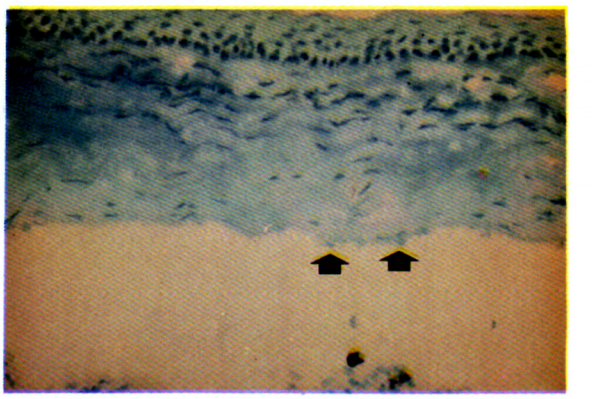

Fig. 7. Immunohistologic staining of rejected corneal graft with human endothelial marker (PHM 5). Endothelium were rarely identified (arrow) but none of them were PHM 5 positive.

cause of dense corneal opacity (OG=3-4) and edema (EG=2-4). New vessels in the cornea grew into transplant almost to the center (NV=3-4) (Fig. 5). Remaining two transplants were also rejected at day 12 (OG=3, EG=2, NV=3). All rejected corneas remained opaque (OG=3-4) by the end of third week.

During the follow-up period, epithelial defect had developed in five (5) eyes, but had spontaneously healed within 3-5 days. Wound disruption occurred in two (2) eyes (excluded from this study). In two eyes, there was a 20-30% hyphema, which was resorbed within 3-4 days.

Examination by light microscope at the end of the third week revealed heavy infiltration of inflammatory cells especially around sutures and neovascularization in rejected transplants (Fig. 6). Endothelial cells were almost destroyed; the remaining few endothelial cells were identified as rat endothelial cells (PHM5 negative) (Fig. 7).

Endothelial denuded group

All eight (8) transplants showed severe corneal edema (EG=2-3) immediately after transplantation. The transplanted cornea became opaque (OG=2-3) two days after the operation (Fig. 8). Two transplants showed temporarily clear at day 6 but again became opaque. All transplants remained opaque during the follow-up period. New vessels began to grow into the transplants at the second week, finally reaching the center at the end of the third week. Epithelial defect which developed in four (4) eyes was spontaneously healed in 2-3 days.

Examination by light microscopy revealed severe infiltration of inflammatory cells in transplants. There were no endothelial cells.

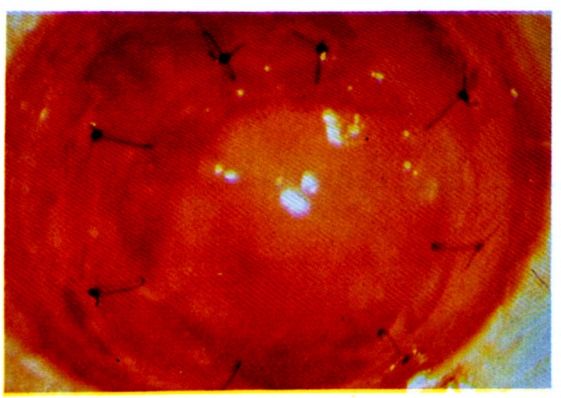

Fig. 8. Primary donor failure of corneal graft in the rat with endothelial denuded graft. two days after transplantation. The cornea became edematous and opaque just after transplantation.

DISCUSSION

It has been demonstrated that cultured, homologous or heterologous, corneal endothelial cells seeded onto the denuded surface of donor corneas will rearrange into monolayer (Gospodarowicz and Greenburg, 1979). Corneas with heterologous endothelial cells which were transplanted in rabbits and cats remained clear prior to rejection (Gospodarowicz et al., 1979; Insler and Lopez, 1991). In these studies, however, inbred animals were not used so the contribution of MHC antigens of corneal epithelium and stroma on the immune rejection reaction was not excluded.

Recent studies have demonstrated the efficacy of the rat penetrating keratoplasty model for studying corneal graft rejection reaction (Moran et al., 1988; Tchah et al., 1991). The rat, unlike rabbits and cats, has inbred lines and its MHC antigens are identified. All isografts remained clear after transplantation. In contrast, homografts with both MHC class I and II antigens disparity were rejected in 84.6% and the result was reproducible (Tchah et al., 1991). This rat model was used in this study.

Lewis rat corneas with human endothelial cells were transplanted to the syngeneic Lewis rat. There was no discrepancy in MHC antigens of the stromal and epithelial cells between the donors and the recipients. Therefore, the immune rejection reaction after such transplantation was induced solely by antigens of transplanted endothelial cells. This suggests that antigens of corneal endothelial cells are strong enough to sensitize a host immune system. There was a possibility of shedding of endothelial cells into the anterior chamber after transplantation and access to the host vascular system directly. But a large amount of endothelial shedding is less likely because of firm attachment to the Descetmet's membrane (Gospodarowicz and Greenburg, 1979)

This study demonstrates that heterologous corneal endothelial transplantation induces immune rejection reaction. But, homologous corneal endothelial transplantation may not induce rejection because antigenic challenge in homologous transplantation is thought be lesser than in heterologous transplantation. This needs further evaluation. However, it is emphasized that a single layer of endothelial cells (less than 2% of total cornea thickness) induced immune rejection reaction. Corneal endothelium can be a major source and target of immune rejection reaction when the cornea is transplanted.

Seeded human endothelial cells were not found in the rejected cornea. Certainly, these cells were the

primary target in the rejection reaction. Maintaining a clear graft for five (5) days after transplantation revealed that the pump function of transplanted human endothelial cells was good enough to deturge the cornea.

One disadvantage of the rat model is that rat endothelium has regenerative power unlike human endothelium (Tuft et at., 1986). This means that rejected endothelium can be replaced by regenerated recipient endothelium. A few rat endothelial cells were identified in a rejected cornea. Eight corneas, however, denuded of endothelium failed to clear after transplantation indicating the limited capacity for endothelial mitosis within three weeks.

In summary, current research indicates that human corneal endothelial cells transplanted to Lewis rats can induce immune rejection reaction, even though they function normally to maintain corneal clarity immediately after transplantation.

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