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BASIC SCIENCE

Establishment of a murine model of chronic corneal allograft dysfunction

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Abstract
Background To establish a murine model of chronic corneal allograft dysfunction (CCAD) that permits molecular evaluation of chronic allograft dysfunction after corneal transplantation.
Methods C57BL/6 (allogeneic), CB6F1 (semiallogeneic) and BALB/c (syngeneic) corneal grafts were transplanted orthotopically to BALB/c recipients and to the BALB/c mice as a control group. The follow-up time was more than 100 days. Graft survival time was monitored. Corneal endothelium was examined by alizarin red and PI/Hoechst stain. $CD4^+$ and $CD8^+$ T lymphocytes were examined by immunohistochemistry. Ultra-structure changes of the grafts were examined by electron microscopy.
Results Median graft survival times were 17 days, 85.5 days, and >100 days in allogeneic, semiallogeneic, and syngeneic groups respectively. Acute rejection episodes were observed only in the allogeneic group. A large amount of $CD4^+$ and $CD8^+$ T lymphocyte infiltration was present in allografts only in the allogeneic group, and few $CD4^+$ and $CD8^+$ T lymphocytes were observed in grafts in other groups. Large amounts of apoptotic and necrotic cells could be seen in the allogeneic group. Endothelial cell density decreased, and few apoptotic cells could be detected in semiallogeneic and syngeneic groups. No apoptotic or necrotic endothelial cells were found in the control group. Ultra-structural characteristic changes mainly included fibrosis formation, endothelium atrophy, and degeneration in failed grafts among transplant groups, as determined by electron microscopy.
Conclusions The changes in semiallogeneic and syngeneic groups after transplantation were similar to those observed during clinical study. Although differences between mouse strains and clinical situations remain to be explored, this murine model provides the basis for understanding the pathogenesis of CCAD.

Keywords Corneal transplantation · Immune rejection · Allograft dysfunction · Model · Mice

Introduction

With the development of matching techniques and the application of new immunosuppressive drugs, acute immune rejection episodes after organ and tissue transplantation have become less frequent. Chronic allograft dysfunction has become the predominant cause of late graft failure [1].

Corneal transplantation has a long history of more than 100 years [2, 3]. Microscope technology has greatly improved the success rate of corneal transplantation [4]. Clinical application of immunosuppressive agents, such as cyclosporin A and FK506, has significantly reduced the frequency of acute rejection [5–12]. The incidence of postoperative immune rejection is reported to range from 9.6%–21% in non-high-risk keratoplasty [13–15]. In 51 to 89 percent of the patients that rejected their transplants, the rejection was reversed after routine immunosuppressant treatment [13, 14, 16–18].

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Even so, the long-term survival rate of corneal grafts is still unsatisfactory. With respect to solid tissue allografts in humans, corneal allograft appears to be very successful, with an overall first-year survival rate as high as 90%. Unfortunately, the overall success rate diminishes to 74% at 5 years and 50% at 10 years [19, 20]. Of greater concern is that the long-term survival rate has not improved over the past decade [21].

Clinically, we found that the corneal graft displayed gradual function deterioration for months to years after transplantation. That is, with no history of rejection, the corneal graft eventually became opaque and unresponsive to corticosteroids. This behavior has been attributed to progressive chronic corneal allograft dysfunction (CCAD) [22, 23], which may represent the leading cause of poor long-term survival rates after keratoplasty.

To date, few studies have investigated CCAD. The reasons may be as follows: 1) lack of allograft involvement in clinical dysfunction, and 2) lack of an ideal CCAD animal model. So, the focus of this study was to develop a model of CCAD that would permit molecular evaluation of chronic allograft dysfunction after corneal transplantation.

In this study, BALB/c and C57BL/6 were bred in our colony to produce semiallogeneic F1 hybrids (CB6F1). Firstly, we used these three strains of mice with gradually weakened immune systems for corneal transplantation. Because alloantigen-specific and non-alloantigen-specific factors both contribute to the development of CCAD, the CCAD model should be conducive to studying both modes of transplantation. This rationale was the theoretical foundation for establishing the CCAD model.

Materials and methods

Animals

Adult BALB/c (H-2d) mice and C57BL/6 (H-2b) mice were obtained from the Institute of Zoology Chinese Academy of Sciences. We also used semiallogeneic CB6F1 (H-b/d), the F1 hybrid of BALB/c (female), and C57BL/6 (male) mice (Fig. 1). All animals were 8 to 12 weeks of age. This study was approved by the Institutional Animal Care Committee, and all procedures were performed according to the Association for Research and Vision in Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Groups

Seventy-two BLAB/c mice were randomly divided into four groups (18 mice per group): (1) group A, an allogeneic group in which corneal grafts of C57BL/6 were transplanted into BALB/c recipients, (2) group B, a semiallogeneic group in which corneal grafts of CB6F1 were transplanted into BALB/c recipients, (3) group C, a syngeneic group in which corneal grafts of BALB/c were transplanted into BALB/c recipients, and (4) group D, a control group, normal BALB/c mice that did not undergo corneal transplantation.

Fig. 1 Semiallogeneic CB6F1 (*left*) mice were obtained from a hybrid of female BALB/c (*middle*) and male C57BL/6 (*right*)

Surgical technique

Full-thickness orthotopic penetrating keratoplasty was performed as previously described, with a few modifications [24]. Mice were anesthetized systemically with an intraperitoneal (IP) injection of 1.33 mg/kg ketamine HCl and 6.68 mg/kg xylazine. Proparacaine HCl ophthalmic solution USP (0.5%; Alcon Laboratories, Fort Worth, TX, USA) was used as a topical anesthetic. Donor grafts and recipient graft beds were scored with 2.5- and 2.0-mm trephines respectively, and the corneas were excised with Vannas scissors. Donor grafts were sewn into place with eight interrupted 11-0 nylon sutures (Mani, Tochigi, Japan), and sutures were removed on day 7 after transplantation. Tobramycin ointment (Alcon, Fort Worth, TX, USA) was applied for 7 days post-operation; immunosuppressive drugs were not used, either topically or systemically.

Clinical evaluation of grafted corneas

Corneal grafts were examined two times once a week with a slit-lamp biomicroscope (Haag-Streit model BQ-900, Switzerland). Graft opacity was scored using a scale of one to five, as previously described [24]. The scoring system used was as follows: 0, clear; 1, minimal, superficial (non-stromal) opacity, pupil margin and iris vessels readily visible through the cornea; 2, minimal, deep (stromal) opacity, pupil margins and iris vessels visible; 3, moderate

stromal opacity, only pupil margin visible; 4, intense stromal opacity, only a portion of pupil margin visible; and 5, maximum stromal opacity, anterior chamber not visible. Corneal grafts were considered as failures after receiving two successive scores of 3. All examinations were performed by two masked observers.

Evaluation of the corneal endothelium

The corneas were collected at 1, 2 and 3 months, and the corneal endothelium was examined by alizarin red and PI/Hoechst stain (Beyotime Institute of Biotechnology, Shanghai, China). Then 1% alizarin red was added to the cornea, which was incubated for 2 minutes at room temperature and then washed three times in normal saline. Then PI/Hoechst33342 was added to the cornea, and incubated for 15–20 minutes at 4°C in the dark. Endothelial cells were observed using a Fluorescence E800 microscope (Nikon, Tokyo, Japan).

Immunohistochemical evaluation of grafted corneas

Allografts from the transplanted groups were collected at 1, 2 and 3 months. The grafts were studied using standard immunohistochemical methods; 6–8-micrometer serial frozen sections of each eye were prepared for immunohistological examination. The tissue specimens were subjected to fluorescent immunohistochemistry for detection of $CD4^+$ and $CD8^+$ T lymphocytes. The primary monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the secondary antibody, fluorescently-conjugated goat anti-rat (mouse) immunoglobulin G, was obtained from Boster Biotechnology (Boster Biotech, Wuhan, China). Primary antibody was omitted in some sections to verify that staining was specific. A Fluorescence E800 microscope (Nikon, Tokyo, Japan) with an attached camera was used to examine the slides and take pictures.

Electron microscopy

The corneas in each group were cut into pieces and flooded with 4% glutaraldehyde in 0.05 M cacodylate buffer for 1 h, post-fixed in 1% osmium tetroxide in veronal acetate buffer for 1 h, and washed overnight with 0.05 M cacodylate buffer containing 0.22 M sucrose. The fixed materials were dehydrated through a series of ethanols. The corneas were placed in amyl acetate. Specimens for transmission electron microscopy (TEM) were stained with 2% uranyl acetate and processed in Spurr's resin. Ultra-thin sections (70 nm) were stained with lead citrate and examined with JEOL JEM-1200 (Tokyo, Japan) electron microscopes.

Statistical analysis

Data were analyzed by SPSS 11.5 software. The Kaplan–Meier method was used to compare the survival curves of the allografts. The log-rank test was used to compare the survival curves between groups. The Mann–Whitney test was used to compare the median survival times between groups. In each case, $P < 0.05$ was considered to be significant.

Results

Clinical evaluation of grafted corneas

Fifty-four BALB/c mice underwent PK surgery; fifty of these were successful. Therefore the success rate was 92.6%. Four failed mice were excluded from the study.

Following the surgery, mild corneal edema and neovascular invasion were observed in all animals. This edema and neovascularization disappeared or lessened within 7–14 days after surgery (Fig. 2a1–d1). At 2–4 weeks post-operation, the graft became opaque, and edema with a large amount of neovascular invasion was observed in the allogeneic group (Fig. 2a2), while corneal neovascular regression and graft clarification were observed in the semiallogeneic and syngeneic groups (Fig. 2b2, c2). At 1–2 months post operation, The grafts were still opaque with subepithelial bullae, and the intensity of neovascular hyperemia was reduced in the allogeneic group. Grafts remained transparent except in six mice, in which we observed stage 1–2 opacity with no hyperemia or inflammation in the anterior chamber in the semiallogeneic and syngeneic groups (Fig. 2b3, c3). At 2–3 months post-operation, the graft was reduced in size but still opaque in the allogeneic group (Fig. 2a4); in thirteen mice in the semiallogeneic group, we observed 2–4 degree graft opacity with no hyperemia (Fig. 2b4). In the syngeneic group, one mouse displayed degree 3 graft opacity, and four mice displayed 1–2 degree graft opacity (Fig. 2c4). Corneas in the control group remained transparent during the follow-up period (Fig. 2d1–4). The median survival times in the four groups were 17.0±5.7, 85.5±16.3, 100±3.8 and >100 d±0 days (P=0.000) respectively (Table 1). The results related to graft survival functions are shown in Fig. 3.

Evaluation of corneal endothelium

The endothelial cell borders of corneal grafts in the allogeneic group were not clear, and could not be counted (Fig. 4a1). PI/Hoechst staining showed that the nucleus was uneven, with many apoptotic (Fig. 4a2) and

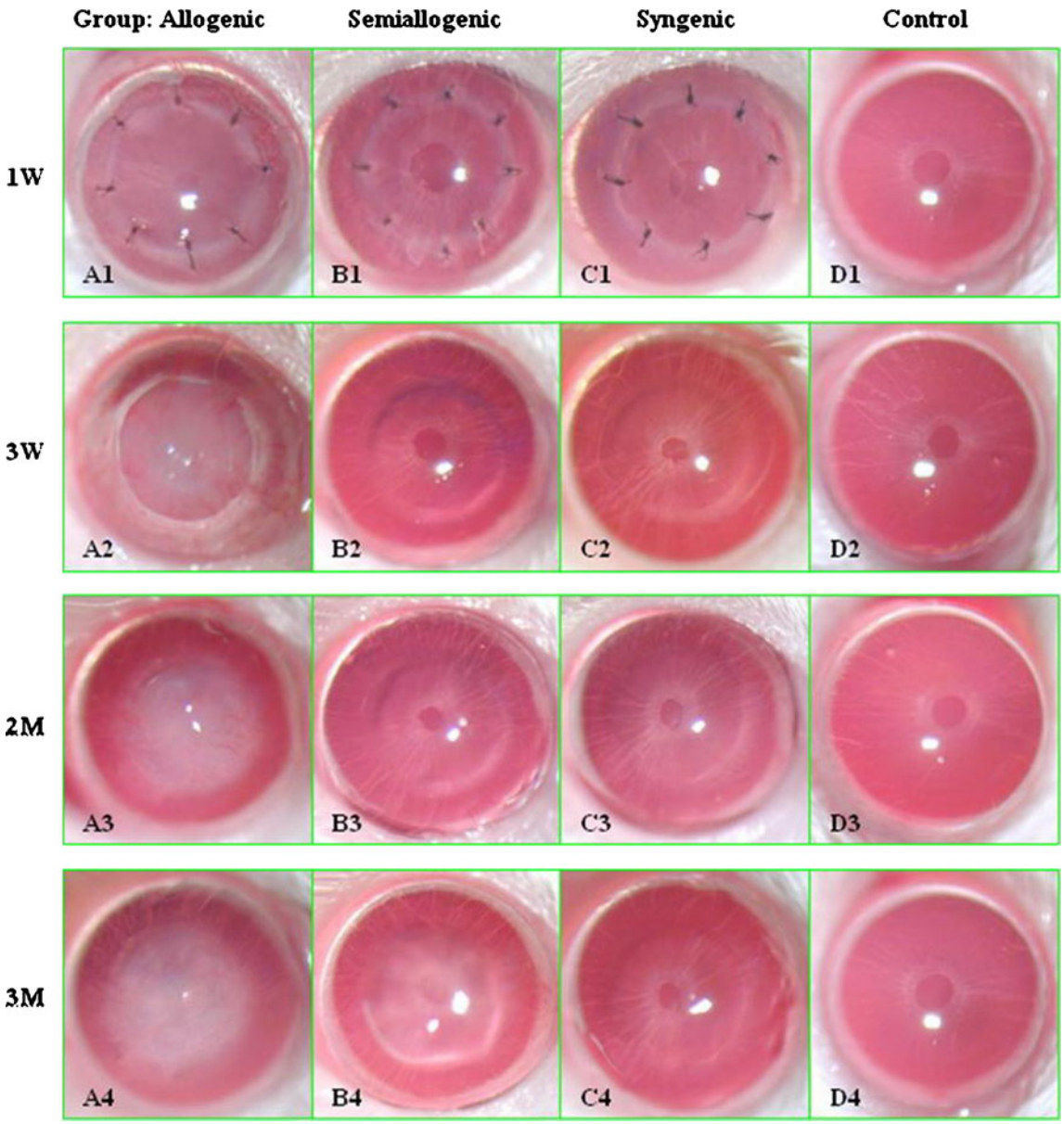

Fig. 2 Clinical evaluation of grafted corneas in the four groups. The graft was subjected to a typical clinical immune rejection episode and became opaque and edematous in the allogeneic group, while the grafts in the semiallogeneic and syngeneic groups both displayed similar clinical manifestations of chronic dysfunction without clinical episodes of immune rejection

necrotic cells (Fig. 4a3) in the allogeneic group. The number of endothelial cells in the semiallogeneic group decreased, and the normal hexagonal structure was lost (Fig. 4b1). Few apoptotic cells could be detected, and no necrotic cells were present in the semiallogeneic group (Fig. 4b2–3). In the syngeneic group, the number of endothelial cells also decreased, and the original hexagonal structure was maintained (Fig. 4c1). PI/Hoechst

Table 1 Graft survival time in all four groups after PK. Survival time differed significantly among groups (F=344.038, P=0.000), except for group C compared with group D

*the quantity of mice that had chronic allograft dysfunction.

Group	Graft survival times (days)	median
Allogeneic	10, 11, 12 (2*), 13, 14 (2*), 17 (3*), 21 (4*), 24 (3*), 28	17
Semiallogeneic	49, 56, 63 (2*), 70, 80, 84 (2*), 90 (2*), 94, >100 (5*)	85.5
Syngeneic	84, >100 (17*)	>100
Control	>100 (18*)	>100

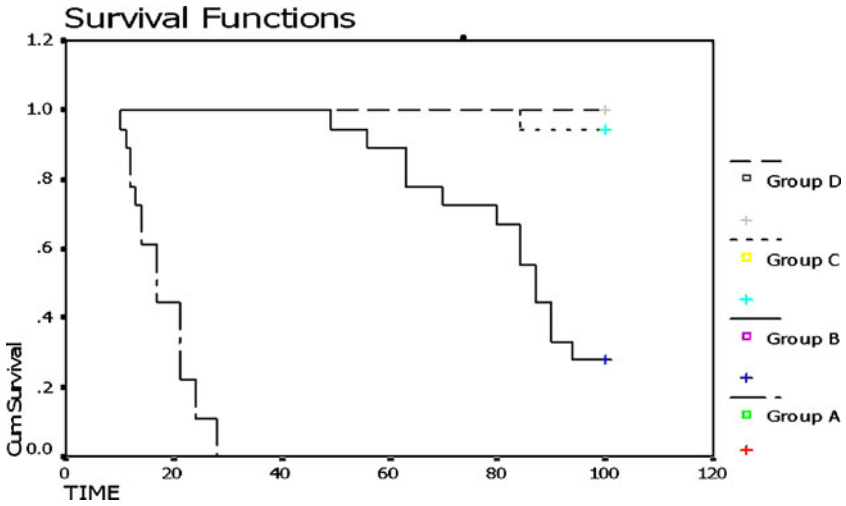

Fig. 3 Graft survival curve for each of the four groups. All grafts in the allogeneic group failed within 3 weeks. In contrast, only some of the grafts in the semiallogeneic and syngeneic groups failed gradually

staining also showed that few apoptotic cells could be detected, and no necrotic cells were present in the syngeneic group (Fig. 4c2–3). Alizarin Red staining of the normal control group showed cells with a normal hexagonal structure; no apoptotic cells or necrotic cells were detected (Fig. 4d1–3).

Immunohistochemistry

Three weeks after transplantation, a large amount of $CD4^+$ (Fig. 5a1) and $CD8^+$ (Fig. 6a1) T lymphocyte infiltration was present in the stroma of the grafts of the allogeneic group. In the semiallogeneic and syngeneic group, little

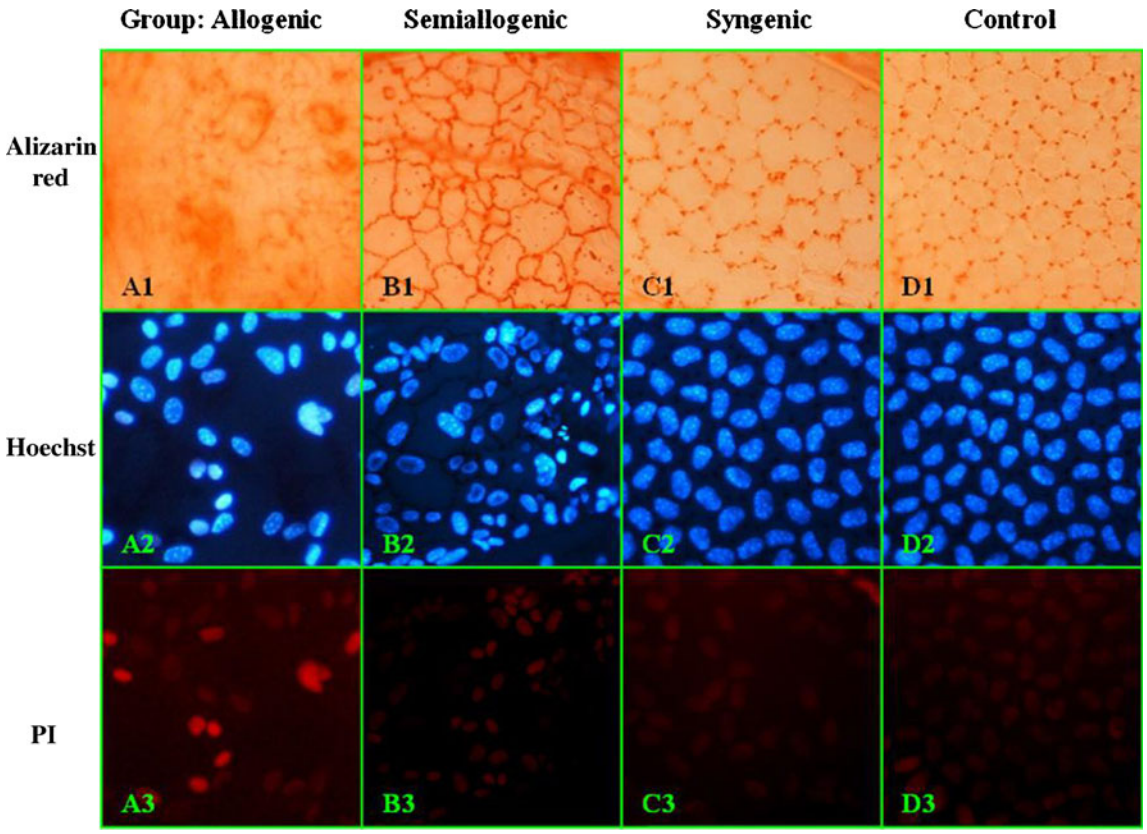

Fig. 4 Endothelial evaluation of grafted corneas in the four groups. PI/Hoechst staining showed many apoptotic and necrotic cells in the allogeneic group. Few apoptotic cells could be detected, and no necrotic cells were present in the semiallogeneic and syngeneic groups. Alizarin Red staining showed that the endothelial cell density decreased in all transplanted groups as well as in the control group

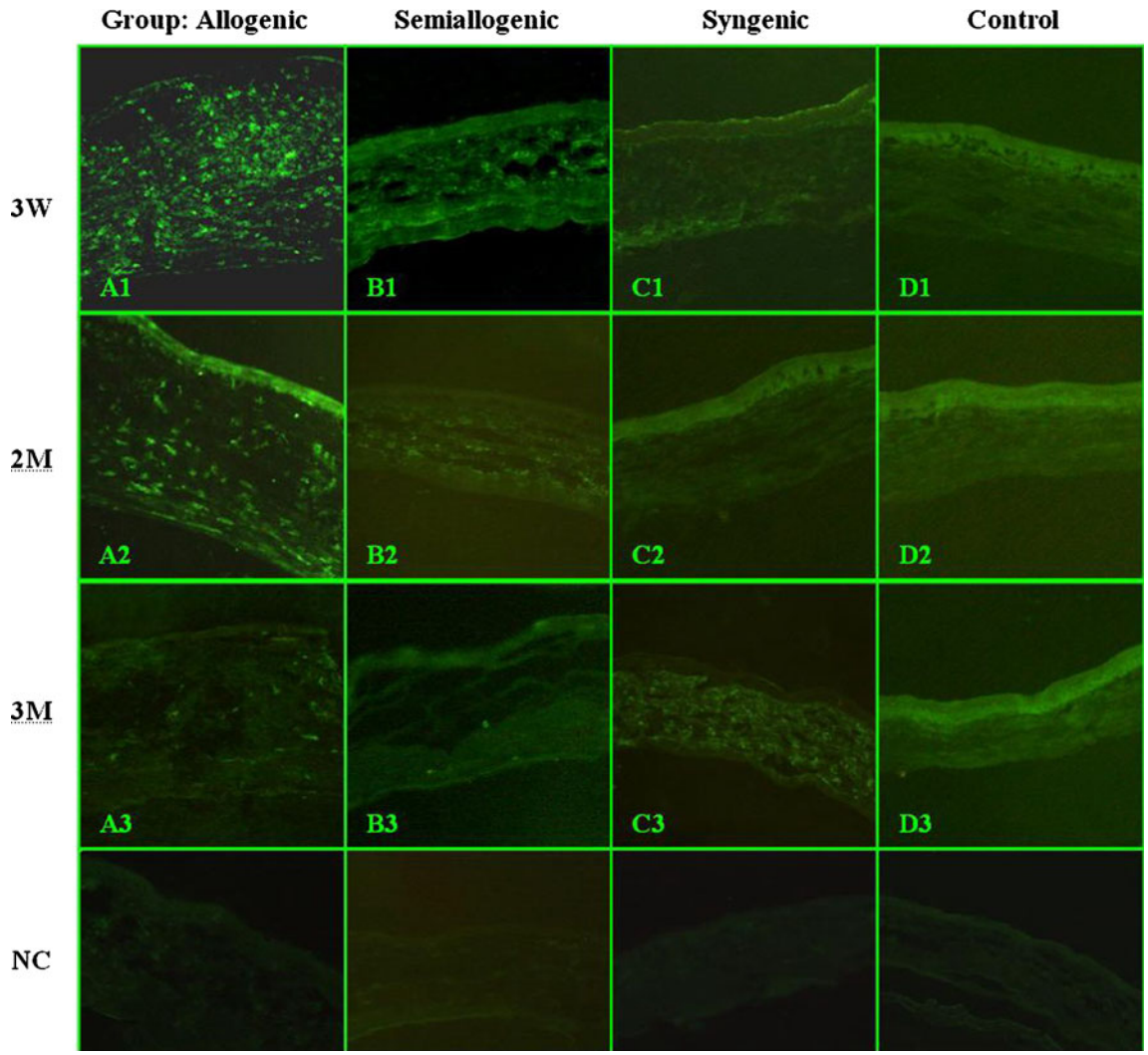

Fig. 5 $CD4^+$ evaluation using immunohistochemistry on grafted corneas from all four groups. A large amount of $CD4^+$ T lymphocyte infiltration was observed in the stroma of the grafts in the allogeneic group. Low levels of $CD4^+$ T lymphocyte infiltration were observed in the semiallogeneic and syngeneic groups. *NC*: negative control

$CD4^+$ and $CD8^+$ T lymphocyte infiltration was detected (Fig. 5b1, C1, 6b1 and c1). High levels of $CD4^+$ and $CD8^+$ T lymphocyte infiltration were observed (Fig. 5a2) in the allogeneic group at 2 months (Fig. 5a2). $CD4^+$ and $CD8^+$ T lymphocyte infiltration was barely detected at 3 months (Fig. 5a3). Extremely low levels of $CD4^+$ and $CD8^+$ T lymphocytes were observed at 2 and 3 months in the semiallogeneic and syngeneic groups (Fig. 5b2–b3, 6b2–b3, 5c2–c3 and 6c2–c3); $CD4^+$ and $CD8^+$ T lymphocyte infiltration was not observed in the control group (Fig. 5d1–d3 and 6d1–d3).

Electron microscopy

In the control group, the corneal stroma was arranged in an orderly fashion, without the invasion of inflammatory cells; Descemet's membrane thickness was even; the endothelial cells contained abundant cell organelles (Fig. 7d). Compared with the control group, corneal stroma fibers were arranged in a disorderly fashion in the allogeneic group (Fig. 7a). Furthermore, large amounts of inflammatory cells invaded the stroma in the allogeneic group. However, in the semiallogeneic and syngeneic groups, there was no clear invasion of inflammatory cells in the stroma (Fig. 7b, c), and Descemet's membrane was uneven. Fibrotic cells could be seen between Descemet's membrane and the endothelium in allogeneic and semiallogeneic groups, but were not found in the syngeneic group.

Discussion

The most successful study of chronic dysfunction after solid organ or tissue transplantation focused on chronic

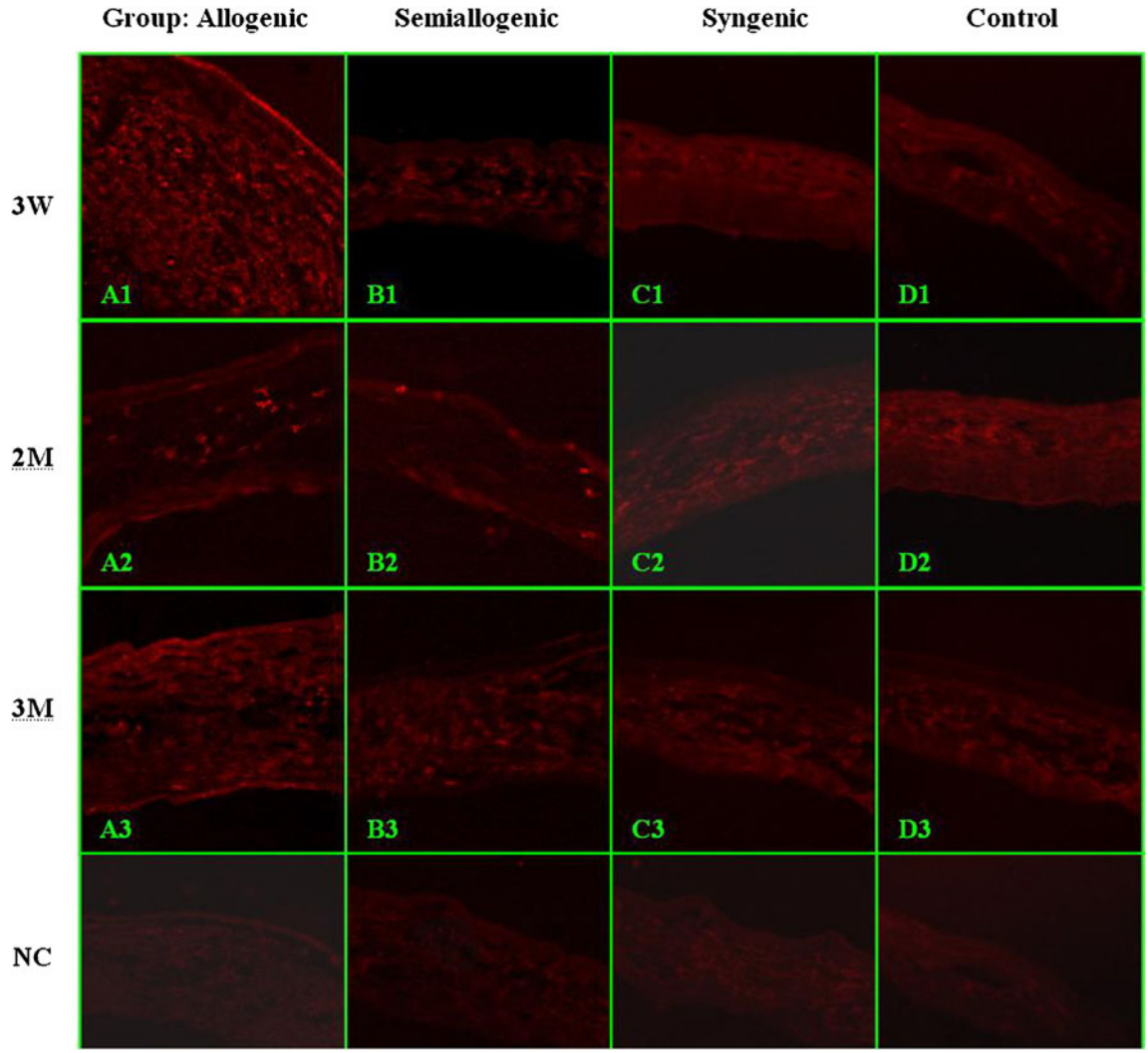

Fig. 6 $CD8^+$ evaluation using immunohistochemistry on the grafted corneas from all four groups. In the allogeneic group, a large amount of $CD8^+$ T lymphocyte infiltration was observed. In the semi-allogeneic and syngeneic groups, low levels of $CD8^+$ T lymphocyte infiltration was detected. *NC*: negative control

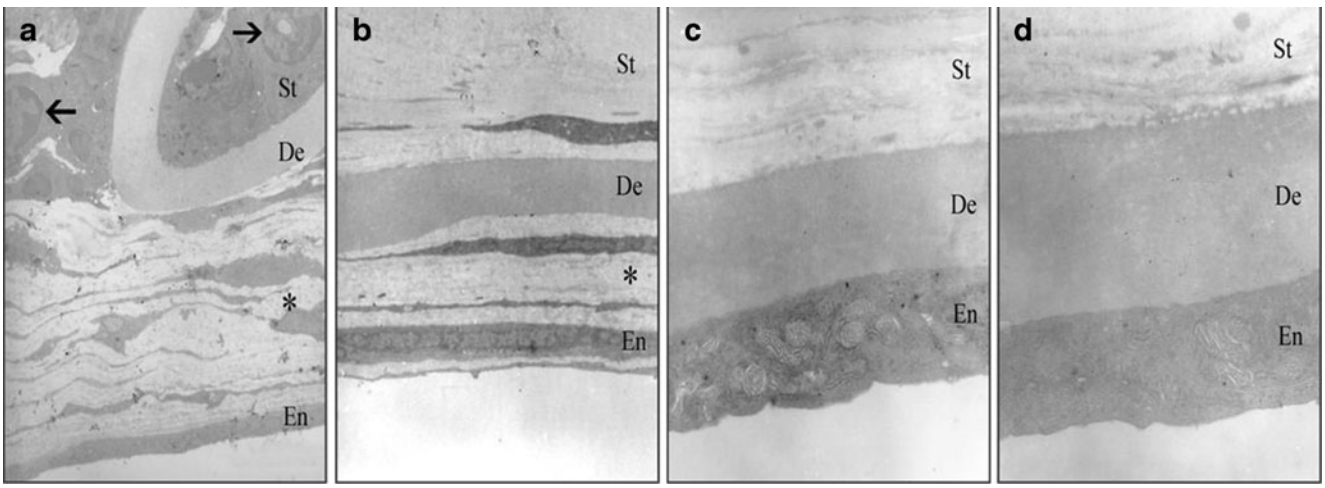

Fig. 7 Ultra-structural examination of all four groups. Compared with the control group, the thickness of Descemet's membrane is uneven. Abnormal lacunae can be seen between Descemet's membrane and the endothelium in the CCAD group. Fibrosis (*star*) is observed between Descemet's membrane (*De*) and the endothelium (original enlargement 1,500). *St*: stroma; *De*: Descemet's membrane; *En*: endothelium

renal allograft dysfunction (CRAD). The CRAD model was established using Fischer 344 (F-344 RT11V1) rat kidney transplanted into Lewis (LEW RT1.1) rats [25–27]. This model had already been applied extensively to study the mechanism underlying CRAD [28–30]. We obtained semi-allogeneic CB6F1 (BALB/c × C57BL/6) mice (H-b/d) from matching female BALB/c (H-2d) and male C57BL/6 mice. Using these three strains of mice, we ultimately obtained a transplantation model with weakened immune function. Both alloantigen-specific and non-alloantigen-specific factors contribute to the development of chronic allograft dysfunction, so in this study the CCAD model should be functional after transplantation.

According to the definition of CCAD [22], the CCAD animal model needs to meet the following conditions: 1) corneal graft displaying gradual deterioration of function after transplantation, eventually becoming opaque, with corneal opacity that was not caused by acute rejection or other disease (e.g., infection), and 2) opaque graft with characteristic histological features. Therefore, we sought to use the CCAD model to investigate the transplantation groups.

The clinical features of acute immune rejection included conjunctival congestion, topical graft edema, opacity, and vascularization during the initial stages. If there was no treatment at this time, the entire corneal graft became edematous and opaque 2 or 3 days later [31, 32]. In this study, acute immune rejection was only detected clinically in the allogeneic group. Although partial grafts became opaque in semiallogeneic and syngeneic groups, we did not find clinical evidence of acute rejection. However, the grafts in the two groups both displayed similar clinical manifestations of chronic dysfunction: the grafts displayed gradual deterioration of function, eventually becoming opaque.

The results of the immunological examination underscored the conclusions afforded by our clinical observations. It has been proven that acute immune rejection is mainly mediated by the cellular immune response after organ or tissue transplantation, and that grafts are infiltrated with large numbers of $CD4^+$ and $CD8^+$ T lymphocytes when acute immune rejection occurs [32, 33], In our study, large numbers of $CD4^+$ and $CD8^+$ T lymphocytes were only found in allogeneic group grafts. Therefore, the main reason for graft opacity in the allogeneic group was acute immune rejection. $CD4^+$ and $CD8^+$ T lymphocytes were not seen or were seen occasionally in the semiallogeneic and syngeneic groups, even when grafts became opaque. Therefore, we can conclude that the graft opacity observed in the semiallogeneic and syngeneic groups was not caused directly by acute immune rejection.

A previous microstructural study in our laboratory revealed certain ultra-structural changes in the failed CCAD grafts—atrophic changes of the endothelium and fibrosis without the infiltration of inflammatory cells [22]. In this study, we found that there were large amounts of inflammatory cells in the stroma, accompanied by cell necrosis and fiber formation when graft opacity was present in the allogeneic group. However, inflammatory cells were not observed in other groups. The cell necrosis and immune cell formation suggest that graft opacity was caused by acute immune rejection. The cell atrophy and fiber formation found in the semiallogeneic and syngeneic groups were similar to the clinical findings observed in grafts after CCAD. These results indicated that the grafts in the two groups underwent similar pathological damage during the course of CCAD.

Clinical studies found that the speed of endothelial cell decline was faster than that observed in the physiological situation, even in one free of immune rejection [34, 35]. In this study, we also found that the speed of endothelial cell decline in transplanted groups was faster than that in the control group. Endothelial cell death in the allogeneic group was caused mainly by necrosis. In the semiallogeneic or syngeneic group, the main reason for endothelial cell death was apoptosis. This finding implied that although grafts in the semiallogeneic and syngeneic groups did not suffer acute immune rejection, the endothelium was in an unsteady state. The cell count dropped gradually, which might eventually lead to chronic allograft dysfunction.

Clinical and laboratory examination did not provide evidence of acute immune rejection during the period of gradual graft failure in the semiallogeneic and syngeneic groups. Microstructural examination found similar ultra-structure changes among various CCAD grafts. Therefore, semiallogeneic and syngeneic groups are suitable as animal models for CCAD study. The former could be used mainly for studying the influence of alloantigen-specific factors on CCAD, and the latter could be used mainly for studying the influence of non-alloantigen-specific factors on CCAD. Although differences between mouse strains and clinical situations remain to be explored, this murine model provides the basis for understanding the pathogenesis of CCAD.

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