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Xenotransplantation of Retinal Pigment Epithelial Cells Into RCS Rats

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Purpose: Successful engraftment of retinal pigment epithelial cells (RPE) to treat RPE-related retinopathy will depend, at least in part, on controlling the immune response. In order to understand this process we evaluated the fate of RPE xenografts in the subretinal space, anterior chamber, and subcutis of nonimmunosuppressed Royal College of Surgeons rats.

Methods: Freshly isolated adult porcine RPE cells were used as xenografts and implanted when recipients were 17 to 21 days old. The extent of photoreceptor rescue by subretinal transplants was determined by counting the maximum layers of surviving photoreceptor nuclei in histologic sections. Cellular immune response was evaluated by immunohistochemistry.

Results: Compared to non- or sham-injected eyes, subretinal xenografts in RPE-transplanted eyes were able to induce a dramatic rescue effect ($P < .01$). However, the effect was not absolute and photoreceptor cell degeneration was only delayed. Xenografts both in the anterior chamber and in the subcutaneous tissue led to an inflammatory cellular infiltration.

Conclusion: RPE xenografts in subcutaneous space and in the anterior chamber are rejected by a delayed but vigorous inflammatory cell infiltration. Subretinal RPE xenografts are protected from a strong cellular rejection, but seem to undergo a slow functional deterioration, reflected by a decline in their capability to rescue adjacent photoreceptors. **Jpn J Ophthalmol 2002;46:36–44**

Key Words: Immunity, RCS rat, retinal dystrophy, retinal pigment epithelial cells, transplantation.

Introduction

The retinal pigment epithelium (RPE) plays a pivotal role in the maintenance of the proper functionality of the eye. It is essential both for the vitality of the retina and for the subsistence of the physio-anatomical barriers.[1] Alteration of this tissue in inherited or acquired ocular diseases, therefore, inevitably leads to blindness.[2–5] Hence, orthotopic transplantation of healthy RPE has been pursued as a potential therapeutic approach to replace impaired or destroyed RPE and to restore vision in retinopathy with presumed RPE dysfunction.[6–9]

When normal syngeneic RPE cells are implanted into the subretinal space of mutant Royal College of Surgeons (RCS) rats, the cells survive and rescue photoreceptor cells otherwise destined to undergo degeneration.[10–15] Yet, despite these encouraging successes, in clinical practice the use of syngeneic or autologous grafts remains elusive. The ultimate goal, therefore, will be to achieve long-term survival of RPE allografts or xenografts.

Because the eye is an immunologically privileged site,[16–18] both allogeneic and xenogeneic intraocular grafts can enjoy a prolonged survival when compared with similar grafts implanted into conventional body sites, such as the skin. However, ocular

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immune privilege is not absolute and, as a consequence, immunologic recognition of allogeneic or xenogeneic tissues can result in rejection of the histoincompatible graft.[19,20] To promote successful engraftment of RPE, it is important to understand and control the process of immune rejection. The long-term aim of this pilot study is to develop a specific immunosuppressive strategy to encounter graft rejection. In order to provide a base we investigated the fate of porcine RPE xenografts in nonimmunosuppressed RCS rats. Xenografting was chosen, because it should display a more obvious immune reaction. Orthotopic transplantation into the subretinal space should provide the information about the immune response and the graft function that is reflected by photoreceptor rescue. Given the transparency of the cornea, grafts were also implanted into the anterior chamber for better clinical observation of the intraocular graft. Additionally, grafts were also implanted into the subcutaneous space in order to determine the immunogenicity of the porcine RPE cells unaffected by an immunoprivileged site.

Materials and Methods

Animals

Dystrophic RCS rats (aged 17 to 19 days) served as recipients. All experimental rats (n = 115) were obtained from our breeding colony at the University of Cologne. Animals were maintained in a common room of the climate-controlled vivarium, where an overhead fluorescent light provided 12-hour cycles of light and dark. Inoculations and clinical examinations of grafted tissue were performed under anesthesia induced by intramuscular injections of ketamine (Ketalar; Parke Davis, Detroit, MI, USA) 0.075 mg/g body weight, and xylazine (Rompun, Bayer, Germany), 0.006 mg/g body weight. At the appropriate time animals were sacrificed under CO_2 anesthesia by cervical dislocation. All experimental procedures conform to the Principles of Laboratory Animal Care, as well as the current German Law on the Protection of Animals.

Preparation of Donor RPE

Donor RPE cells were prepared from porcine eyes. The methods used for securing animal tissue were humane and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The enucleated eyes were transported to the laboratory in ice-cold normal saline and processed under aseptic conditions for cell preparation. Briefly, the eyes were dissected circumferentially posterior to the ora serrata. After gentle removal of the vitreous and the retina, the RPE cells were released from posterior eyecups by treatment with trypsin 0.25% and ethylenediaminetetraacetic acid (EDTA) 0.02%. RPE cells were washed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM HEPES.

Implantation of RPE

Recipient rats received general anesthesia. For implantation of freshly isolated RPE into the subretinal space, the eyelids were kept open and the eyeball held steady with forceps. A 0.3-mm penetrating wound was made at the posterior portion of the wall of the eye using a microsurgical knife with a 15° angle. For implantation in the anterior chamber, a similar wound was made on the cornea of the eye. Implantation into the subcutaneous space was performed into the ear pinna. To implant the cells, a 10-μL Hamilton Microliter Syringe was used and about 1.5 μL of Hanks Balanced Salt Solution (HBSS) containing approximately 5×10^4 RPE cells was slowly injected via the wound into the anterior chamber, subretinal space or subcutaneous space. One group (n = 20) animals served as negative control and received no transplant. A further control group (n = 20) received a subretinal sham injection of HBSS containing no cells. RPE cells were transplanted into the subretinal space, anterior chamber and subcutaneous space of 75 RCS rats subdivided into groups of equal number.

Clinical Examination

Clinical examination was performed in order to evaluate the general appearance of both the host eyes and the grafts. Once weekly, rats were anesthetized and their eyes examined with a dissecting microscope equipped with a 35-mm camera. In order to view the posterior segments of the eyes, the pupils were dilated with 0.5% mydriacil and 2.5% phenylephrine hydrochloride and the fundus visualized through a coverslip applied as a contact lens.

Histological Examination

On the appropriate days (Figure 2) the recipients were sacrificed and the explanted tissue fixed overnight at 4°C in PLP fixative. The PLP fixative consisted of lysine phosphate buffer (0.2 M lysine monohydrochloride, 0.1 M disodium hydrogen orthophosphate adjusted to pH 7.4, and 0.02 M sodium periodate) and a paraformaldehyde-dextrose solution (5% dextrose and 8% paraformaldehyde) mixed to a final concentration of 0.25% paraformaldehyde. Dehydration

Jpn J Ophthalmol
Vol 46: 36–44, 2002

was followed by paraffin embedding and serial 5-μm sectioning. Conventional histochemistry and immunohistochemistry were preceded by deparaffination and rehydration of the specimens.

A panel of antibodies was used for the immunohistochemical detection of pan-T-cells (KI-T 1R, 1:500, BMA, Augst, Switzerland), of macrophages (ED1, 1:150; BMA, Augst, Switzerland), of immunoglobulins IgG1 and IgG2b (MARG1-2 and MARG2B, 1:2500; IK, Frankfurt, FRG), of B-cells (HIS-24, CD45D, 1:150; IK), of CD 8 (OX-8, 1:500; IK) and of MHC II (0X-6, 1:250; IK). Incubation (60 minutes at room temperature) with phosphate-buffered saline (PBS) containing 20% normal rabbit serum (NRS) and 5% bovine serum albumin (BSA) to block unspecific binding, was followed by the application of the mouse derived antibodies (overnight at +4°C). After repeated washing with PBS the specimens were incubated for 60 minutes with the biotinylated secondary antibody (rabbit anti-mouse IgG, 1:200; DAKO, Glostrup, Denmark) diluted in PBS containing 3% NRS and 2% BSA and subsequently with Streptavidin-AP (30 minutes, 1:50; DAKO). As negative controls, sections were incubated with PBS containing 3% NRS and 2% BSA but no primary antibody. The sections were washed in PBS and incubated with the second antibody (rabbit anti-mouse IgG). Positive control testing was performed on rat thymus.

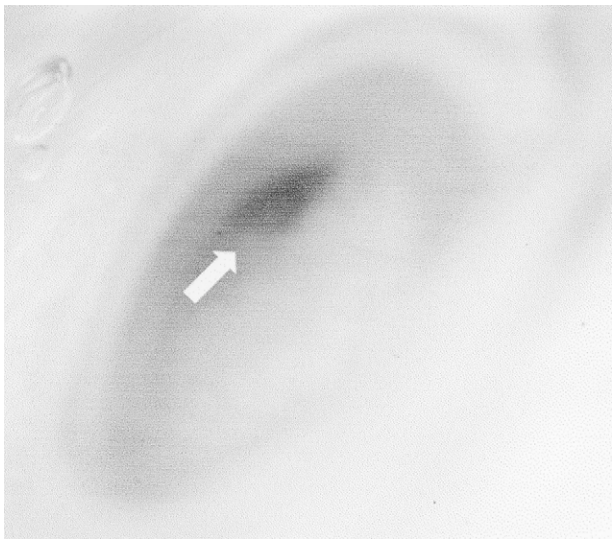

Figure 1. Aspect of the fundus of Royal College of Surgeons rat that received retinal pigment epithelial xenotransplant 4 weeks previously. Arrow indicates pigmented graft, localized underneath retina. No signs of inflammation are detected.

Statistics

Photoreceptor cell degeneration was measured by counting the cell layers in the outer nuclear layer (ONL). At the time of transplantation the ONL consists of approximately 17 cell rows. This number was set equal to 100%, and retinal degeneration was characterized by the percentage of photoreceptor cell layers surviving thereafter. The mean number/percentage (with standard deviation) of surviving layers of photoreceptor nuclei in untreated (Control), sham-injected (Sham), and transplanted (RPE) eyes at the same time points were collected. Statistical analysis of the averaged results was done by a two-tailed Student *t*-test for the comparison of two groups at one survival time. The resulting data was assumed to be significant if $P < .05$.

Photographs

A 35-mm camera system attached to a Zeiss dissection microscope (Stemi 2000; Zeiss, Göttingen, FRG) was used to photograph the graft. Micrographs of sections were taken with a 35-mm camera system attached to a Zeiss research microscope (Zeiss).

Results

Fate of Subretinal Transplants

Subretinal implantation of RPE cells caused a retinal detachment that gradually subsided in the following days, allowing visualization of the pigmented graft. No clinical evidence of inflammation was detected (Figure 1).

Histological examination disclosed the previously described retinal dystrophy in RCS rats. The progressive retinal degeneration is associated with the loss of photoreceptor outer segments and ONL and the accumulation of subretinal debris starting from the second postnatal week. At the time of transplantation, when recipients were 17–19 days old, the ONL consisted of approximately 15–17 rows of cells. One month after birth the ONL was already reduced by approximately 50% and almost vanished 1 month later (Figure 2). As described previously,[21] the surgical trauma provokes a short-lived rescue effect. The ONL of sham-injected eyes was 70% preserved at 1 month after birth, but similar to the naive eyes, these eyes also lost the ONL 2 months after birth (Figure 2). In contrast, implanted porcine RPE cells induced a dramatic rescue effect, preserving approximately 60% of the ONL in the second postnatal month (Figure 2). Some of the transplanted eyes even displayed photoreceptor outer segments at this time (Figure 3A). However, retinal degeneration progressed and

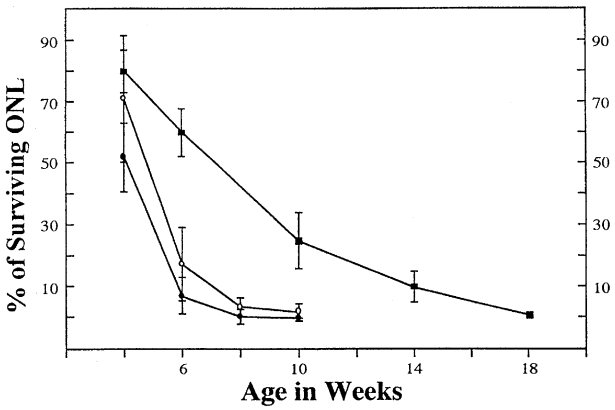

Figure 2. Graph showing mean number (with standard deviation) of surviving layers of photoreceptor nuclei in untreated (•), sham-injected (○) and retinal pigment epithelial-transplanted (■) eyes. Sample size at each time point was n = 5. Number of photoreceptors in eyes of transplanted animals compared to control or sham-injected eyes was statistically greater until the third month ($P < .01$), but declined afterwards.

the ONL receded completely in the third month after birth (Figure 2). This was associated with the infiltration of the retina by large pigmented cells (Figure 4). No additional inflammatory cells could be detected. Immunohistological analysis of untreated RCS rat retina disclosed ED1 positivity, presumably[22,23] in activated retinal macrophages, infiltrating the outer nuclear layer and the debris zone (Figure 5A). RCS rat eyes that received subretinal RPE transplants were negative for lymphocyte markers, IgG and MHC class II. However, the large pigmented cells that infiltrated the retina in the late phases (Figure 4) disclosed ED1 positivity (Figure 5B).

Fate of Anterior Chamber Transplants

After implantation into the anterior chamber, the original cloudy mass of suspended cells formed conglomerates that adhered mostly on the iris. Clinical examination revealed no prominent signs of inflammation. The aqueous humor appeared clear, and no vessels were seen at the sites of graft attachment (Figure 6). Over time the size of the pigmented grafts appeared to be reduced.

Histological examination showed that within 3–4 weeks the pigmented RPE grafts were obviously infiltrated by inflammatory cells (Figure 7A). In the following weeks cellular infiltrates retreated, less pigmented cells were found and the pigmented cells appeared enlarged (Figure 7B). Immunohistochemical analysis revealed that the cellular infiltrate was mostly characterized by ED1-positive cells and both B- and T-lymphocytes (Figures 8A, B). Large pig-

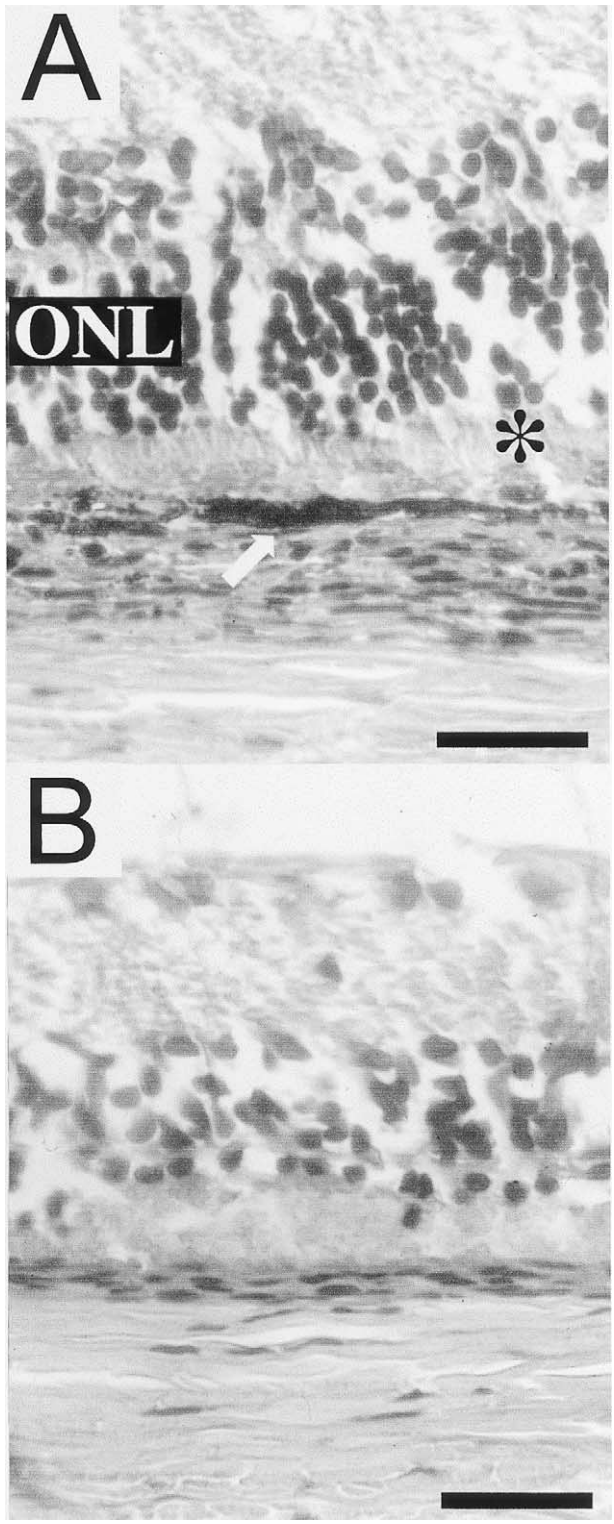

Figure 3. Light microscopy of hematoxylin eosin-stained Royal College of Surgeons rat retina from 6-week-old animal receiving porcine retinal pigment epithelial xenografts (arrow) on 17th postnatal day. (**A**) Site of engraftment. Outer nuclear layer (ONL) and photoreceptor outer segments (asterisks) are almost preserved. (**B**) Shows same eye as in (**A**) at site far from graft. ONL displays only one photoreceptor cell layer. Bars = 30 μm.

Jpn J Ophthalmol
Vol 46: 36–44, 2002

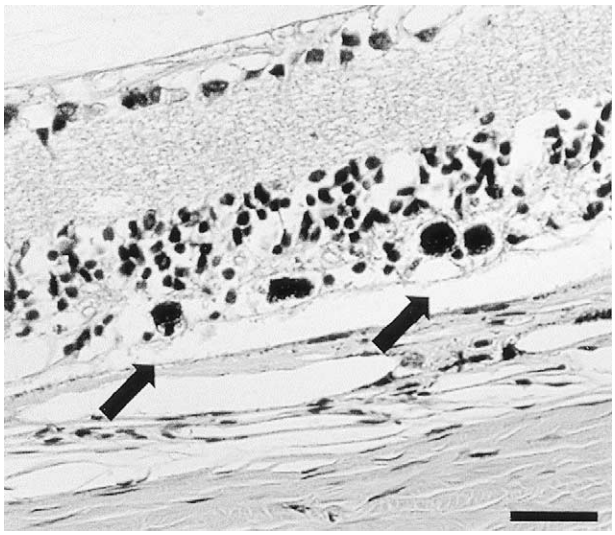

Figure 4. Light microscopy of hematoxylin eosin-stained Royal College of Surgeons rat retina from a 14-week-old animal receiving porcine retinal pigment epithelial xenografts on 17th postnatal day. Site of engraftment displays large pigment-loaded cells infiltrating retina (arrows). Bar = 30 μm.

ment-loaded cells in the late phases could be identified as ED1-positive macrophages (Figure 8C).

Fate of Subcutaneous Transplants

The subcutaneous grafts in the ear pinnae could be well-monitored. The implantation-dependent tissue trauma subsided rapidly within 1 or 2 days. During inoculation time there were no dramatic signs of inflammation as redness or swelling at the implantation site (Figure 9). Over time, pigmented grafts, however, paled slightly.

Histologically there were no signs of an acute rejection. However, starting within the first 2 weeks after implantation, an increasing infiltration of inflammatory cells became obvious. The cellular infiltration surrounding the pigmented RPE graft peaked around the third or fourth week (Figure 10). In the following weeks and months the cellular infiltrates slowly retreated, and the number of pigmented cells was reduced. Immunohistochemical analysis revealed that the cellular infiltrate was composed mostly of ED1-positive cells and both B and T lymphocytes (Figure 11). Large pigment-loaded cells in the late phases were identified as ED1-positive macrophages. No deposition of IgG could be detected.

Discussion

Therapeutic transplantation of RPE is likely to become one of the major achievements in ophthalmol-

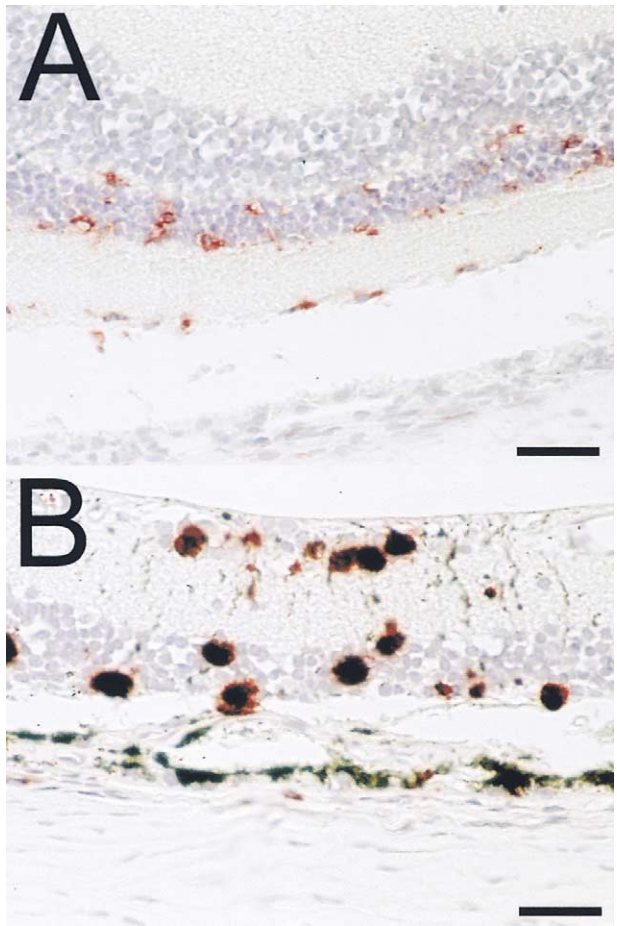

Figure 5. Immunohistochemical staining of Royal College of Surgeons rat retinas. (**A**) Specimen obtained from 4-week-old naive animal showing infiltration of outer nuclear layer by ED1-positive cells and subretinal debris zone. (**B**) Specimen from 14-week-old rat that received orthotopic retinal pigment epithelial transplant, showing ED1-positive pigmented cells infiltrating retina. Bars = 30 μm.

ogy. Although encouraging progress has been made in the development of transplantation techniques and tissue preservation, several obstacles still need to be overcome to achieve the goal of successful long-term engraftment.[24,25] The immune response associated with graft rejection represents one of these obstacles. Therefore, it is necessary to understand and possibly control immune rejection.

Intraocular transplantation occupies a special status. Fundamental studies have demonstrated that the eye is an immune-privileged region, compared to conventional sites, that allows intraocular transplants to enjoy a prolonged survival.[16–18] Within this context it is understandable that orthotopic RPE transplants both of allo- and xenogeneic origin might survive for an extended time without obvious im-

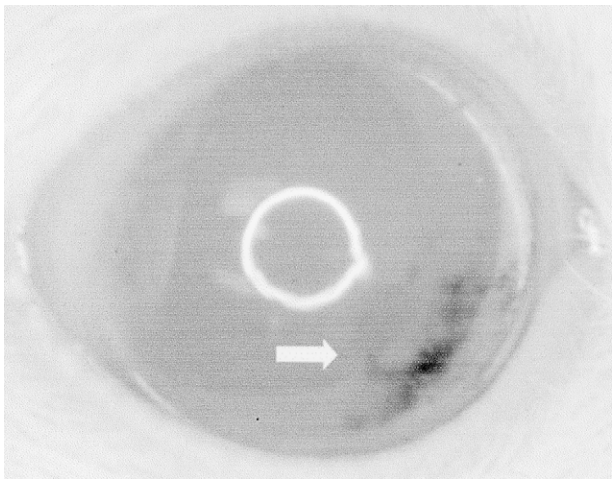

Figure 6. Aspect of anterior segment of Royal College of Surgeons rat that received retinal pigment epithelial xenotransplant into anterior chamber 4 weeks previously. Injected cell suspension aggregated to several pigmented grafts localized on iris (arrow). No clinical signs of inflammation were detected.

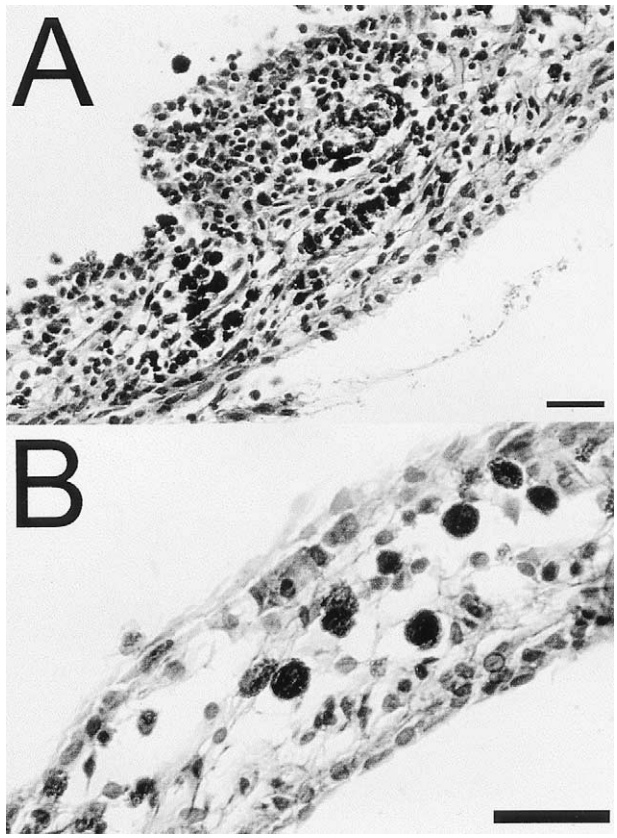

Figure 7. Light microscopy of hematoxylin eosin-stained Royal College of Surgeons rat eyes that had received porcine retinal pigment epithelial xenografts into anterior chamber 4 weeks (**A**) or 14 weeks (**B**) previously. One month after implantation graft site displays obvious cellular infiltrate (**A**), which dissolves in following weeks leaving large pigmented cells within iris stroma (**B**). Bars = 30 μm.

mune response. However, present investigations show contradictory results, which might be based on different animal models, techniques, and observation time.[10,19,20] Furthermore, it is conceivable that the immune-privileged status of the eye will cease within a pathological environment, such as upon disruption of the blood—retinal barrier. First clinical trials give evidence that even fetal RPE transplants, which are supposed to be less immunogenic, are rejected when the blood—retinal barrier is disrupted, as happens in the wet form of age-related macular degeneration (AMD).[6,7] Because AMD is going to be the major focus for RPE transplantation, it is imperative to formulate an immunosuppressive strategy.

The long-term aim of this pilot study is to develop a specific immunosuppressive method to suppress graft rejection. For this we needed to establish a positive control that could be used later to compare the efficacy of future therapy. Because a xenograft should display a more obvious immune reaction, we choose to transplant porcine RPE cells into the RCS rat model.

The RCS rat suffers from a well-characterized, early onset, and progressive form of photoreceptor cell degeneration. Substantial data show that this condition is caused by abnormal RPE cells. Grafting of normal rat RPE cells has a rescue effect on the dystrophic retinas. It has been demonstrated before that human fetal RPE xenografts in RCS rats can rescue photoreceptor cells.[13] All rats in the mentioned study were immunosuppressed with daily injections of cyclosporine A. The authors sacrificed the animals 4 weeks after transplantation and demonstrated a delay in photoreceptor degeneration in transplanted animals. Transplanted regions displayed approximately four rows of photoreceptor cells, whereas in nontransplanted areas or in naive animals the ONL had almost vanished by this time. In our study orthotopic porcine RPE xenotransplants in nonimmunosuppressed hosts conceivably enjoyed the immunoprivileged status of the subretinal space. Four weeks after transplantation, porcine grafts induced an even greater rescue effect (Figure 2). Transplanted areas displayed approximately 10 rows of photoreceptor cells still expressing outer segments (Figure 3). The retinal degeneration process was delayed until 2 months after transplantation. At this time the ONL had almost disappeared. There was no

Jpn J Ophthalmol
Vol 46: 36–44, 2002

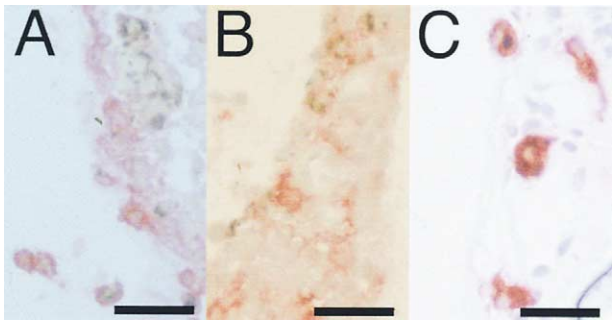

Figure 8. Immunohistochemical staining of Royal College of Surgeons rat eyes that had received porcine retinal pigment epithelial xenografts in anterior chamber 4 weeks (**A**/**B**) and 14 weeks (**C**) previously. Iris stroma was infiltrated by CD45R-positive cells (**A**) and ED1-positive cells (**B**/**C**). In late phase pigmented cells were ED1-positive (**C**). Bars = 20 μm.

obvious inflammatory infiltration, but the retina was invaded by pigment-loaded cells (Figure 4). These were ED1-positive and therefore probably retinal macrophages[22,23] (Figure 5). No other inflammatory cells could be detected. An effect induced by a humoral immune response cannot be excluded.

Besides orthotopic transplantation into the subretinal space, we choose two additional different transplantation sites. These were the anterior chamber and the subcutaneous space. The anterior chamber was chosen as a transplantation site because it is, like the subretinal space, an immunoprivileged region and because the transparency of the cornea allows easy visualization of the graft (Figure 6). Surprisingly, the RPE xenografts in the anterior chamber were not protected as expected, but were affected by an inflammatory cell infiltration (Figure 7). Within 3–4 weeks the graft site was infiltrated by lymphocytes and macrophages (Figure 8). Finally, the inflammatory reaction subsided, leaving singular pigment-loaded cells that could be identified as macrophages (Figure 9). Because the anterior chamber is an immunoprivileged site protected by the so-called anterior chamber associated immune deviation (ACAID),[17,18] we need to ask to what extent the ACAID in RCS rats might be defective. Several factors are known to disrupt the mechanisms of ACAID. One of these factors is an altered blood—ocular barrier. This condition seems to apply also in RCS rats. Essner and coauthors described that intrave-

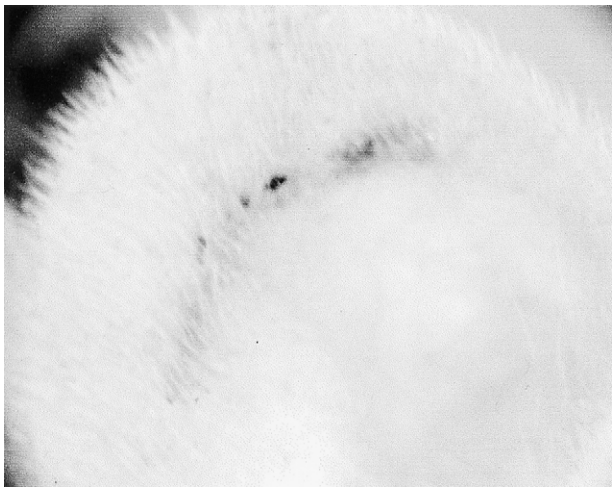

Figure 9. Aspect of ear pinna of Royal College of Surgeons rat that had received a retinal pigment epithelial xenotransplant 4 weeks previously. Pigmented graft is localized in subcutaneous space. No macroscopic signs of inflammation were detected.

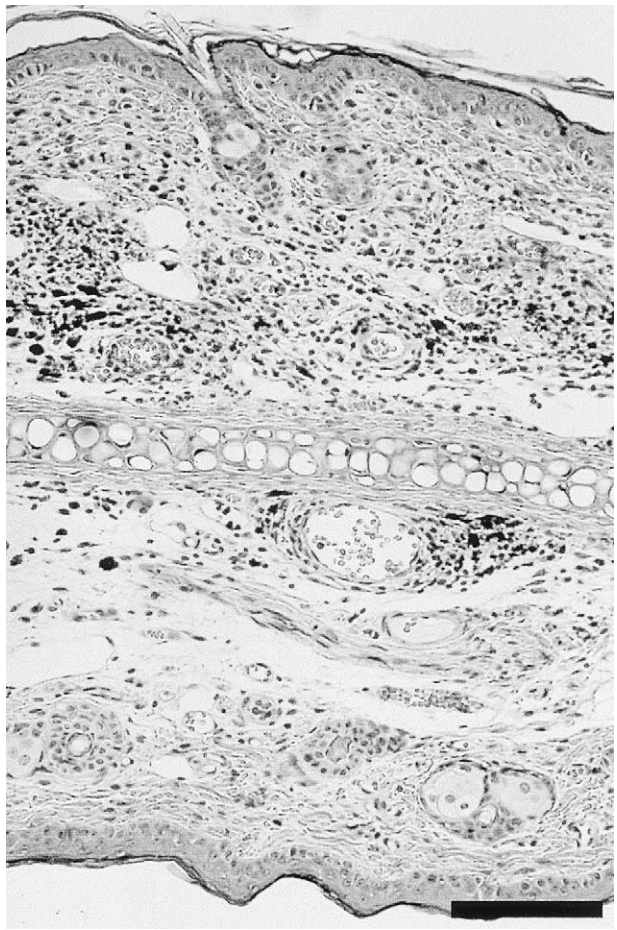

Figure 10. Light microscopy of hematoxylin eosin-stained specimen from Royal College of Surgeons rat ear that had received subcutaneous porcine retinal pigment epithelial (RPE) xenografts 4 weeks previously. Inflammatory cell infiltrate surrounds pigmented RPE cell graft. Bar = 60 μm.

nously injected microperoxidase and horseradish peroxidase are extravasated from the outer retinal capillaries of RCS rats from approximately the 11th week of age and older.[4] The dystrophic RCS rat is not only characterized by a progressive retinal degeneration but also by changes affecting the anterior part of the eye (eg, iris and ciliary body).[26,27] It is, therefore, conceivable that the blood—ocular barrier of the anterior chamber is altered, too, affecting ACAID.

In order to determine the immunogenicity of the porcine RPE cells unaffected by an immunoprivileged site, ie, the eye, grafts were also implanted into the subcutaneous space. We expected that this type of xenografting would be discordant and that transplantation at a conventional site would induce a hyperacute rejection. However, the rejection of the subcutaneous RPE xenografts occurred as a delayed type cellular response. The rejection was not accompanied by clinically obvious inflammatory signs, such as swelling or redness (Figure 10). Nevertheless, the immune response was characterized by a slow cellular infiltration, which consistently peaked around the third or fourth week after transplantation (Figures 10 and 11). The inflammatory infiltration persisted until only a few pigmented cells were left at the engraftment site. These cells could be identified as macrophages that had phagocitized pigment granules from the original RPE cells. Because the grafts were notably not affected within the first 2 weeks, it might be supposed that naturally occurring xeno-antibodies are absent in this combination. However, the implantation of porcine skin into the ear pinnae of RCS rats induced a more vigorous reaction that occurred within a few days (data not shown). Therefore, we assume that the "milder form" of rejection associated with the RPE cells might well be based on a different cause. RPE cells can produce immunosuppressive cytokines, such as transforming growth factor β.[28] This might lead to an immunosuppressive microenvironment, which can delay but not completely suppress the immune response and graft rejection.[29]

In summary, this study demonstrates that porcine RPE xenografts can replace dystrophic RPE cells in RCS cells, as is reflected in photoreceptor cell rescue. Retinal degeneration, however, will progress. Although trophic mechanisms cannot be excluded, activated macrophages seem to be involved. An infiltration by other inflammatory cells can be excluded. However, porcine RPE xenografts can induce a delayed cell-mediated graft rejection when implanted at a conventional site, such as the skin. Interestingly, RPE cell grafts in the anterior chamber, which should be protected by ACAID, are rejected in a similar fashion.

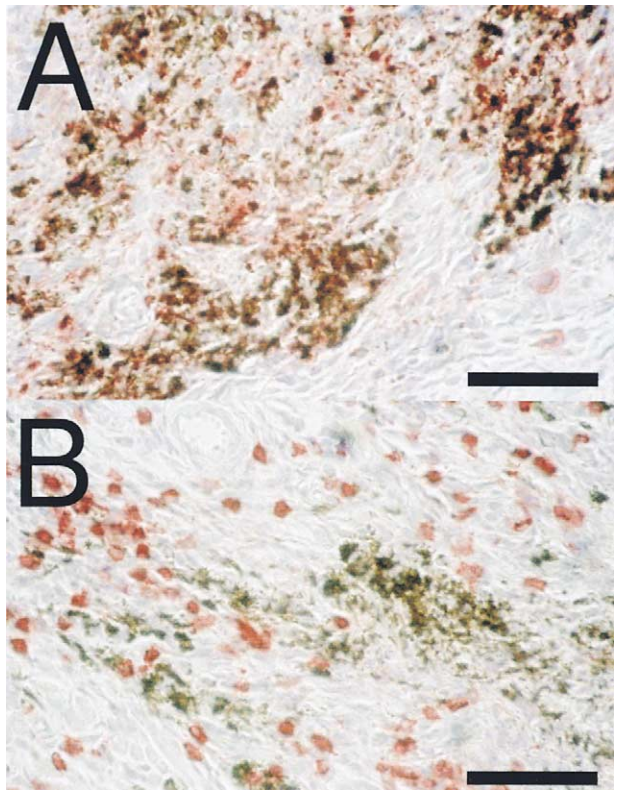

Figure 11. Immunohistochemical staining of Royal College of Surgeons rat ear that had received porcine retinal pigment epithelial xenografts in subcutaneous space 4 weeks previously. Transplants were infiltrated by ED1-positive (**A**) and CD45R-positive cells (**B**). Bars = 30 μm.

Although the proper interpretation and clinical relevance of this study is possibly limited by differences between species, origin of the grafts (allo- or xenografts), and by distinct interactions in different pathologies, it is conceivable that RPE xenografts can replace dystrophic RPE cells, but will be rejected when the immune privilege of the eye is disrupted. This study provides a base for RPE graft rejection. The understanding of the mechanisms accounting for RPE graft rejection is necessary in envisioning an immunosuppressive strategy.

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