Cytokine Gene Expression after Subretinal Transplantation

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Abe, T., Takeda, Y., Yamada, K., Akaishi, K., Tomita, H., Sato, M. and Tamai, M. *Cytokine Gene Expression after Subretinal Transplantation.* Tohoku J. Exp. Med., 1999, **189** (3), 179–189 —— Transplantation study of neural retina, retinal pigment epithelial (RPE), or iris pigment epithelial (IPE) cells have been performed not only in animal model but in human age-related macular degeneration, and some of the findings reported with cystoid macular edema may have been due to graft rejection. In this investigation, we examined cytokine gene expression by reverse transcriptase-polymerase chain reaction at the transplanted subretinal space. Transplantation was performed in normal Royal College of Surgeon's rats using cultured human RPE and rat IPE. They were followed without immunosupression. Gene expression for melanogenesis of transplanted human RPE was observed only in the early days after transplantation. Rat interleukin (IL)-1α, -1β, -2, -6, interferon γ, and tumor necrosis factor α (TNFα) genes were also expressed after the early days of transplantation. Cytokine expression was observed not only after cell transplantation but also after vehicle-only injection, which was considered a reaction to the surgical trauma. However, statistically significant amount of expressions of IL-1α, -1β, and -6 were observed after the early days of transplantation of human RPE or IL-1α, -1β, and TNFα of rat IPE, if we compare them to vehicle-only injection. These cytokines may play an important role for the local reaction after transplantation. ———— cytokine; transplantation; retina; RT-PCR © 1999 Tohoku University Medical Press

Transplantation study of neural retina, retinal pigment epithelial (RPE) (Li and Turner 1991; Sheedlo et al. 1991; Lavail et al. 1992), or iris pigment epithelial (IPE) (Tamai 1996; Rezai et al. 1997) cells have been performed using animal model, such as Royal College of Surgeon's (RCS) rats. The cells used for transplantation were rat RPE cells for iso or allograft (Li and Turner 1991; Sheedlo et al. 1991) and human (Little et al. 1996) RPE cells for xenograft. The results of the transplantation have shown good preservation of photoreceptor cells and have

reported no evidence of rejection. However, xenograft transplantation of RPE with collagen sheets in the subretinal space or anterior chamber demonstrated well-preserved histochemistry of the retina, even though the electroretinographic findings reported decreased amplitudes (Bhatt et al. 1994; Tamai 1996). Recently, the transplantation of fetal RPE cells also has been evaluated in human age-related macular degeneration (AMD), and some of the findings reported with cystoid macular edema may have been due to graft rejection (Peyman et al. 1991; Algvere et al. 1994, 1997). These results indicated that histochemical examination alone is not enough to understand local reaction to transplantation in the eye.

Fealy et al. (1995) reported that allografts with inflammatory cytokines at the transplanted area, showed evidence of rejection, presevation injury, or mild inflammation. Conversely, retinal degeneration in mice have been reportedly rescued by intravitreal injection of several types of cytokines (Lavail 1992). Li and Turner (1991) also reported that photoreceptor rescue of RCS rats was observed by injection with vehicle only in the early days after transplantation. These results suggest that some local factors, such as cytokines, may be generated and may have some influence at the lesion.

In this study, we examined whether or not cytokine genes were expressed at the transplanted area using normal RCS rats.

MATERIALS AND METHODS

Preparation of cultured human RPE or Long Evans rat IPE cells

Human RPE cell cultures were established as we previously reported (Durlu and Tamai 1997). In brief, after removing the anterior segment and vitreous, the eye cups were incubated with trypsin (0.05%)/ethylenediamine tetraacetic acid (EDTA) (0.53 mM) (Gibco BRL, Bethesda, MD, USA) solution in Hank's balanced salt solution (HBSS) (Gibco)- for 30 minutes at 37°C in 5% CO_2. After separation of the neural retina, the eye cups were washed twice with HBSS. By performing pipeting under the dissecting microscope, we could collect RPE in 20% fetal bovine serum (FBS), F-12 medium (Gibco). Next, the RPE cells were seeded at 37°C in 5% CO_2 in modified polysterene dishes (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 1×10^4 cell/ml in 20% FBS in F-12 medium. These cells were examined with anti-cytokeratin pan (a monoclonal antibody mixture; Sigma, St. Louis, MO, USA) to confirm that the cells were epithelial in origin. Long Evans rat IPE cell cultures also were established by the same methods as above except that the pigment epithelium were mechanically detached from the stroma after 1 hour incubation with 0.25% trypsin in Dulbecco's solution at 37°C. Cell number was determined with use of a Burke-Turk hemocytometer.

The methods for transplantation

RPE, IPE, and culture medium were transfered into the subretinal space of

normal RCS rats, weighting of 250–300 g, as reported elsewhere (Yamaguchi et al. 1993). In brief, host rats were anesthetized with an injection of pentobarbital sodium (60 mg/kg) followed by an injection of atropine sulfate (0.4 mg/kg). An incision was made through the superior eyelid to expose the dorsal surface of the eye. Through that incision, a 2 μl suspension of variable RPE or IPE cells (apporoximately 8×10^4 cells) was injected into the subretinal space using a 32-guage needle attached to a 10-μl Hamilton syringe. Sham-transplanted control animals were injected with 2 μl of F-12 medium without FBS (vehicle only) into the subretinal space through the same surgical procedure. All experiments were performed in the left eye of RCS rats and a total of 4 rats were used for each experiments. These rats were followed without immunosupression.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

An area, about 2 mm in diameter including retina, choroid, and sclera, where RPE, IPE, or vehicle was transplanted, was cut out by scissors, and mRNA was extracted from these regions using oligo dT cellulose (Pharmacia Biotech Inc., Uppsala, Sweden), as reported previously (Abe et al. 1996). In brief, the cells from the lesions were suspended in extraction buffer (4 M guanidinium thiocyanate and 0.5% N-lauroyl sarcosine), and cleared cellular homogenate by centrifugation, was mixed with oligo dT cellulose (Pharmacia Biotech Inc.). The oligo dT cellulose was washed by high salt buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5 M NaCl) for several times followed by low salt buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1 M NaCl) for several times, and then mRNA was eluted by pre-warmed elution buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). First strand cDNA was generated by random hexadeoxynucleotides at 0.2 μg in each reaction, which was catalyzed by Moloney Murine Leukemia Virus reverse transcriptase (Pharmacia Biotech Inc.). With the use of a thermocycler (Perkin Elmer, Norwalk, CT, USA), PCR was carried out in 50 μl of reaction mixture; 20 μM of each primer; 200 mM each of dATP, dCTP, dGTP, and TTP; 50 mM of KCl; 10 mM of Tris-Cl (pH 8.3); 1.5 mM of $MgCl_2$ and 0.001% gelatin; and 2.5 units of Taq polymerase. Reaction cycles were 28, 30, 33, 35, and 40. The temperature settings for PCR were 94°C for 1 minute for denaturation, 60°C for 2 minutes for annealing, and 72°C for 2 minutes for polymerization. Negative controls were performed either without reverse transcriptase or no samples. In each case, amplified DNA was separated in 1.5% agarose gel (SeaKem, FMC BioProducts, Rockland, ME, USA) containing 0.05 mg/ml ethidium bromide. DNA was visualized with use of an ultraviolet transilluminator.

Semi-quantitative PCR

Semi-quantitative PCR was performed, as we reported previously (Abe et al. 1998a). Briefly, PCR was performed under the conditions described, with variable cycles (28, 30, 33, 35, and 40 cycles). The PCR products were separated on

1.5% agarose gel and photographed for further analysis; the photographs were quantitatively analyzed by NIH image analyzer on the computer screen. Under the exponential phase of the PCR products, the quantities of the PCR products were compared with each other. The β-actin was used as an internal control. Analysis of variance and Dunnett test were performed at $p = 0.05$.

Primer preparation

The primer sets for human tyrosinase gene amplified 276 bp, human tyrosinase-related protein-1 (TRP-1) 364 bp, (Abe et al. 1996) rat interleukin (IL)-1α 623 bp, rat IL-1β 303 bp, rat IL-2 342 bp, rat IL-6 614 bp, rat interferon (IFN)γ 288 bp, rat tumor necrosis factor (TNF)α 295 bp (Clontech, Palo Alto, CA, USA), and β-actin 313 bp. For tyrosinase: 5'-TGT CTG TAG CCG ATT GGA GG-3' (sense direction) and 5'-CAA GGC ATT GTG CAT GCT GC-3' (antisense direction), for TRP-1: 5'-ATG TCT GTG ATA TCT GCA CGG-3' (sense direction) and 5'-TGA AGA CTT CGA ACA GCA GG-3' (antisense direction), for rat IL-1β: 5'-GAG GTG CTG ATG TAC CAG TTG GGG AAC T-3' (sense direction) and 5'-TGA GCT GAA AGC TCT CCA CCT CAA TG-3' (antisense direction), for β-actin: 5'-CTA CAA TGA GCT GCG TGT GG-3' (sense direction) and 5'-CGG TGA GGA TCT TCA TGA GG-3'(antisense direction). Primers for rat IL-1α, rat IL-2, rat IL-6, rat TNFα, and rat IFNγ were purchased from Clontech and they were specific for rat cytokines. All primers were designed to expand at least one exon between each primer to avoid the possibility of amplifying the sequences of the gene itself.

RESULTS

The epithelial origin of cultured human RPE and cultured rat IPE cells was confirmed by immunohistochemistry using anti-cytokeratin antibody as we reported previously (data not shown)(Abe et al. 1998b). Both types of cells used for transplantation showed an expression of cytokeratin (about 90%). In the indicated cultured condition of human RPE, tyrosinase and TRP-1 genes were expressed as we previously reported (Abe et al. 1998b) as long as the passage was less than 6. Cultured human RPE cells at passage 4 were used for transplantation study. Cultured rat IPE cells at passage 7 were also used for transplantation.

After transplanting these cells in the subretinal space of the normal RCS rats, the expression of the human genes for melanogenesis was examined by RT-PCR. We found that human tyrosinase and TRP-1 genes were amplified at the transplanted regions after 3 days of transplantation (Fig. 1). However, these gene expressions were extremely decreased after 7 days or 30 days of transplantation. β-Actin gene was amplified well and seemed to show no differences among samples (Fig. 1).

Expressions of the genes for rat IL-1α, -1β, -2, -6, IFNγ, and TNFα at the
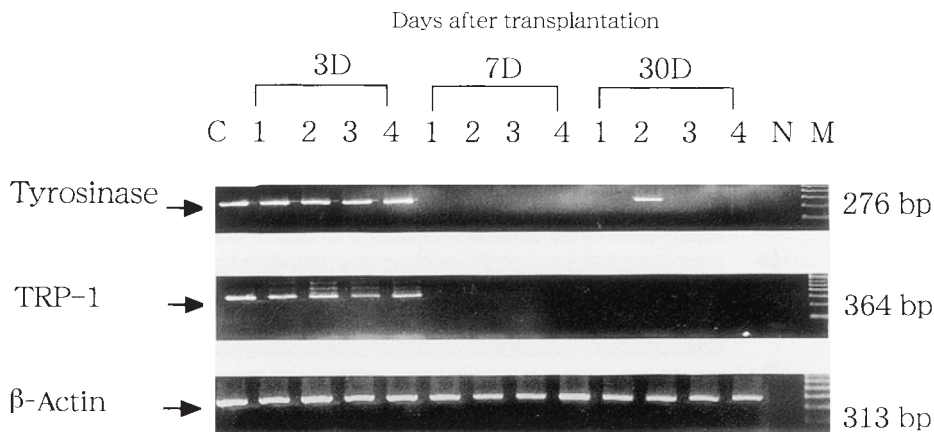
Days after transplantation

Fig. 1. The results of RT-PCR of human genes for melanogenesis after transplan-
tation of cultured human RPE.   Tyrosinase indicates 276 bp PCR product of
tyrosinase gene, TRP-1; tyrosinase-related protein-1 364 bp, and β-actin; 313
bp. Cindicates the results of cultured human RPE cells used for transplanta-
tion, each number of the upper lane; each number of normal RCS rats used for
transplantation, N; negative control, M; markers of 100 bp and increment of
100 bp each, 3D, 7D, and 30D; days after transplantation.   The methods for
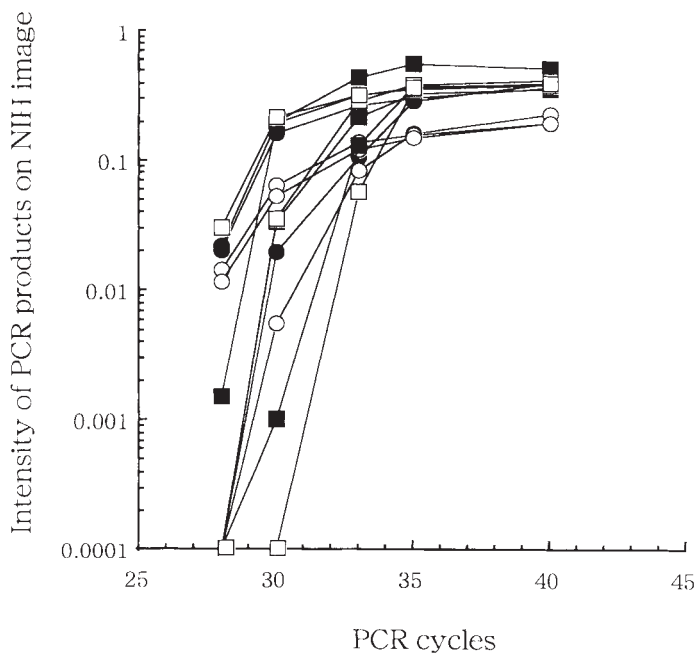transplantation are described in the text.

Fig. 2. The results of RT-PCR of β-actin with total 12 times examination in each
cycles were shown.   Open circles indicate the results of untransplanted
normal retina and underlying tissue; solid circles, vehicle-only injection; open
square, cultured rat IPE cells; and solid square, cultured human RPE cells.

transplanted regions also were examined by semi-quantitative RT-PCR.   Fig. 2
shows the results of RT-PCR with variable cycles of β-actin among untransplant-
ed normal retina and underlying tissue, vehicle-only injection, cultured rat IPE
cells, and cultured human RPE cells (3 samples each, total 12 samples in each
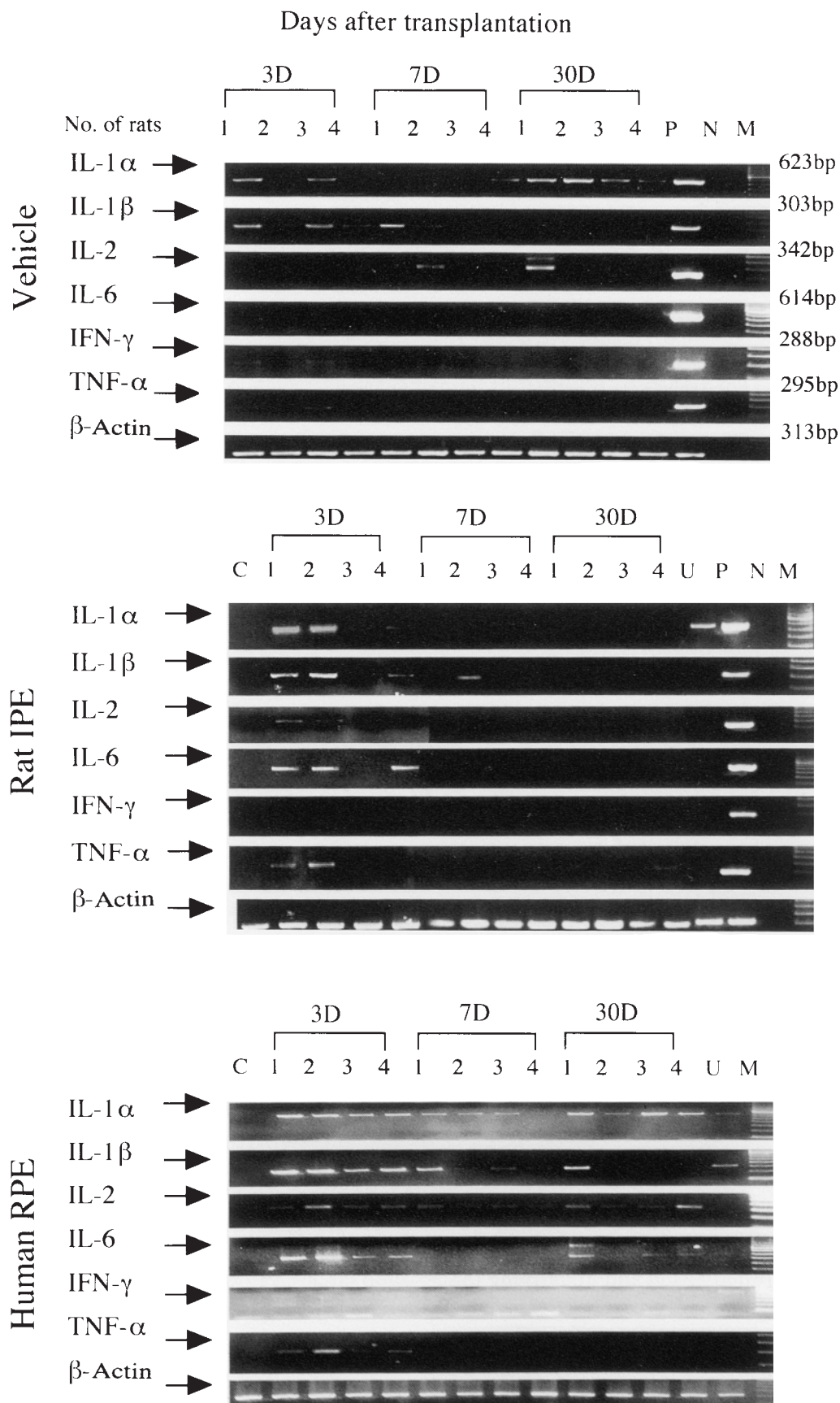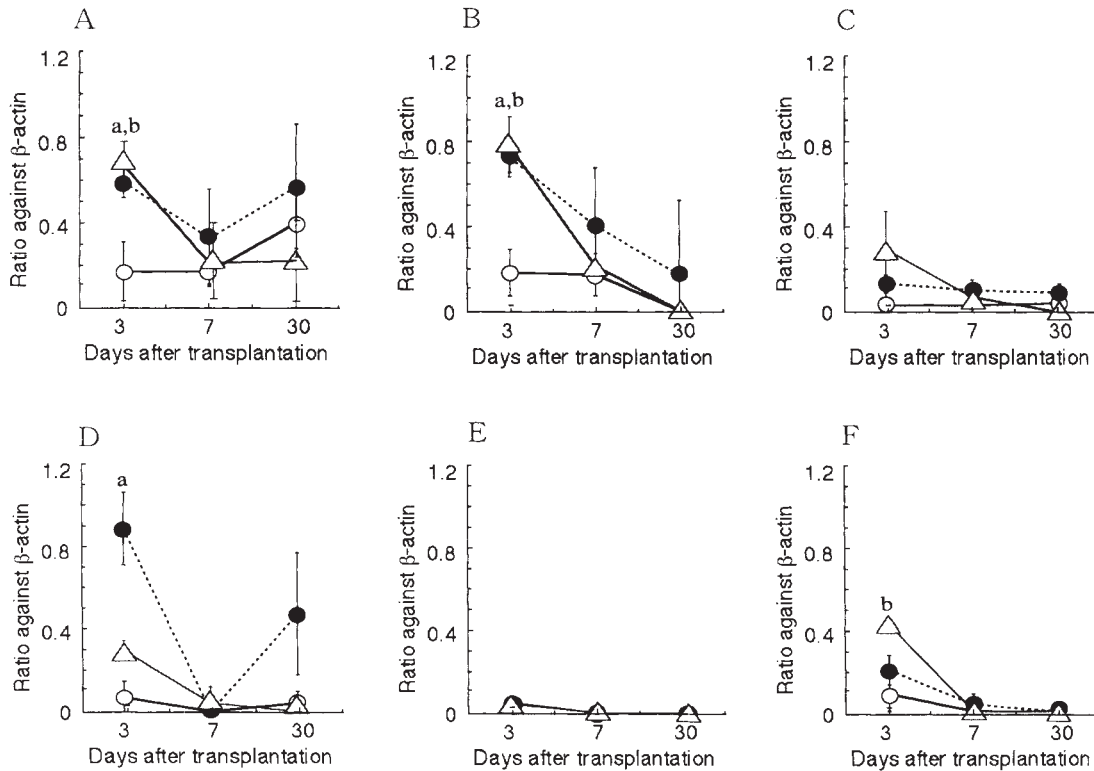
Days after transplantation

Fig. 3

Fig. 4. The results of semi-quantitative RT-PCR for rat IL-1α (A), -1β (B), -2
(C), -6 (D), IFNγ (E), and TNFα (F) are shown. Numbers of Y axis; ratios
of each cytokine gene expression to the internal control of β-actin, numbers of
X axis; each day examined after transplantation. Closed circles indicate
cultured human RPE cells; slashed triangles, cultured rat IPE; and open
circles, vehicle only. Statistically significant transcription is indicated by
[a](human RPE) and [b](rat IPE).

cycle). These results were quantitatively analyzed by NIH image. We
confirmed that products were amplified exponetially and decided to use 30 or 33
cycles for analyzing each cytokine transcription against β-actin. Fig. 3 showed
the results of rat IL-1α, -1β, -2, -6, IFNγ, TNFα and β-actin of vehicle only
(upper), cultured rat IPE (middle), and cultured human RPE (lower). Extreme-
ly low expression of rat IL-2, IFNγ, and TNFα were also observed. The
multiple bands of IL-2 gene at 7 or 30 days after transplantation of vehicle only
(upper) revealed artifact by sequence analysis. We could not amplify these

Fig. 3. The results of RT-PCR for rat interleukin (IL)-1α, -1β, -2, -6, IFNγ, and TNFα
after transplantation of vehicle only (upper), Long Evans rat IPE cells (middle), and
human RPE cells (lower). Cindicates the results of cultured rat IPE (middle), or
human RPE (lower) cells used for transplantation, each number of the upper lane; each
number of normal RCS rats used for transplantation, U; untransplanted normal retina
and underlying tissue, P; positive control, N; negative control, M; markers of 100 bp
and increment of 100 bp each, 3D, 7D, and 30D; days after transplantation. The
primer sets for rat IL-1α gene amplified 623 bp, rat IL-1β 303 bp, rat IL-2 342 bp, rat
IL-6 614 bp, rat IFNγ 288 bp, rat TNFα 295 bp, and β-actin 313 bp. Positive and
negative controls were not always shown.

cytokine genes from cultured rat IPE or human RPE cells used for transplantation (C in Fig. 3 middle and lower, respectively). Expressions of IL-1α and/or -1β also were confirmed in untransplanted normal retina and underlying tissue (U in Fig. 3). These results were also evaluated by NIH analyzer for IL-1α, -1β, -2, -6, IFNγ, and TNFα against β-actin (Fig. 4). The genes for rat IL-1α, -1β, -2, -6, IFNγ, and TNFα were amplified after 3 days of transplantation of cultured human RPE, cultured rat IPE, and vehicle-only. However, expressions of IL-1β, -2, -IFNγ, and TNFα were gradually decreased after 7 or 30 days of transplantation. Conversely, expression of IL-1α and IL-6 decreased at 7 days of transplantation and then they were tend to increase again after 30 days of transplantation of cultured human RPE cells, although they were not statistically significant (Fig. 4). However, statistically significant amount of expression of the genes for IL-1α ($p = 0.0004$), -1β ($p < 0.0001$), and -6 ($p = 0.0009$) were observed after 3 days of transplantation of cultured human RPE cells, if we compare the results with that of vehicle only (a, Fig. 4). Statistically significant amount of expression of IL-1α ($p = 0.0002$), -1β ($p < 0.0001$), and TNFα ($p = 0.0017$) also was observed after 3 days of transplantation of cultured rat IPE cells, if we compare the results with those of vehicle only (b, Fig. 4).

Discussion

In spite of advanced surgical techniques, some retinal diseases such as AMD result in poor visual prognosis. One reason for this poor prognosis may be damaged RPE due to the background condition or surgical abrasion of the underlying normal RPE (Tamai 1996). There have been reports of many trials providing normal RPE or IPE cells in the subretinal space to rescue photoreceptor degeneration of RCS rats (Hammer and Yinon 1991; Li and Turner 1991; Lavail et al. 1992; Sheedlo et al. 1995; Tamai 1996; Whiteley et al. 1996) whose original condition contributed to dysfunction of the phagocytosis of the RPE (Bok and Hall 1971). Recently those methods were extended to human AMD patients using fetal RPE cells (Algvere et al. 1997). Even though photoreceptors have been rescued in model RCS rats by transplantation of variable species, some clinical trials of human AMD were suspected to be caused by local rejection (Algvere et al. 1994, 1997). Our results also showed that the essential gene expression for melanogenesis of the transplanted human RPE was markedly influenced at the subretinal space of normal RCS rats after 7 days of transplantation. No expression was observed after 7 days of transplantation, although the cells used for transplantation were xenografts.

The results of the rat cytokine gene expression after transplantation resembled those of previously reported cytokine expression in other organ transplantation (Dallman et al. 1991; Martinez et al. 1992; Fealy et al. 1995; Krams et al. 1995). These cytokine gene expressions were observed after transplantation of xenograft (cultured human RPE), allograft (cultured rat IPE), and vehicle-only

injection. The cytokine expression after vehicle alone may reflect a response to surgical trauma. Strong expression such as IL-1, IL-6, and TNFα against surgery (transplantation) or polytrauma have also been reported (Soulillou 1995). Our findings also showed statistically significant amount of expression of IL-1α, -1β, -6, and TNFα at 3 days after transplantation. IL-1α immunoreactivities are reportedly peaked in lesioned brains between 9-12 days after injury (Tchelingerian et al. 1993). Although the expression level of IL-1α appeared higher after 30 days than 7 days of transplantation in our experiments (the level was not statistically significant), peak expression may be observed after 7 days. We examined only limited numbers of cytokine expression and used normal RCS rats, but not dystrophic RCS rats. So the difference may influence the expression of each cytokine. However, the fact that a small, but significant rescue of the dystrophic photoreceptor of RCS rats by injection with vehicle only in the early days after transplantation (Li and Turner 1991) may be explained by some of these cytokine expressions. This speculation also was supported by the fact that one of the cytokines, basic fibroblast growth factor, was reported to rescue photoreceptor degeneration in RCS rats (Faktorovich et al. 1990).

As reported by Fealy et al. (1995) cytokines behave in a time-dependent fashion at or in graft rejection. So absent or low expression of some cytokines examined here may not preclude its involvement in the course of inflammatory reaction. We also do not know which cell type expresses each cytokine gene, because we performed only RT-PCR. Further examination such as in situ hybridization will be necessary.

Although the present studies were performed using normal RCS rats but not dystrophic RCS rats and methods of transplantation were performed by a trans-scleral approach, the amplification of these cytokine genes by RT-PCR in or around the transplanted region of rats suggested that these cytokines may play an important role at the transplanted area.

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