Local Immunosuppression Prolongs Survival of RPE Xenografts Labeled by Retroviral Gene Transfer

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PURPOSE. To determine whether local immunosuppression with Cyclosporin A can influence the survival of human fetal retinal pigment epithelium (RPE) xenografts in the rabbit’s subretinal space.

METHODS. Cultured human fetal RPE cells were transduced with the gene for green fluorescent protein (GFP) using a lentiviral vector. The RPE was transplanted into the subretinal space of rabbits that received intravitreal cyclosporine either by weekly injections (0.25–0.5 mg) or by slow release (approximately 2 μg/d) from a capsule sutured into the vitreous cavity after prior cryopexy. The transplanted RPE was followed by GFP fluorescence scanning laser ophthalmoscopy and by histology of the transplant site.

RESULTS. RPE xenografts in eyes receiving intravitreal cyclosporine survived longer (several months) than they did in control eyes without cyclosporine. Survival was as long with slow release capsules as it was with weekly intravitreal injections at much higher concentrations of cyclosporine.

CONCLUSIONS. Local immunosuppression of the eye with cyclosporine prolongs the survival of RPE xenografts in the subretinal space of rabbits, implying that rejection involves activated T lymphocytes. Local immunosuppression with slow release capsules is as effective as weekly injections at much higher concentrations.

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into the subretinal space of rabbits, anesthetized with ketamine (20 mg/kg intramuscularly [IM]) and rompun (10 mg/kg, IM). The cells selected for transplantation were followed in culture after transduction with lentivirus to assess their GFP fluorescence. The percentage of cells expressing GFP ranged from 40% to 80%. The transplanted cells were not sorted for fluorescence before transplantation but were used directly after they were removed from the culture plate by trypsinization, washed with balanced salt solution, and concentrated by gentle centrifugation. A glass pipette with a tip diameter of 100 to 150 μm was used to inject the cell solution subretinally under microscopic control. Twenty-two rabbits received a transplant, occupying a space less than a millimeter in diameter, in each eye adjacent to the myelinated region of the optic nerve, which provides a landmark to find the transplant site by SLO.

Fourteen rabbits received an intravitreal injection of Cyclosporine A (CSA) weekly. Five rabbits received 0.25 mg/wk and nine rabbits 0.5 mg/wk. The solutions were obtained by volumetrically diluting a stock solution of CSA, 50 mg/ml. This is a standard solution for the intravenous administration of cyclosporine, which contains 0.65 g/ml castor oil as a solvent and 33% ethyl alcohol as a preservative. The opposite eye received an intravitreal injection of an equal volume of a balanced salt solution in the controls.

Eight rabbits were used to study the effect of slow release capsules containing only cyclosporine. The slow release capsule was sutured through the sclera at the pars plana 2 weeks after local cryopexy. Slow release capsules have been found to deliver approximately 2 μg of cyclosporine daily into the vitreous chamber. The other eye received a device consisting of polymers only without cyclosporine.

The rabbits were followed weekly by biomicroscopy, indirect ophthalmoscopy, and scanning laser ophthalmoscopy (SLO) using infrared, red and argon blue light, and fluorescence filtering for fluorescein emission. Survival of the transplant was judged by the presence of GFP fluorescence in the transplant area. When no fluorescence was detected, the transplant was considered to have been rejected. At this point the rabbit was killed and the eyes removed for histology. Three rabbits were killed at 2 to 3 weeks after all fluorescence had disappeared. One rabbit was euthanatized while there was still strong GFP fluorescence, and this rabbit was not considered in the survival data. The eyes were fixed in either 3% buffered glutaraldehyde or 4% paraformaldehyde in phosphate-buffered saline at pH 7.2. The former eyes were processed for Epon embedding and thin sectioning and the latter for cryosectioning and fluorescence microscopy. For cryomicroscopy, the paraformaldehyde-fixed sections were immersed in ornithine carbamoyltransferase compound and frozen by dry ice. Sectioning was performed on a Leica 1850 cryotome (Leica Instruments, Nusslach, Germany). Sections were mounted on gelatinized glass slides with fluoromount-G and examined by fluorescence microscopy.

The animals were treated in conformity with the Declaration of Helsinki, the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**RESULTS**

Figure 1A shows a patch of human fetal RPE in culture viewed by white light transillumination. The patch (asterisk) is heavily pigmented with nondividing RPE. Along the edge of the patch migrating, dividing cells can be seen with much less pigmentation (arrowheads). Figure 1B shows the patch of RPE in the presence of an additional strong blue light that induces GFP fluorescence. Despite their dense pigmentation, the fluorescence is intense enough to make GFP-expressing cells quite visible. Some cells (small arrows) are fluorescing more strongly than others. Many cells are fluorescing too dimly to be seen in the presence of the transilluminating white light but can be identified and counted by fluorescence illumination alone. The fraction of GFP-fluorescing cells in this patch was estimated to be approximately 70%. The fraction of GFP fluorescence obtained by lentiviral transduction varied from 40% to 80%. This fluorescence remained stable in vitro.

Figure 2 shows the appearance of a transplant site in an eye with a slow release capsule of cyclosporine at 2, 3, 4, and 12 weeks after surgery. The upper set of photographs shows the appearance of the transplant in blue light. The transplant site is a darker structure just to the left of the highly reflecting myelinated nerve fibers at the optic nerve head of the rabbit. There are several small, highly reflecting structures close to the perimeter of the transplant that are not always visible because they depend in large part on the angle of illumination (e.g., they are not very visible in the photograph at 4 weeks after transplantation surgery). The lower set of photographs in Figure 2 shows the fluorescent appearance of the retina. Here relatively small, bright structures about the size of single epithelial cells (i.e., approximately 10–30 μm) can be seen within and along the perimeter of the transplant. They do not correspond to the reflecting structures seen along the perimeter of the transplant in blue light, described previously. These GFP fluorescent structures remained similar in size, position, and brightness for the entire 12-week period after transplantation.

Figure 3 shows a transplant in the opposite eye, in which there was only a polymer device without cyclosporine. The transplant site is a darker elliptical structure, which has a dark vitreal band extending to the retinotomy. The transplant is adjacent to the myelinated optic nerve, seen on the right in the photograph (arrow) taken at 12 weeks after transplantation surgery. The lower set of photographs in Figure 3 shows the fluorescent appearance of the retina. At 2 weeks after transplantation, numerous small fluorescent cell-like spots can be seen throughout the transplant site. At 3 weeks after transplantation, many of these fluorescent spots have disappeared. At 4 weeks after transplantation, all the fluorescent spots have disappeared, which was our criterion for rejection. This is also the appearance at 12 weeks, although at that point some fluorescence is seen adjacent the transplant site. These fluorescent spots are slightly larger, less bright, and more amorphous than the GFP fluorescence. These structures show a yellow fluorescence in histologic sections and appear as lipofuscin-like particles in the host RPE layer by electron microscopy.

Figure 4 shows a transplant that has survived for 14 weeks after transplantation. In the upper set of photographs, the reflected blue light image shows the transplant site adjacent to the myelinated optic nerve. The dark spot on the right edge of the transplant is the retinotomy. In the lower set of photographs, the fluorescence of the retina shows many brightly
FIGURE 1. Color photographs showing a patch of human fetal RPE in vitro (A). The darkly pigmented cells within the patch (asterisk) have not divided; lightly pigmented, migrating cells are visible along the edge of the patch (arrowheads). (B) The same patch viewed with both fluorescence and transillumination. Green fluorescence of GFP is evident in a large proportion of the stationary cells in the patch. Some cells (arrows) fluoresce more strongly than others. (C) A frozen section of rabbit retina shows GFP fluorescent xenografts on the host RPE layer. There is a weak yellowish fluorescence considered to be lipofuscin surrounding black cytoplasmic melanin in the host RPE (arrow). (D) A frozen section of rabbit retina, viewed with both fluorescence and transillumination, shows GFP-fluorescent xenografts on top of the nonfluorescent host RPE layer (arrow at the basal margin of this layer). (E) An Epon section of a transplant site, which shows rejection. There is a dense concentration of monocytes within the subretinal space (asterisk) and the adjacent choroid (C) directly under a disrupted host RPE layer (an arrow shows the basal edge of this layer). The neural retina (NR) has few monocytes. The inflammatory response is extremely local to this area of retina. (F) An Epon section of a transplant site, which shows another example of rejection. There are two foci of monocytes in the subretinal space (asterisks) and the adjacent choroid (C). An arrow shows the basal edge of the host RPE layer. The neural retina (NR) has virtually no monocytes.
Figure 1. (Continued)
fluorescent spots, about the size of single retinal epithelial cells, within the transplant site. These fluorescent spots remain similar in size, shape, position, and brightness over time. This eye received 0.25 mg/wk of cyclosporine intravitreally.

Figure 5 illustrates a transplant that also survived for more than 7 weeks after transplantation in an eye that contained a slow release capsule of cyclosporine. In this case the retina is shown in blue, fluorescence, and infrared images. The blue light image (upper set) shows the transplant site below the myelinated optic nerve fibers. There is a vitreal band extending to the retinotomy. The transplant site is visible as a horizontal structure with a light demarcation line around it. The fluores-

Figure 2. SLO views of a transplant site at 2, 3, 4, and 12 weeks after transplantation seen by reflected blue light (upper set) and fluorescence (lower set). The transplant, located adjacent to the myelinated optic nerve fibers, is seen as a darker area surrounded by a light demarcation line in blue light and in fluorescence as a structure containing bright pointlike structures, which are considered to be GFP-expressing transplanted RPE. This eye had a slow release capsule of cyclosporine.

Figure 3. SLO views of the transplant site in the opposite eye of that shown in Figure 2. This eye had a capsule without cyclosporine, and the transplant shows evidence of rejection at 3 weeks. The upper row shows the transplant in reflected blue light; the transplant is a darker structure surrounded by a lighter demarcation line. It is adjacent to the myelinated optic nerve fiber layer seen only in the rightmost photograph (arrowhead). The lower row shows the fluorescent view. At 2 weeks, GFP-fluorescing cells are visible within the transplant. At 3 weeks, the number of fluorescing cells is reduced. At 4 weeks all fluorescence has disappeared. At 12 weeks there is also no fluorescence within the transplant; some lipofuscin-like fluorescence has developed outside the transplant site (at the upper left).
cent view (middle set) shows the GFP fluorescent transplant cells within the transplant site. These cells retain their shape, position, and brightness for the entire 7-week period. The infrared view (lower set) reveals a dark band extending through the entire transplant site. This darkness is considered to represent the added density of the transplanted RPE cells.

**FIGURE 4.** SLO views of a transplant site in an eye receiving weekly injections of cyclosporine (0.25 mg). The *upper row* shows the retina in reflected blue light. The transplant is visible by a faint demarcation line and mottling of the RPE layer; the retinotomy site is seen as a dark spot adjacent to the myelinated optic nerves on the right side of each photograph. The *lower row* shows the fluorescent view. GFP fluorescent cells can be seen for 14 weeks after transplantation.

**FIGURE 5.** SLO views of a transplant site in an eye with a slow release capsule containing cyclosporine at 2, 3, and 7 weeks after transplantation. The *upper row* shows the retina in reflected blue light, the *middle row* in fluorescence, and the *lower row* in infrared light. GFP-fluorescing cells can be seen for the entire time. The infrared view shows a darker reflected image within the transplant site produced by the increase due to the xenograft.
within the subretinal space, only a fraction of which exhibit GFP fluorescence.

This hypothesis was supported by the histology where transplanted cells identified by their GFP fluorescence could be seen on top of the host RPE layer (Figs. 1C, 1D). When rejection occurred, GFP fluorescent cells were no longer found in the retina, and when the transplant site was examined within several weeks after the fluorescence had disappeared, there was always clear evidence of rejection within the transplant site. The rejection was characterized by intense collections of monocytes in the choroid and focal areas of host RPE and photoreceptor damage (Figs. 1E, 1F). Many more monocytes were always found in the choroid adjacent to the transplant site than in the neural retina. The longer the time between the disappearance of the GFP fluorescence in the retina and the euthanatization of the rabbit, the less was the amount of monocytes detectable around the transplant site. In some cases, the major evidence for rejection was disruption of the host RPE layer and loss of photoreceptors at the transplant site.

Figure 6 compares the survival of the xenografts in the presence and absence of local cyclosporine immunosuppression. Based on the criterion of complete loss of GFP fluorescence as an indicator of rejection, locally immunosuppressed transplants survived longer than those without immunosuppression. Fifty percent of the RPE xenografts survived for at least 5 weeks when associated with local immunosuppression. Without immunosuppression, 50% of the xenografts disappeared in less than 1 month. The mean graft survival time for the controls, slow release CSA, and 0.25 and 0.5 mg CSA weekly groups was 4, 11, 10, and 9 weeks, respectively. The slow release of cyclosporine was as effective as repeated intravitreal injections of much greater concentrations. Survival of a transplant in one eye did not appear to be related to its survival in the other eye. One RPE xenograft survived for at least 10 weeks without immunosuppression, whereas all the others disappeared within 5 weeks. The loss of GFP fluorescence occurred relatively quickly, disappearing within the course of 1 week. In some cases, there was a more gradual loss of fluorescence, and this seemed more common in the transplants that survived the longest. All the transplants surviving for 15 weeks or longer had lost much of the fluorescence they originally had.

**DISCUSSION**

These results indicate that local administration of cyclosporine prolongs the survival of human fetal RPE xenografts in the subretinal space of rabbits. This supports the hypothesis that classic rejection plays a role in the survival of foreign transplants in the subretinal space. It implies that a T cell-mediated response must be involved, because cyclosporine immunosuppresses mainly, if not exclusively, by inhibiting the calmodulin-dependent phosphatase, calcineurin in T cells. That a cellular form of rejection is involved in subretinal xenograft rejection is supported by the histology, which usually showed an intense monocyte infiltration within and around the transplant site. This cellular reaction appears to diminish with time after rejection. There is a report of only mild rejection of human RPE xenografts in the rabbit and also evidence of tolerance of a fraction of human RPE grafts to the monkey for as long as 6 months. We have also observed survival of one transplant for more than 2 months without immunosuppression, whereas all the other control transplants rejected within weeks after transplantation. These differences imply that several factors are involved in the viability of these grafts.

What is interesting is that immunosuppression can be effective at a local level and with relatively low total concentrations of cyclosporine. Local immunosuppression does not entirely eliminate rejection of these xenografts, however, because virtually all disappear with time despite immunosuppression. This may be because of either the inability of local immunosuppression to completely stem the rejection process or of other factors that appear to influence the survival of these xenografts.

RPE allografts in the subretinal space seem less prone to rejection than xenografts. In mice, RPE allografts in the subretinal space survive longer than those placed in the conjunctiva and in addition induce a cell-mediated suppression of delayed hypersensitivity to graft antigen. There are reports that RPE allografts survive and continue to rescue photoreceptors from degeneration in the Royal College of Surgeons rat for relatively long times. On the other hand, there is evidence that such allografts in rats are slowly rejected but in an atypical, noninflammatory manner. In rabbits, RPE allografts have been reported to survive with or to degenerate slowly without cyclosporine immunosuppression. Small human RPE allografts have been reported to survive when placed periliminally, but larger ones, especially those placed over areas where the blood–brain barrier has been disturbed, are rejected without immunosuppression. That local cyclosporine immunosuppression could prolong the survival of RPE allografts would seem to be a reasonable expectation, but evidence that a slow allograft rejection in the rabbit subretinal space appears unaltered by systemic immunosuppression is evidence to the contrary. Further research will be needed to determine whether there are cyclosporine-resistant mechanisms mediating allograft and xenograft rejection in the subretinal space.

**Figure 6.** The survival time of each transplant as judged by the complete absence of GFP fluorescence at the transplant site. The ordinate represents the percentage of transplants that survived, and the abscissa represents the time after transplantation surgery in weeks. The controls received no CSA immunosuppression. The other three groups received either 0.25 or 0.5 mg CSA by weekly intravitreal injection or by a slow release vitreal capsule.
Such research is greatly expedited by having an in vivo monitor, which provides a means to track the behavior and survival of these retinal transplants, noninvasively. It would be much more difficult and time-consuming to follow the fate of these transplants relying on postmortem histology alone. The use of the fluorescent marker GFP allows the same transplant to be followed over time, providing clues to the onset and extent of the rejection process. We expect that even greater improvements in the resolution of SLO imaging will provide even more powerful means of following rejection and survival in the living retina in the future.

References