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MORPHOLOGICAL ASPECTS RELATED TO LONG-TERM FUNCTIONAL IMPROVEMENT OF THE RETINA IN THE 4 YEARS FOLLOWING rAAV-MEDIATED GENE TRANSFER IN THE RPE65 NULL MUTATION DOG

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1. INTRODUCTION

Retinal gene transfer studies performed in our laboratories in groups of 4-42 month-old RPE65 null mutation dogs have shown remarkable functional improvement following a single unilateral subretinal injection of a recombinant adeno-associated virus vector, serotype 2, rAAV2/2, cDNA for dog RPE65 with a cytomegalo virus (CMV) promoter (Narfström et al, 2003a,b, 2005, Ford et al, 2003). The purpose of the present investigation was to further assess long-term therapeutic effects of the gene transfer performed in a group of young RPE65 null mutation dogs and to correlate the functional effects of therapy to morphologic findings up to 4 years following the gene transfer. In addition, retinas of affected dogs were examined for histologic correlates of spots and color changes seen by ophthalmoscopy in both treated and untreated affected dogs.

2. MATERIALS AND METHODS

Gene transfer surgeries were performed during a 2-year period (2001-2002) in a subgroup of 4-11 month old dogs, derived from an initial pedigree of Swedish Briard-Beagle dogs. Details regarding the procedures and short- and long-term follow-up studies have been reported (Narfström et al, 2003a,b, 2005, Ford et al, 2003). Injections were

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performed using standard aseptic microsurgical procedures with a two-port entry into the pars plana of the eye, the first port for a light pipe and the second for a hand-drawn glass micropipette. In most cases 100 micro-liters of the rAAV.RPE65 construct was injected subretinally into the right eye and similar volumes of the rAAV.GFP construct into the left eye.

Post-operative studies included clinical ophthalmic, behavioural and ERG studies in all treated dogs as previously described (Narfström et al, 2003a). Four affected dogs that had undergone gene transfer were used for angiographic studies 2 years following the gene transfer, using indocyanine green (ICG) and fluorescein (FL) (Seeliger et al, 2006). For this, dogs were anesthetized with Medetomidine and Ketamine, pupils were dilated with 1% Isopto-Atropin, and 2 and 4 ml of ICG and FL, respectively, were injected into the cephalic vein. A Heidelberg SLO was used for fundus visualization and photography throughout the angiography procedure. At variable time periods the 4 affected treated dogs and 2 untreated affected dogs were euthanized and both eyes fixed for light- and electron microscopy. The eyecups were immersion fixed in fresh, cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Eyecups were prepared and sectioned using standardized methods for our lab (Narfström et al, 2003a). Plastic sections from three sets of eyes; from two affected dogs, treated 0.8 and 2.5 years previously, and from one affected dog that was not treated were used for light microscopic studies, including morphometric studies performed at the Lions Eye Institute, University of Western Australia. Sections from three sets of eyes; from two affected dogs, treated 3.7 and 4 years previously, and from one dog that was not treated, were sent to the Department of Ophthalmology, University of Zurich, for electron microscopy. For the morphometric studies lipid granules per 80 microns of retina were counted as well as rows of photoreceptor nuclei in 3 serial sections obtained from the following: at the injection site, in the treated area adjacent to the injection site, and in the peripheral area of the fundus. Sections were obtained from similar locations from both eyes. For the ultrastructural studies the treated and untreated eyes were compared, through examination of 12 regions from comparable areas of each eye.

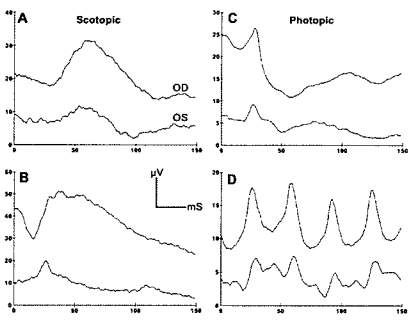

Figure 1. Results of bilateral ERG recordings from treated eye (OD) and untreated eye (OS) 4 years after subretinal gene transfer in an RPE65 null mutation dog treated at age 11 months. Scotopic recordings were performed after 2 hours of dark adaptation using -2.0 log cd.s/m^2 (A) and 0.6 log cd.s/m^2(B), respectively, of white light stimuli. After 10 minutes of light adaptation (30cd/m^2) photopic responses were obtained at 0.0 log cd.s/m^2 at 5.1 Hz (C) and 30 Hz (D), respectively.

3. RESULTS

ERG responses improved within 4-6 weeks after surgery in all treated dogs. Improved ERG responses were observed throughout the 4-year follow-up period (data not shown). Following the early enhancement of ERG amplitudes, there were successive declines mainly of scotopic low light intensity (rod) responses during the follow-up period, while scotopic high intensity (mixed rod and cone responses) and photopic single flash (cone) responses, as well as 30 Hz flicker responses (cone and inner retinal function) remained better preserved. At termination of the study the ERG responses were still improved (Fig. 1) in

comparison to pre-operative responses; barely recordable for both eyes.

Fig. 2 shows an example of angiography performed in one of the long-term studied affected treated dogs. A normal vascular pattern was observed in the fundus without leakage from the retinal vasculature. At the injection site, however, marked changes were seen, changes that could not be readily visualized by regular ophthalmoscopy (Fig. 2A). ICG showed a circular area of hypofluorescence (Fig. 2B), while FL showed hyperfluorescence in the same area (Fig. 2C).

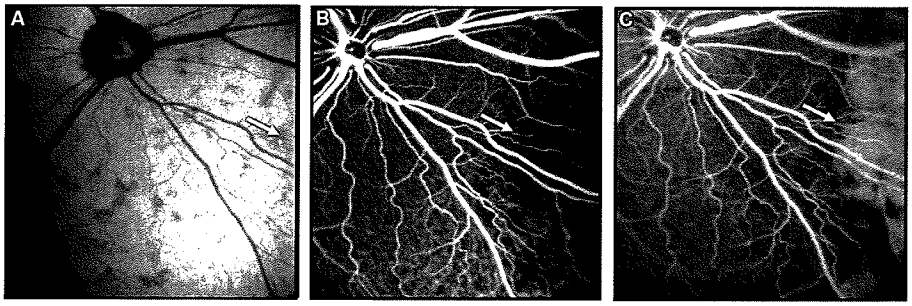

Figure 2 A. Fundus of affected treated dog 2 years after subretinal injection of the gene construct in the inferior central part of the fundus, the area shown (arrow) using SLO and infrared light in A, using Indocyanine Green IV in B and Fluorescein IV in C.

Morphometry showed that the rAAV-RPE65 treatment reduced the RPE lipoid inclusion content specifically in the injected area, shown in Fig. 3A. As seen in Fig. 3B, there was a preservation of photoreceptors in the treatment region adjacent to the injection site of the gene transfer treated eye in comparison to outside the treated area and in comparable areas in the contralateral eye. No major differences were found in amount of inclusion bodies and photoreceptor numbers when areas outside of the injection region were compared for treated and untreated eyes.

Funduscopic studies have shown an increased spotting of the fundus with age both in untreated and in gene transfer treated affected dogs. These spots could be described as irregular dark lesions and whitish, pimple-like specks, as shown in Fig. 4A. Fig. 4B shows the ultrastructure of the peripheral non-treated area of the fundus of the treated eye of the same dog from which the image in Fig. 4A was obtained: the eye was enucleated 4 years post-op. The photoreceptor layer is completely degenerated in the most peripheral parts of the fundus, the RPE cells are bulging, filled with a multitude of pigment and lipoid laden inclusions. Further, there is migration of large macrophage-like cells to the

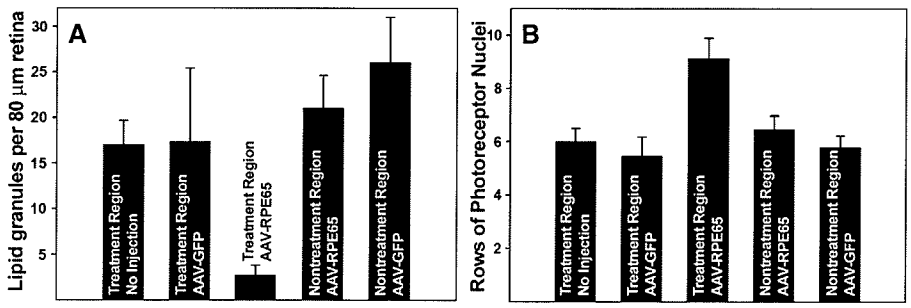

Figure 3. Morphometry of different fundic regions of treated and fellow eyes of affected dogs that had undergone gene therapy. Lipid granules and rows of photoreceptor nuclei were counted and compared in different regions.

inner retina. The latter cells are likewise filled with inclusion bodies similar to those observed in the RPE cells. Electron microscopy of the treated region in another dog studied 3.7 years after the gene therapy is shown in Fig. 5. Long and slender photoreceptor outer and inner segments are seen with only slight reduction of the number

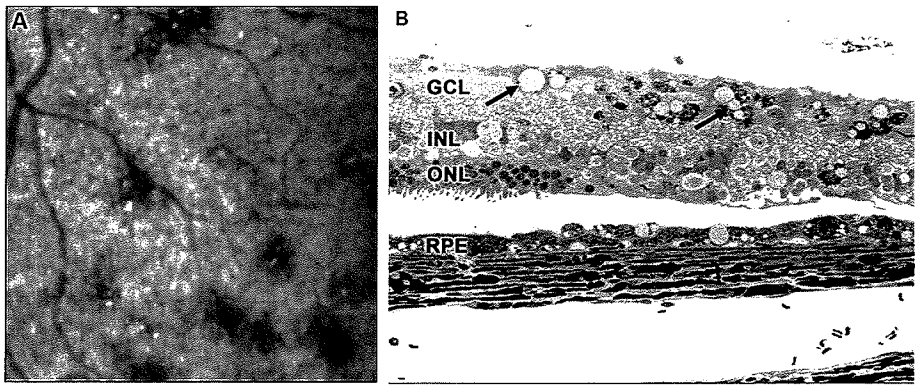

Figure 4 A. High magnification, using SLO and infrared light, of the central part of the fundus in the treated area of a dog euthanized 4 years after gene therapy. Note the pigmented spots and the pimple-like whitish specks. B. 0.5 micron light microscopic section of the same treated eye shown in A. The peripheral retina shows severe degenerative changes with distinctly swollen pigment epithelial cells that contain photoreceptor debris and lipid droplets. The entire outer nuclear layer is missing, the inner retina reveals large pigment laden phagocytic cells (arrow), clinically known as "bone spicules". Original magnification 40X. RPE: Retinal pigment epithelium, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.

of outer nuclear layer nuclei. Inner retina is preserved and mainly normal appearing. In the RPE small amounts of lipid droplets are observed.

4. DISCUSSION

Clinically, surgeries in the dogs followed up to 4 years were successful: all regained functional vision 4-6 weeks after surgery. Long-term studies using simultaneous bilateral ERGs showed that improved retinal function was preserved during the entire follow-up period. The differences between dark- and light-adapted ERGs suggest that cone function was preserved better and longer than rod function following RPE65 gene therapy. The successive decline of rod function correlated with the reduced numbers of photoreceptor cell nuclei in the retina found 4 years following the gene therapy. Eventually, the number of photoreceptor nuclei became similar when non-treated regions in both eyes were compared. In the treated eye, specifically in the bleb area, but outside of the direct injection site, ultrastructure showed areas with well preserved photoreceptor cell morphology. In these areas phagosomes were observed in the RPE, indicative of active disc shedding. The well-preserved retina was observed to encircle the specific injection site, in which photoreceptor outer segments were short and stubby, although inner segments were more normal appearing. The focal degenerative changes in the outer segments were most probably due to the trauma induced by the neuroretinal penetration by the injection needle in combination with forceful expression of fluids subretinally. The RPE cells in this area also appeared more flat than normally and slightly changed from their normal cuboidal structure. These findings corroborated the hypo- and hyperfluorescence, using ICG and FL, respectively, observed in the same area in all dogs included in the present study. Focal degenerative changes in RPE cells would cause

hyperfluorescence using FL angiography. Likewise, an accumulation of degenerative debris in the outer retina, especially in the subretinal space, would cause hypofluorescence using ICG for the evaluation procedure. Severe degenerative changes in the outer retina have been shown to follow more short-term studies of experimental retinal detachments in human, cat and rabbit, respectively (Guerin et al, 1993, Lewis et

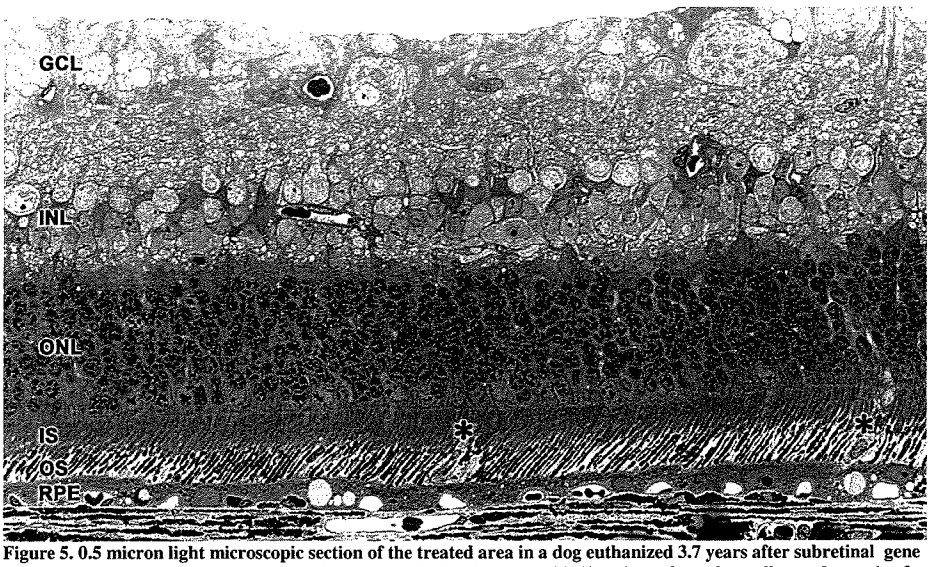

Figure 5. 0.5 micron light microscopic section of the treated area in a dog euthanized 3.7 years after subretinal gene therapy. Photoreceptors appear regularly structured, the pigment epithelium is moderately swollen and contains few lipid droplets and conspicuous nucleoli. Two phagocytic cells appear in the region of outer segments (*). Original mag.: 40X. RPE: Retinal pigment epithelium, OS: outer segments, IS: inner segments, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.

al, 2002, and Ivert et al, 2002). Long-term studies of focal detachments such as those produced through the subretinal gene transfer as performed in the present study has, to the authors' knowledge, not been previously reported. It appears that subretinal injection can have permanent deleterious effects at the injection site. Thus, if the approach to therapy is extended to humans, injection directly under the macula should be avoided.

The area of greatest therapeutic benefit appeared to be restricted to the region of the retina immediately surrounding the injection site. Outside of this region there was progression of the retinal degeneration. With advancing age, migration of giant macrophage-like cells into the inner retina was observed, even in treated eyes. It is possible that these were migrating RPE cells, given their rounded appearance, the latter due to the accumulation of partly pigmented and granular as well as lipoid-like inclusions in these cells. There was, further, a successive degeneration of photoreceptor cells that occurred, with complete atrophy of photoreceptors in peripheral parts of the retina of the gene transfer treated eye, 4 years after surgery, the dog then approximately 5 years old. This indicates that although there is a long period during which gene transfer can be effective in rescuing retinal function, eventually retinal degenerative changes would make such therapeutic intervention fruitless.

It is clear that it is only a small region, less than the actual injection area that recovers structurally after subretinal gene therapy. However, restoration of the ability of the RPE in this area to synthesize 11-cis retinoids may result in at least partial functional recovery in other parts of the retina. Eleven-cis retinoids, in particular 11-cis retinal, are

likely to reach areas of the retina outside of the treatment region via the subretinal space. The production of 11-cis retinal by cells in the area in which transgene expression occurred was apparently large enough to elicit ERG responses up to approximately 50% of those of normal dogs and to provide long-term functional vision. Although restoration of RPE65 gene function in a small area of the RPE may provide 11-cis retinal to a broad region of the retina, RPE cells lacking functional RPE65 still accumulate lipid droplets that may lead to functional impairment of these cells and secondary degeneration of the overlying retina. This degeneration likely accounts for the progressive functional decline in the treated eyes. Therefore, permanent restoration of retinal function by RPE65 gene transfer will likely be restricted to the area of the retina directly overlying regions of the RPE that express the transgene.

Because permanent damage to the retina was observed at the site of subretinal injection, future studies should be directed towards the elucidation of less traumatizing treatment regimes for gene therapy that deliver the transgene to wider areas of the RPE. It is clear from the successful gene transfer studies now performed in three different strains of RPE65 null mutation dogs (Acland et al, 2001 and 2006, Narfstrom et al, 2003 a and 2005, Le Meure et al, 2006) that retinal gene therapy is feasible and that long-term alleviation of visual impairment is possible.

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