CHAPTER 44

CONE SURVIVAL: IDENTIFICATION OF RdCVF

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1. INTRODUCTION

The foremost cause of irreversible blindness in major retinal diseases is photoreceptor degeneration. In animal models as well as in human retinal hereditary dystrophies, the mutations described since 1990 affect mainly coding sequences for structural proteins (peripherine, Rom 1) or components of the phototransduction cascade (rhodopsin, cGMP-dependent phosphodiesterase) found in the rod outer segments.[1,2,3] The mechanisms leading to programmed cell death of these cells are still hypothetical.[4] In addition to this direct rapid rod loss, delayed cone loss is seen in clinical situations and was described in 1978 in the "retinal degeneration" (rd) mouse model.[5] Their loss is responsible for the major visual handicap because cones are essential for diurnal, colour and central vision.[6] This secondary loss of cone photoreceptors does not have any obvious explanation since cones are generally not directly affected by the genetic anomaly found in these diseases.

In several models leading to selective rod loss, such as transgenic mice[7] or mice carrying a spontaneous mutation,[8] secondary cone loss is observed whereas the causal abnormality is not directly incriminated in their degeneration. In certain studies the link between rod loss and cone drop-out is still hypothetical. The cellular interactions involved in cone survival have never been the subject of a systematic experimental approach, and can be amply justified through the major perspectives in fundamental neurobiology and therapeutic outcomes. Taking into account the multiple cone functions, preservation of this population would open an original avenue of therapeutic investigation which would enable a considerable limitation of functional consequences for the patients.

2. ROD SECRETE FACTOR(S) ABLE TO PROMOTE CONE SURVIVAL

Our approach is based on the observation that in retinal pathologies (mutant animal models and patients) rod degeneration precedes that of cones. Our efforts initially focused

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on selective rod replacement, obtained by use of a vibratome sectioning method. Our first results demonstrated that when pure photoreceptor layers were transplanted to the subretinal space in 5 week old *rd1* mice [at this stage very few rods remain (<0.02%) whereas about 30% of the cones are still present] the transplant induces a significant increase in the number of surviving cones (an average increase of 30%, $p < 0.001$) compared to that of non-treated congenic retina.[9] This trophic effect can be detected distant from the transplant, suggesting the existence of a diffusible factor liberated by the transplanted cells. Tests carried out *in vitro* on *rd1* retina and wild type retina co-cultures confirmed this hypothesis.[10] This study showed that about 40% of cones normally lost during the sixth postnatal week are saved when cultured in the presence of rod-rich samples. This effect is photoreceptor specific since transplants of inner retina exert no beneficial effects on cone survival.[11,12] The neuroprotective activity (40-50% increase in viability) on cones is heat labile and has an apparent molecular weight larger than 15 kDa.[13] These experiments indicate that the activity is carried out by protein(s): the Rod-dependent Cone Viability Factors (RdCVFs).

Taken together, these assays show the existence of at least one trophic factor secreted by rods that is essential for cone survival. Rod degeneration in *rd1* mice and RP patients might hence lead to survival factor deprivation and consequently progressive cone loss. A key implication is that prevention of apoptotic rod death could lengthen cone survival. Such a mechanism, which has never been proposed prior to our work, provides a justification for the numerous strategies aimed at preserving non-functional rods, since these will protect cones.[14-19] In humans, diagnosis is often made at late stages, and in the absence of residual rods only PR transplantation (mainly rods) could restore the expression of these factors allowing to block or reduce secondary cone degeneration.[19] The potential clinical importance of such factors is obvious, cone loss representing the main cause of visual handicap in RP and AMD. Of importance, since cone loss is a late-onset downstream event and occurs independently of the specific mutation expressed by rods, a potentially broad number of patients could benefit from such therapy.

The observed trophic effect of rod transplants on cone survival suggests that non-functional rod protection could be sufficient to preserve cone viability.[10,12,19,20]

3. IDENTIFICATION OF RdCVF

Our systematic approach to characterize factors involved in cone viability has led to identification of a new gene (RdCVF) encoding a secreted protein for which we will explore its functions.

We postulate that the degeneration of rods of the *rd1* mouse retina is leading to the loss of expression of secreted protein factor(s) essential for cone viability. This mechanism of cone degeneration is also likely in human retinas affected with RP.[25] The identification of the genes encoding these Rod-dependent Cone Viability Factors is a prerequisite to a therapy aimed at preventing the secondary loss of cones and of vision.

To identify the RdCVF genes, we used a systematic strategy based on a functional assay using cone–enriched cultures. We developed a high throughput cone-enriched culture system using chicken retina.[26,27] Contrarily to the mammals, birds have retinas dominated by cones. In these cultures, the primary postmitoting cells (60-80% cones) are degenerating over a period of few days. An increase in cell survival was observed when cultured in the presence

of conditioned media isolated from wild-type mouse[13]. The viability activity on chicken cone, as for RdCVF, is heat labile has an apparent molecular weight larger than 15 kDa. The chicken embryo retinal culture system is an easy, reproducible and high throughput cone viability assay.[27]

We constructed an expression library from wild type mouse retina and tested all the genes for their potential to promote chicken cone survival by expression cloning methods. Briefly, pooled by 100 clones from the expression library were transfected into a cell line (COS-1). The conditioned media from the COS-1 tranfected cell were added to primary chicken cone cells seeded into 96 well-plates. After 7 days, viable cell counts from the cone-enriched cultures were measured using in house high content screening methods and compared to that of empty library vector. Twenty-one hundred pools, corresponding to 210,000 individual clones, were screened. A Pool (number 939) contained twice as many living cells as the negative controls. By limiting dilution clone 939.09.08 was isolated and shown to contain a 502-bp insert with an open reading frame encoding a putative 109-amino acid polypeptide. We named this gene Rod-derived Cone Viability Factor. The novel gene carries many characteristics of the postulated therapeutic gene:

1) Purified recombinant RdCVF protected chicken cones in a dose dependent manner.
2) RdCVF, when transfected into COS-1 cells exerts its survival activity on cones from *rd1* retinal explants, the model of the degenerative disease.
3) Purified recombinant RdCVF protected mouse cones when injected into the sub-retinal space on the *rd1* mouse.
4) RdCVF protein is detected in conditioned media from wild-type retina and COS-transfected cells.
5) RdCVF messenger RNA and protein expression is largely decreased in a rod-less mouse retina (the degenerated *rd1* retina).
6) RdCVF is expressed by pure cultures of photoreceptors from mouse (97% rods).
7) Immunohistochemistry demonstrated that RdCVF localized in the photoreceptor layer of the retina with a more intense staining in the extracellular matrix surrounding cone cells.
8) RdCVF antibodies are able to block the neuroprotective effect generated by wild-type conditioned media.

In addition, RdCVF expression was found to be restricted to the retina. RdCVF encodes for two polypetides of 17 and 34 kDa by alternative splice, the longer form being extended in its C-terminal region. Both forms have a limited homology with the thioredoxin family and the gene was named accordingly Txnl6 (Thioredoxin-like-6) in the databases. We could not demonstrate any thiol-oxidoreductase activity for the isolated polypeptide (the 17 kDa form). The founder member of the thioredoxin family, Trx-1 has been isolated originally as the adult T-cell leukemia-derived factor[28] a factor secreted by cells by a mechanism that does not involve a signal peptide sequence,[29] a signal also absent in RdCVF.

4. CONCLUSION

Mutation-independent therapies offer a means of slowing down or even stopping photoreceptor degeneration process in the medium term. They would be applicable to most patients regardless of the genetic defect and limit their handicap. Such therapies are based

on the use of either transfer of antiapoptotic genes to the photoreceptors or, more readily, delivery of neuroprotective factors. The endogenous rod-derived cone viability factors (RdCVFs) are some of the most appropriate neuroprotective candidates. In addition, the development of eye delivery methods using genetically engineered and encapsulated cells implanted directly into the vitreous offers a new means of long-term controllable and reversible neuroprotective treatment. Finally, progress in the understanding of endogenous neurogenesisn will perhaps provide new possibilities of treatment in the late stages of RP.

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