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ScienceDirect
Experimental Eye Research 86 (2008) 734–745
EXPERIMENTAL EYE RESEARCH
www.elsevier.com/locate/yexer

Generation of transparency and cellular organization in lens explants

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Received 7 November 2007; accepted in revised form 29 January 2008
Available online 3 February 2008

Abstract

The lens grows via the proliferation and differentiation of lens epithelial cells into lens fibres. This differentiation process, thought to be controlled by factors present in the vitreous fluid, generates tightly-packed, parallel-aligned fibre cells that confer transparency to the lens. Using lens epithelial-cell explants we examined how explant orientation and growth factor treatment can affect cellular arrangement and explant transparency. Fibre cell differentiation was induced in lens explants by culturing cells with fibroblast growth factor (FGF) or bovine vitreous. Cell shape and arrangement was investigated using confocal microscopy, electron microscopy, immunofluorescence and in situ hybridization. Explant transparency was measured using light microscopy. Confocal microscopy demonstrated that explant orientation determined cellular arrangement, irrespective of the differentiation stimuli used. In explants where epithelial cells were confined between their normal basement membrane (the lens capsule) and the base of the culture dish, the cells became elongated, thin and parallel-aligned. In contrast, in explants cultured with cells directly exposed to the culture media the cells appeared to be shorter, globular and haphazardly arranged. FGF initiated the differentiation of most lens epithelial cells; however, abnormal cellular morphologies developed with subsequent culture of the cells. As a result, the transparency of these explants decreased with prolonged culture. Interestingly, explants cultured with vitreous (i) did not develop abnormal cellular morphologies, (ii) contained two distinct cell types (retained epithelial cells and newly differentiated fibre cells) and (iii) remained transparent throughout the lengthy culture period. In summary, we have developed a culture system that generates a transparent tissue with a cellular arrangement resembling that of the lens *in vivo*. We have shown that while FGF and vitreous initiate differentiation within this system, better maintenance of fibre cell integrity, more appropriate regulation of molecular events, and better maintenance of explant transparency was achieved in the presence of vitreous. This system offers an opportunity to further investigate the process of lens fibre cell differentiation as well as a means of better identifying the factors that contribute to the development of tissue transparency *in vitro*.

Keywords: lens; explant; fibre differentiation; fibroblast growth factor; vitreous; transparency

1. Introduction

The crystalline lens is a transparent tissue composed of epithelial and fibre cells which are surrounded by a thick basement membrane, the lens capsule. The cuboidal lens epithelial cells are contained as an anterior monolayer while the elongated fibre cells form the bulk of the lens (Mann, 1964; McAvoy, 1980). This cellular arrangement is maintained by the polarized distribution of the two ocular fluids, the aqueous and the vitreous, which are respectively found anterior and posterior to the lens equator (Coulombre and Coulombre, 1963). Growth of the lens occurs largely through the coordinated differentiation of epithelial cells located at the lens equator, into secondary fibre cells via the influence of factors supplied by the vitreous (Coulombre and Coulombre, 1963).

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0014-4835/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.exer.2008.01.020

As part of this process, epithelial cells elongate in parallel with each other to produce a tightly packed tissue with limited extracellular space (Kuwabara, 1975; Kuszak, 1995). It is this close packing of fibre cells, in combination with their unique composition, that is thought to confer transparency to the lens (Asano et al., 1995; Benedek, 1983; Delaye and Tardieu, 1983; Trokel, 1962).

Numerous studies conducted *in vivo* and *in vitro* have provided compelling evidence that the fibroblast growth factor (FGF) family plays an important role in normal mammalian lens development (Lovicu and McAvoy, 2005). For example, over-expression of FGF family members in transgenic mice has shown that some (Lovicu and Overbeek, 1998; Robinson and Overbeek, 1995; Robinson et al., 1998), though not all (Stolen et al., 1997), are capable of initiating inappropriate differentiation of the lens epithelial monolayer. Other transgenic studies, in which truncated FGF receptors were over-expressed, have shown that lens fibres require FGF signalling during early and late phases of differentiation and possibly for survival of the terminally differentiated fibres (Chow et al., 1995; Robinson et al., 1995; Stolen and Griep, 2000). Further studies have shown that when the genes coding for the FGF receptors normally present in the lens were deleted, fibre differentiation does not occur (Zhao et al., 2003).

Experiments performed *in vitro* using explanted lens epithelial cells have shown that both recombinant FGF (Le and Musil, 2001; Lovicu and McAvoy, 2001), as well as FGFs purified from ocular fluid (Schulz et al., 1993) or other sources (Chamberlain and McAvoy, 1989; Lovicu and McAvoy, 1989, 1992), are capable of stimulating epithelial cells to undergo changes characteristic of lens fibre differentiation *in vivo*. These changes include cell elongation, expression of fibre specific proteins and accumulation of complex membrane specializations (Chamberlain and McAvoy, 1989; Le and Musil, 2001; Lovicu and McAvoy, 1989, 2001; Schulz et al., 1993). However, little has been reported on the ability of FGFs to reproduce, *in vitro*, the unique cellular arrangement characteristic of the lens *in vivo*. Similarly, few studies have reported on the functional properties of the differentiating lens explants such as, their ability to transmit light (O'Connor and McAvoy, 2007). For the present study, we investigated the influence of explant orientation on the cellular arrangement of differentiating cells in individual FGF-treated explants. Furthermore, the ability of FGF and vitreous to induce fibre differentiation was investigated, with an emphasis on their ability to maintain explant transparency during the culture period.

2. Materials and methods

All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.1. Vitreous collection

Bovine eyes were placed on ice immediately after harvesting and were then transported to the laboratory. The ocular fluids were then collected within approximately 6 h of the eyes being harvested as previously described (Schulz et al., 1993). Briefly, aqueous fluid was collected using a sterile syringe and 22G needle inserted into the anterior chamber through the central cornea, after which the anterior portion of the eye was removed with the lens attached. Vitreous fluid from twenty eyes was collected and pooled using a sterile syringe without a needle. The pooled vitreous was homogenized using a syringe and 19-gauge needle, aliquoted, and stored at −20 °C for up to 4 weeks.

2.2. Tissue culture

Rat lens epithelial cell explants were prepared based on methods previously described (McAvoy and Fernon, 1984). Briefly, postnatal day 10 Wistar rats were euthanized before their eyes were removed and placed in pre-warmed, serum-free medium 199 (M199) with Earle's salts supplemented with 0.1% bovine serum albumin, 0.5 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml Amphostat (all from Trace Scientific, NSW, Australia).

Using a dissecting microscope and jewellers-forceps, the eyes were immediately torn at the optic nerve to release the lens. Lenses were placed in pairs into pre-warmed M199 and equilibrated at 37 °C, 5% CO_2 for approximately 15 min. Epithelial cell explants were collected by gently tearing the lens capsule adjacent to the posterior suture and slowly removing the lens fibre mass. Explants were pinned to the culture dish with either the capsule in contact with the culture dish and the cells directly bathed by the media (i.e. standard explant), or alternatively, with the cells in contact with the culture dish and the capsule directly bathed by media (i.e. inverted explant). In order to obtain a uniform population of epithelial cells, the peripheral regions of the explants were trimmed away to ensure that any cells already stimulated to differentiate *in vivo* were not present during the culture period. Explants were cultured in M199 containing either 50, 100 or 150 ng/ml human recombinant FGF2 (Peprotech, Rocky Hill, USA), or in bovine vitreous diluted 1:1v/v with M199. The culture media (including growth factors or vitreous) was changed every fifth day of culture.

2.3. Confocal microscopy

The cellular arrangement of differentiating cells within explants was analysed under the confocal microscope using the membrane stain DiOC6 (3,3′-dihexyloxacarbocyanine iodide, Molecular Probes, Eugene, USA) and the nuclear stain EtBr (ethidium bromide, Sigma, St. Louis, MO, USA). Explants were fixed for 20 min with 10% neutral buffered formalin (NBF), washed in phosphate-buffered saline (PBS) and stained overnight at 4 °C with 5 μg/ml DiOC6 and 30 μg/ml EtBr in 70% ethanol. Stained explants were washed repeatedly at room temperature with PBS before being mounted in 10% PBS in glycerol. Images of stained explants were obtained using a Bio-Rad Confocal Scanning System and accompanying software (LaserSharp2000, Bio-Rad, Hercules, CA, USA).