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Review

Corneal epithelial cell cultures as a tool for research, drug screening and testing

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Abstract

Understanding of visual system function and the development of new therapies for corneal diseases and damages depend upon comprehension of the biological roles of the tissue. The *in vitro* cultivation of corneal epithelial cells and cell lines derived from them has become a powerful tool to analyze and understand such issues. Currently, researchers have developed well-defined and precisely described culture protocols and a collection of corneal epithelial cell lines. These cell lines have been obtained through different experimental approaches: (1) the ectopic expression of oncogenes, (2) the inactivation of p16 and p53 pathways and hTERT expression, and (3) the spontaneous establishment after serial cultivation of cells. The advantages or disadvantages for these approaches are discussed. In conclusion, the availability of several culture protocols and immortalized cell lines that express corneal epithelial phenotype will be useful for investigating issues such as gene regulation and tissue development, or for validating alternative methods in toxicology.
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Keywords: corneal epithelial cells; cell culture; cell immortalization; cell differentiation; drug screening; *in vitro* assay

1. Introduction

Understanding of visual system function and the development of new therapies for ocular diseases and damages depend upon comprehension of the biological roles of the tissues that constitute the eye. The cornea is the first and most powerful refracting medium in the eye. Light passes through the transparent cornea on its way to retina. It has a greater curvature than the rest of the eyeball and a refractive power of approximately 42 dioptres. The cornea constitutes the anterior surface of the eye, and consists of three cellular layers (corneal epithelium, stroma and Descemet's endothelium), which are separated from each other by two thin, acellular layers (the Bowman's and Descemet's membranes) (Hogan et al., 1971).

The anterior surface of mammalian cornea is covered by a stratified squamous epithelium, which is contiguous to conjunctival epithelium through a transitional zone called the limbus (Hogan et al., 1971). Compared with other stratified squamous epithelia such as epidermal and esophageal, the corneal epithelium shows distinctive characteristics that make it unique. The first resides in its structure; given that corneal epithelium lies onto an extremely flat stroma with no rete ridges, individual basal cells undergo an exceptionally excessive demand to maintain the highly integrated balance between proliferation, differentiation, and cell death (Lu et al., 2001; Lavker et al., 2004). The second consists in its apparent lack of protection; since corneal epithelium is totally unpigmented, it is considered that all its basal cells are exposed to damage by external agents such as solar radiation. In spite of such conjecture, corneal carcinomas are exceedingly rare, being most of them associated to the limbal zone (Garner, 1989; McKelvie et al., 2002; Miller et al., 2005). In view of evidence, it has been proposed that in corneal epithelium, the stem cells and their early precursors are exclusively circumscribed to the narrow vascularized rim that separates the cornea from the conjunctiva: the limbus (for extensive reviews see Wolosin et al., 2004; Lavker et al., 2004; Chee et al., 2006).

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Several *in vitro* systems have been used to analyze issues such as corneal epithelial wound healing, the control of gene expression during corneal cell differentiation, and the screening of drugs, chemicals or agents prior to final testing in animal models. Among them, the most important are those assays on isolated bovine corneas (Muir, 1985), isolated eyes (Burton et al., 1981; Maurice and Singh, 1986; Doughty, 1997), or in cultured epithelial cells (Jumblatt and Neufeld, 1986; Simmons et al., 1987; Bracher et al., 1987; Grant et al., 1992).

Whereas experimental models vary from isolated cells to animals, cell culture has been used to substitute or complement the Draize eye irritation test to assess pharmacological and toxicological effects of chemicals. The Draize assay was developed at mid-twentieth century (Draize and Kelley, 1952; Draize et al., 1944), and was incorporated into FDA regulation in 1964. This method has been questioned since its performance and reproducibility are limited (York and Steiling, 1998). Among its limitations, the instillation of a fixed amount of a test compound onto the rabbit eye is not necessarily consistent and realistic (Prinsen, 2006). Moreover, the high number of variables involved in the assay, including tear production, blink frequency, time contact and dosage depend on the chemical nature of the compound (liquid, pastes or solid), reduces the ability of the test to predict the potential irritation caused by chemicals (Prinsen, 2006; Vinardell and Mitjans, 2007). Despite criticisms, this *in vivo* test has been used in the clinical setting for a long time and remains as the reference protocol. Thus far, no *in vitro* alternatives have been validated as a replacement (Ubels and Clousing, 2005; Secchi and Deligianni, 2006). However, possible alternatives to assess eye irritation potential are among others: the low volume eye test (LVET) which instillates directly on the rabbit eye cornea 10 μl of the test material (Gettings et al., 1996, 1998), the red blood cell (RBC) hemolysis assay which is particularly valid for the study of surfactants (Pape et al., 1987), and the chorioallantoic membrane (CAM) test, which shows a good linear correlation with the *in vivo* assays (Lagarto et al., 2006; Macián et al., 1996).

1.1. Corneal epithelial cell culture

The ocular surface is a highly structured system whose complexity makes difficult to develop alternative tests with similar physiological, structural and metabolic characteristics. For these reasons, animal tests have been difficult to eliminate. While animal testing is required by law to guarantee minimum safety standards for the licensing of drugs and chemicals, the regulations to perform animal tests in basic research are variable depending on the country and on the application of ethical principles and guidelines (Gruber and Hartung, 2004; Rusche, 2003). Such regulatory variation has led to concerns on animal welfare, and prompted a growing pressure to minimize pain and distress to laboratory animals. Therefore, cultivation of cells offers many potential advantages in drug and compound testing. In particular, cell culture opens the possibility to decrease the number of animal experiments and increases the potential for manipulation of the environment or cellular properties.

Moreover, since corneal epithelium is the first structure to show trauma after exposure to diverse agents (Maurer et al., 1997; Furrer et al., 2000; Pitts et al., 1987, 1977), cultivation of corneal epithelial cells becomes an excellent alternative for testing of ocular drugs and formulations. Such test systems require the organization of cells into an epithelial stratified structure, because corneal epithelium is the barrier that limits permeation of compounds into the eye tissues. From the perspective of drug discovery and design, corneal cell cultures are also crucial given that they can be used to accelerate the identification of compounds with favorable pharmacokinetic properties and to evaluate structure–absorption and structure–metabolism relationships on a large scale. Large-scale cell cultivation permits the development of new *in vitro* tests, required to evaluate minute quantities of the numerous compounds produced through the modern combinatorial and automated methods of drug synthesis. Therefore, a maximal predictive information should be gained from the *in vitro* use of small quantities of the test substances, a goal only accessible through cell culture assays. In spite of these advantages, cell culture systems also show limitations, mainly related with the complexity of the mechanisms involved in the response of ocular tissues to an irritation (blinking, tear fluid, inflammation, etc.).

To use cell culture as a substitute of animal models or other *in vitro* assays, researchers must address a series of limitations to assure the long-term survival and a stable expression of differentiated phenotypes in the cultured cells. For epithelial cells, this was possible after landmark papers by Rheinwald and Green (1975a,b) and Hennings et al. (1980). In the first approach, keratinocytes grown in the presence of 3T3-feeder layers form stratified colonies with the same basic organization as normal epidermis, proliferation takes place in the basal layer and the cells undergo terminal differentiation as they move through the suprabasal compartment (Rheinwald and Green, 1975a,b; Sun and Green, 1976; Watt, 1983). The second method relies upon the absence of 3T3-feeder cells, and on the growth of cells in medium containing a low Ca^{2+} concentration, consequently, cells grow as a monolayer because desmosome assembly is inhibited (Hennings et al., 1980; Hennings and Holbrook, 1983; Watt, 1984; Watt et al., 1984; Poumay and Pittelkow, 1995). Although differentiation of stratified epithelial cells, cultivated by either of these methods, can deviate from that of normal epithelium, such difference results from phenotype modulation by environmental conditions (Sun and Green, 1977; Doran et al., 1980; Lavker and Sun, 1983; Stanley and Yuspa, 1983; Pillai et al., 1990). So far, both methods have been successfully used for growth, subcultivation and cloning of different epithelial cell types such as urothelial (Wu et al., 1982; Phillips and Rice, 1983; Surya et al., 1990; Southgate et al., 1994; Zhang et al., 2001), esophageal (Banks-Schlegel and Harris, 1983; Resau et al., 1990), or conjunctival (Wei et al., 1993; Gipson et al., 2003; Ang et al., 2004; Paladino et al., 2004).

After the establishment of conditions for the long-term serial culture of keratinocytes in the presence of 3T3-feeder cells, mammalian corneal epithelial cells were successfully

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