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Antibiotics delay *in vitro* human stem cell regrowth

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ABSTRACT

Stem cell line from human limbal area was established to study *in vitro* cell growth and response to the toxic effects of antibiotics used in ophthalmology in terms of cell migration rates and structure of interphase chromatin. Recovery from cellular damages caused by ophthalmologic antibiotics was mimicked by an *in vitro* scratch model and followed by time-lapse microscopy, scanning electronmicroscopy and chromatin image analysis. Experiments revealed that broad spectrum antibiotics, chloramphenicol (0.5–1.0 mg/ml) and rifampicin (0.1–0.2 mg/ml), corresponding to concentrations in common clinical practice, slowed down the regeneration process. Results show that nuclei of naturally occurring limbal cells contain the same intermediates of chromatin condensation as seen in mammalian tumor cells and follow the common pathway of chromosome condensation. These intermediates included decondensed veil-like chromatin, fibrillary chromatin, supercoiled ribbon, chromatin bodies, early linear forms and metaphase chromosomes. Upon chloramphenicol and rifampicin treatment characteristic distorsions took place in the intermediates of chromosome condensation. Damaging effects in limbal stem cells in the presence of chloramphenicol or rifampicin indicate that ophthalmologic treatment with antibiotics should be used cautiously.

1. Introduction

The cornea transmitting the light to the retina is covered by epithelial cells and surrounded by a narrow band of limbal cells. Similarly to other epithelia, the epithelium of the cornea is maintained by stem cells that migrate on the corneal surface centripetally as differentiated cells (Kinoshita et al., 1981; Tseng, 1989; Sun et al., 2010). Stem cell population for the corneal epithelium is located at the area known as limbus forming a narrow zone between the cornea and the bulbar conjunctiva and the sclera and mediate corneal renewal and repair (Secker and Daniels, 2009). (Fig. 1). The highest clonogenicity, a feature of limbal stem cells, is found in the smallest keratinocytes. The diameter of these small cells located in the limbal basal epithelium is 10.1 ± 0.8 μm (Romano et al., 2003). This limbal stem cell population is responsible for the renewal of the corneal epithelium. Loss of limbal function may result in painful blindness (Ahmad, 2012). The isolation of limbal stem cells remained a challenging task due to their colony forming potential, restricted availability of donor corneal tissue for corneal renewal and repair and by the low probability of survival of grafted tissue upon keratoplasty (Majo et al., 2008; Albert et al., 2012). The clonal growth of limbal stem cells was stimulated by fetal bovine serum (Kruse and Tseng, 1993), but presented the risk of transmission of animal-borne viruses or acquisition of antigenic substances on the cell membrane during culturing limbal stem cells (Baylis et al., 2011; Shortt et al., 2007). The risks of cultivating cornea limbal epithelial stem cells have been eliminated by animal material-free medium (Albert et al., 2012). Beside the possibility of rejection of transplanted limbal cells, during the wound healing there is a further considerable risk, namely the infection, which is normally minimized by autologous serum, prophylactic topical antibiotics, steroid and nonsteroid eyedrops (Dua et al., 2010). Oral steroids and oral cyclosporin were used to prevent rejection and graft failure after penetrating keratoplasty (Frucht-Pery et al., 1988).

After limbal stem cell transplantation topical forms of corticosteroids, e.g. betamethasone 0.1% eye drops and ointments, antibiotics such as 0.5% chloramphenicol drops (Kheirkhah and Karimian, 2010) or chloramphenicol with prednisolone 0.5%

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eyedrops have been used (Dua and Azuara-Blanco, 2000). Among the semisynthetic antibiotics, rifampicin has been applied as a 1% ointment to prevent the growth of *Chlamydia trachomatis* and *lymphogranuloma venerum* (Fraunfelder and Meyer, 1982) through the inhibition of DNA-dependent RNA polymerase (Becker, 1972; Becker et al., 1970). Data regarding the cellular toxicity of antibiotics are scarce. Thus, before their medical use it is reasonable to test and compare the *in vitro* damaging and beneficial balance of antibiotics.

In this paper we describe the isolation of limbal stem cells for *in vitro* toxicological tests. Limbal cells were obtained as a small part of the limbal collar that was subjected to cornea transplantation and regeneration. From the primary culture human limbal stem cell line (Hu/Li) was established consisting of immortal cells. The cell growth and regeneration process of limbal stem cells was tested in the presence of two broad spectrum antibiotics, chloramphenicol (0.5–1.0 mg/ml) and rifampicin (0.1–0.2 mg/ml). Structures of condensing chromosomes of stem cells have not been isolated earlier. Chromatin structures of healthy limbal cells resembled closely to other mammalian tumor cells visualized earlier (Banfalvi et al., 2006). Genotoxicity specific chromatin changes induced by antibiotics caused the delayed regeneration of the damaged limbal cell monolayer.

2. Materials and methods

2.1. Materials

DABCO (1,4-diazobicyclo-(2,2,2)-octane), chloramphenicol, rifampicin, Penicillin–Streptomycin–Neomycin antibiotics (PSN-375963) were from Sigma–Aldrich, Budapest, Hungary. 2,6-diamino-2-phenylindole (DAPI) was the product of Braunschweig Chemie (Braunschweig, Germany). Dextran T-150 was purchased from Pharmacia-Biochemicals (Uppsala, Sweden). Colcemid (N-methyl-N-deacetyl-colchicine) was the product of Boehringer (Mannheim, Germany). The molecular formula of chloramphenicol is $C_{11}H_{12}Cl_2N_2O_5$, chemical name: D-*threo*-(−)-2,2-Dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl] acetamide, molecular weight: 323.13 D. The chemical formula of rifampicin is (3-(4-Methyl-piperazinyliminomethyl)-rifamycin), molecular formula: $C_{43}H_{58}N_4O_{12}$, molecular weight: 823.0 D. Rifampicin is a naphthohydroquinone spanned by an aliphatic ansa chain (Tomiyama et al., 1996). In an attempt to eliminate bacterial contamination, PSN antibiotics were used as a sterile-filtered solution containing penicillin, streptomycin and neomycin. PSN antimicrobial antibiotics are distinguished from ophthalmic antibiotics (chloramphenicol, rifampicin). Antibiotics in this publication refer to chloramphenicol and rifampicin, unless otherwise noted.

Sterile stock solutions of antibiotics, 5 mg/ml chloramphenicol in physiological saline and 1 mg/ml rifampicin in sterile physiological saline were obtained from the Pharmacy of Medical Center, University of Debrecen, prepared according to the Formulae Normales Edition VII, National Institute of Pharmacy, Budapest. Further dilutions from stock solutions were made with Dulbecco's Modified Eagle's Medium Nutrient Mixture (DMEM-HAM'S F12) (Sigma–Aldrich, Budapest, Hungary) supplemented with 2 mM L-glutamine, 23 mM sodium bicarbonate, 10% Fetal Bovine Serum (FBS) and 1% PSN. Fetal bovine serum and collagenase type IV (160 units/mg) were bought from GIBCO BRL, Life Technologies (Gaithersburg, MD). Collagenase is a unique protease with the ability to degrade the triple-helical native collagen fibrils commonly found in connective tissue such as skin and other epithelial tissues and is preferentially used in stem cell technology. Betadine antiseptic solution (Povidone-iodine, 10%, topic solution, equal to 1% available iodine) was obtained from Egis Pharmaceuticals PLC, Budapest, Hungary. Eusol-C corneal storage medium containing 143 μg/ml gentamicin was the product of Alchimia SRL, Padova, Italy.

Antifade Medium consisted of 90% glycerol, 2% (w/w) DABCO, 20 mM Tris–Cl, pH 8.0, 0.02% sodium azide and 25 ng/ml DAPI for blue fluorescent total staining of DNA. Hypotonic Buffer for reversible permeabilization contained 9 mM HEPES, pH 7.8, 5.8 mM dithiothreitol, 4.5% dextran T-150, 1 mM EGTA and 4.5 mM $MgCl_2$. Swelling Buffer consisted of 50 mM KCl, 10 mM $MgSO_4$, 3 mM dithiothreitol and 5 mM $NaPO_4$, pH 8.0. Fixative solution contained methanol : glacial acetic acid (3:1).

2.2. Establishment of human limbal stem cell line (Hu/Li)

Removal of fibroblasts from stem cell culture Stem cells of the limbus form a narrow zone between the cornea and the bulbar conjunctiva (Fig. 1). First we have cultivated the corneal disc under standard conditions. The observed spread of the corneal disc was accounted for by the fibroblasts that migrated from the corneal matrix, attached to surface of the flask, pulled and flattened the corneal disc (Video 1). To avoid the fibroblast supply by the cornea, the stem cell rich limbal collar was isolated and subjected to collagenase IV digestion and further growth of limbal cells. The viability of fibroblasts is known to last only for 5–6 days. After one week only the limbal stem cells were growing. The stem cell culture was tested by four tumor and two stem cell markers. Tumor and hematopoietic stem cell negative as well as cytokeratin 19 epidermic marker positive tests confirmed the limbal origin of our cell culture (Table 1).

Isolation of limbal stem cells Detailed description of isolation was as follows. Corneal epithelial limbus was obtained from the enucleated eye of the cadaver of a 56-year-old female patient, whose eye was intact previously. We followed the European Community (EC) Tissues and Cells Directive 2004/23/EC on presumed consent practice for tissue collection. All examinations and sample collections were conducted according to the tenets of the Declaration of Helsinki and followed the guidelines of our Institutional Ethics Committee. The isolation procedure followed strict guidelines (Albert et al., 2012). The corneal disc was stored in Eusol-C storage liquid at 4 °C. For medical transplantation the central corneal disc was used in full thickness. For *in vitro* experiments the remaining

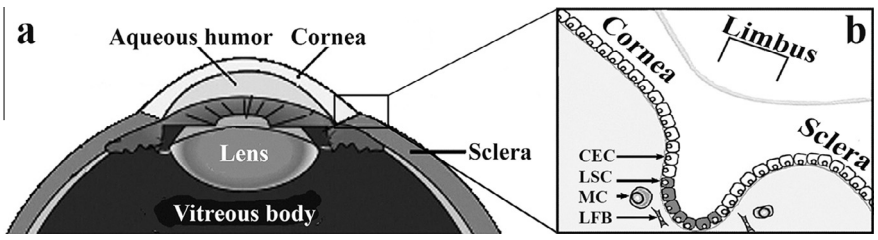

Fig. 1. Schematic view of isolation of limbal cells. (a) Limbal part of the human eye (boxed). (b) Magnified view of limbus. CEC = corneal epithelial cell, LSC = limbal stem cell, MC = melanocyte, LFB = limbal fibroblast.