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ScienceDirect
Experimental Eye Research 84 (2007) 655–662
EXPERIMENTAL EYE RESEARCH
www.elsevier.com/locate/yexer

Isolation, purification and cultivation of conjunctival melanocytes

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Received 2 October 2006; accepted in revised form 3 December 2006
Available online 9 February 2007

Abstract

The purpose of the present study was to develop methods for isolation, purification and cultivation of human conjunctival melanocytes. Conjunctiva excised from donor eyes or corneal rims was subjected with various enzyme digestion methods or by the enzyme-microdissection method. Cells were cultured with F12 medium supplemented by fetal bovine serum, basic fibroblast growth factor, isobutylmethylxanthine and cholera toxin. Contaminant cells were eliminated by a selective cytotoxic agent, geneticin. Both trypsin digestion and dispase-microdissection methods provided pure conjunctival melanocyte cultures with high cell yields, good viability and rapid growth rate. Melanocytes isolated with dispase-microdissection method showed better viability and growth capacity. Cells grew well, could be passaged for 5–10 generations and divided 20 times in vitro. They maintained a constant melanin content per cell and produced measurable amounts of melanin in vitro. Melanogenesis correlated with the degree of pigmentation of the eyes (iris color). This method provides a valuable source of large numbers of human conjunctival melanocytes, which can be used to study their biological behavior, to compare with the epidermal and uveal melanocytes; and to compare them to their malignant counterparts in the exploration of the pathogenesis of conjunctival melanoma.

Keywords: conjunctival melanocytes; cell culture; dispase; melanogenesis; conjunctival melanoma

1. Introduction

There are two different types of ocular melanocytes: conjunctival and uveal melanocytes. Conjunctival melanocytes (CM) are located in the conjunctiva, which covers the pericorneal surface of the anterior portion of the eye and the posterior surface of the eyelid. Uveal melanocytes are located inside the eye, in the iris, ciliary body and choroid. Both CM and uveal melanocytes, as well as epidermal melanocytes, are derived from the neural crest. However, uveal melanocytes are located in the stroma of uvea (derived from neural crest) and are adjacent to neuroectoderm-derived pigment epithelial cells. CM are located in the basal layer of conjunctival epithelium, which are derived from surface-ectoderm. CM transfer melanin to conjunctival epithelium, but uveal melanocytes never actively transfer melanin to any other cell type. Therefore, CM show some functional similarity to epidermal melanocytes.

Conjunctival melanocytes are involved in the pathogenesis of various eye diseases, the most important of which is conjunctival melanoma (Brownstein, 2004; Seregard and Kock, 1992; Seregard, 1998; Shields et al., 2004; Tuomaala et al., 2002). Recently epidemiological and molecular biological studies indicate that conjunctival melanoma is different from uveal melanoma and similar to cutaneous melanoma, e.g., the occurrence of conjunctival melanoma is related to the sunlight exposure and mutation of BRAF gene, whereas the uveal melanomas are not (Bergman et al., 2002; Cohen et al., 2003; Cruz et al., 2003; Edmunds et al., 2003; Gear et al., 2004; Goldenberg-Cohen et al., 2005; Hu, 2005; Inskip et al.,

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doi:10.1016/j.exer.2006.12.003

2003; Rimoldi et al., 2003; Singh and Topham, 2003; Spendlove et al., 2004; Stang et al., 2005; Yu et al., 2003). The difference between the conjunctival and uveal melanomas may be determined by the different environmental and cellular factors of the conjunctival and uveal melanocytes.

The methods for isolation, cultivation and in vitro study of uveal melanocytes were established by us in the early 1990s (Hu et al., 1993a,b). Methods for isolation and cultivation of CM have not been previously reported. Very little is known concerning the biological behavior of these cells and the pathogenesis of conjunctival melanoma. The purposes of the present study were to develop methods for isolation, purification and cultivation of CM.

2. Materials and methods

2.1. Donor eyes and cornea–sclera rims

Ten donor eyes were obtained from New York Eye Bank for Sight Restoration. The donor eyes had been enucleated within 16 h of death, were stored at 4 °C in a wet chamber, and were used within 48 h after the death of the donor. Forty-two cornea–sclera rims (the cornea had been removed for cornea transplantation) were obtained from New York Eye Bank for Sight Restoration, Lions' Eye Bank of Long Island, Northwest Louisiana Lion's Eye Bank and South Dakota Lion's Eye Bank. The rims had been dissected within 16 h of death, were stored at 4 °C in Optisol solution, and were used within 6 days after the death of the donor. All tissues were obtained with pre-mortem consent in accordance with the laws and regulations in place in the various jurisdictions. Optisol is a medium developed for donor cornea preservation. The base medium is Minimum Essential Medium and TC-199 medium with additional constituents, including 2.5% chondroitin sulfate, 1% dextran, precursors of adenine triphosphate and various amino acids, vitamins and antioxidants.

2.2. Isolation of CM

2.2.1. Enzymatic digestion method

There was a ring of conjunctiva attached to the sclera in the donor eyes or the rims, usually 3–5 mm wide. The superficial layer of conjunctiva was dissected from the underlying tissues under a stereo-microscope, washed with Ca^{++} and Mg^{++}-free Hank's solution (Gibco, Life technologies, Grand Island, NY), and used for enzymatic digestion. Three different enzymes were used:

(1) Trypsin digestion: the conjunctiva was cultured with 0.25% trypsin solution (Gibco) at 37 °C for 1.5 h and shook gently every 15 min. Trypsin activity was stopped by adding culture medium with 10% fetal bovine serum (FBS, Gibco). The cells released were collected, centrifuged, resuspended with culture medium, and plated into Falcon culture flasks (Becton Dickinson, Franklin Lakes, NJ).
(2) Trypsin–EDTA digestion: the conjunctiva was treated as above except that trypsin solution was substituted by trypsin (0.05%)–EDTA (0.02%) solution (Gibco).
(3) Dispase digestion: dispase II (1.10 unit/mg, Gibco) was dissolved in Ca^{++} and Mg^{++}-free Hank's solution to obtain dispase solution (0.2%). The conjunctiva was treated as above except that trypsin solution was substituted by dispase solution.

2.2.2. Enzymatic digestion with microdissection method

The conjunctiva and underlying sclera were dissected from the donor eyes or the rims, put in a culture dish, immersed with 0.2% dispase solution and cultured at 37 °C for 1.5 h. Culture medium with 10% FBS was added to stop dispase activity. The cut edge of the sclera underlying the conjunctiva was stabilized with microdissection forceps. An iris spatula was then used to gently scrape the conjunctival epithelium with melanocytes, which appeared as a transparent sheet under a stereoscopic microscope. This could be removed from the underlying tissue as a complete sheet or as several small sheets. The cells released were collected, centrifuged, resuspended with culture medium, and plated into Falcon culture flasks.

2.2.3. Comparison of results of different isolation methods

(1) Trypsin vs. trypsin–EDTA: the conjunctiva dissected from a rim was cut into two equal pieces and treated with trypsin solution and trypsin–EDTA solution separately. Cells isolated by these two different methods were plated into two separate culture dishes. The number of isolated and attached cells (as described below) for these two groups were calculated and expressed as number per eye separately for comparison.
(2) Trypsin vs. dispase: the conjunctiva dissected from a rim was treated as above except that trypsin–EDTA solution was substituted by dispase solution.
(3) Trypsin vs. dispase-microdissection: the conjunctiva was dissected from one half of the circumference of a rim and treated with trypsin solution. The conjunctiva with the sclera was dissected from the other half of the circumference and treated by dispase-microdissection method. Cells isolated by these two different methods were plated to two wells. The number and the growth capacity of cells obtained from these two different methods were measured for comparison.

2.3. Culture of CM

The isolated cells were incubated in a CO_2-regulated incubator in humidified 95% air/5% CO_2 atmosphere. HU16 medium was used for the cultivation, which was the Ham's F12 Nutrient Mixture supplemented by 10% FBS, 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, PeproTech, Rocky Hill, NJ), 0.1 mM isobutylmethylxanthine (IBMX, Sigma–Aldrich, St. Louis, MO), 10 ng/ml cholera