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Human mesenchymal stem cells differentiate into keratocyte-like cells in keratocyte-conditioned medium

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ABSTRACT

Culturing corneal keratocytes is difficult because keratocytes growing in a monolayer rapidly lose their stellate morphology and cease to express keratocyte markers such as keratocan, lumican and aldehyde dehydrogenase 1 family, member A1 (ALDH1A1). Conversely, mesenchymal stem cells (MSCs) can be easily expanded in cell culture, and they have a variety of differentiation pathways. We studied the feasibility of using MSCs as a source for corneal tissue engineering. Based on the observation that keratocytes have MSC-like properties, similar to bone marrow-derived MSCs (BM-MSCs), we hypothesized that MSCs would differentiate into corneal keratocyte-like cells in keratocyte-conditioned medium (KCM). We measured changes in the expression of keratocyte markers through quantitative real-time polymerase chain reaction (qRT-PCR) and found that human MSC's cultured in KCM expressed both keratocan and ALDH1A1. Western blot analysis demonstrated that human MSCs cultured in KCM steadily increased their expression of lumican and ALDH1A1, while they lost expression of α-smooth muscle actin (α-SMA). Immunocytochemistry indicated that human MSCs grown in KCM acquired characteristics similar to those of keratocytes. These results suggest that KCM can direct human MSCs to differentiate into keratocyte-like cells.

1. Introduction

Corneal keratocytes are mitotically quiescent cells with a flat, dendritic morphology (Poole et al., 1993). Keratocytes form extensive cellular networks (Petridou and Masur, 1996; Ueda et al., 1987) and synthesize collagens (Birk et al., 1981) and keratin sulfate proteoglycans such as lumican, keratocan, and mimecan (Funderburgh and Conrad, 1990; Funderburgh et al., 1991, 1993). In artificial corneal tissue engineering, it is important to have the ability to expand keratocyte numbers by subculture while maintaining their specific characteristics and functions. However, when cultured in plastic dishes of serum-containing media, keratocytes rapidly lose their dendritic morphology and acquire a fibroblastic shape (Beals et al., 1999; Jester et al., 1996), while initiating the expression of α-smooth muscle actin (α-SMA), a marker of myofibroblasts (Masur et al., 1996). Thus, a culture system to achieve *ex vivo* expansion of human corneal keratocytes while maintaining their characteristic dendritic morphology and the expression of keratocan, even in the presence of serum, was developed by growing keratocytes on the stromal matrix of a human amniotic membrane (AM) (Espana et al., 2003).

Keratocytes are mesenchymal-derived cells of the corneal stroma that originate from neural crest cells before differentiating into mesenchymal cells (Hayashi et al., 1986). Keratocytes retain neural crest cell progenitor properties, and they are not terminally differentiated even at late embryonic stages. Rather, under suitable conditions they maintain their plasticity and multipotentiality similar to non-cranial neural crest cell progenitors (Lwigale et al., 2005). Mesenchymal stem cells (MSCs) can differentiate into a variety of cell types, including cardiomyocytes and vascular endothelial cells (Choong et al., 2007; Du et al., 2005). MSCs are easily isolated from bone marrow and have a great capacity for self-renewal (Pittenger and Martin, 2004). MSCs derived from adult tissues are a progenitor cell source for tissue engineering and regenerative medicine (Tuan et al., 2003). Liu et al. reported that in mice umbilical MSCs transplanted into corneas with keratocyte dysfunction improved corneal transparency and stromal thickness. The transplanted MSCs assumed a keratocyte phenotype including dendritic morphology and quiescence with the expression of keratocan, lumican, and CD34 (Liu et al., 2010).

Conditioned medium is used to induce cellular differentiation. When conditioning cells are cultivated in medium they secrete mediator substances that are preserved after the original conditioning cells are removed, generating so-called "conditioned medium." When target cells are grown in this conditioned medium

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with conditioning cell mediator substances they develop the characteristics of the conditioning cells. Cellular differentiation can be prompted by such exposure to conditioned medium (Sensenbrenner et al., 1980; Kwiatkowski et al., 1998; Baer et al., 2009). We previously reported that corneal epithelial cell-conditioned medium can stimulate the differentiation of human MSCs into cornea-like epithelial cells (Shin et al., 2007). Therefore, we hypothesized that cytokines and other growth factors in keratocyte-conditioned medium (KCM) would direct the differentiation of MSCs to keratoctye-like cells. MSCs have more potential than keratocytes as a source for tissue engineering because MSCs can be easily isolated from bone marrow and expanded in culture. Based on previous reports that human keratocytes maintain their characteristic morphology and expression of keratocyte markers on an AM stromal matrix even in the presence of serum (Espana et al., 2003) and the fact that keratocytes and MSCs have similar characteristics, we investigated whether MSCs could differentiate into keratocyte-like lineages using KCM and growth on an AM. To investigate the possible differentiation of MSCs into keratocyte-like cells, we analyzed keratocyte marker (keratocan, lumican, aldehyde dehydrogenase 1 family, member A1 (ALDH1A1)) and MSC marker (α-SMA) expression using qRT-PCR, western blotting, and immunocytochemical staining.

2. Materials and methods

2.1. Isolation of human keratocytes

Human donor corneal tissue was obtained and stored in Optisol-GC (Bausch & Lomb) for less than three days. Human keratocytes were isolated from the corneal stroma by sequential collagenase digestion. Both the limbus and central corneal tissues were washed with phosphate-buffered saline (PBS) containing 5% penicillin/streptomycin. Descemet's membrane, including the endothelium, was peeled off, and the stromal tissue was cut into 6–8 pieces and incubated with a mixture of 2.4 U dispase II (Sigma–Aldrich, Milano, Italia) in Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12 (DMEM/F12) medium for 2 h at 37 °C. In order to avoid scraping the epithelium and Descemet's membrane, which can cause lamellar keratocyte apoptosis, the dispase digestion method was used. The stromal pieces were then placed in a conical centrifuge tube with 5 mL DMEM/F12 containing 0.1% collagenase I (Sigma), incubated at 37 °C, and shaken for 20 min. Following shaking, the stromal pieces were agitated by pipetting and separated from the collagenase solution using a cell strainer (BD Falcon, USA). Collagenase digestion was continued by incubation with a second aliquot of collagenase for 40 min, a third aliquot for 1 h, and a final collagenase incubation for 2 h. Each collagenase incubation step was followed by the collagenase separation procedure described above. At the end of each of the four collagenase digestion periods, the cells were collected by low-speed centrifugation (1200 rpm for 5 min) and suspended in 10 mL DMEM/F12. The viability and appearance of the cells was assessed by trypan blue exclusion using an inverted microscope and hemocytometer. In the initial experiments, cells from the second and third collagenase digestion were resuspended in DMEM/F12 containing 10% FBS. The keratocyte-containing cell suspension was then seeded on the stromal side of an AM.

2.2. Primary culture of keratocytes on an amniotic membrane

Human AM preserved in DMEM and pure glycerol (1:1) at −80 °C (Bioland, Ochang, Korea) was thawed and incubated in a solution of versene and ethylenediaminetetraacetic acid (EDTA) (1:1) for 30 min at 37 °C, and the amniotic epithelium was removed from the AM using a scraper. Epithelium-free AM was tightly spread or placed on a 3 cm × 3 cm piece of stainless mesh with the stromal matrix facing upwards. The suspended keratocytes extracted from the corneal tissues were seeded at 1×10^5 cells/mL on 2.5 cm × 2.5 cm denuded AM. Cells were cultured on the AM for 15 days in a medium containing DMEM/F12 supplemented with 10% FBS, and the medium was replaced every two to three days.

2.3. Collection of keratocyte-conditioned medium

Conditioned medium was produced by culturing human corneal keratocytes on an AM as described above. Confluent stromal matrix cells growing on an AM were washed with PBS before adding 13 mL of DMEM/F12 supplemented with 10% FBS. The medium was then harvested after two days and centrifuged. The supernatant was collected by filtration through a 0.22 μm filter and used as KCM, which was directly transferred onto MSC cultures.

2.4. Culture of human mesenchymal stem cells

Bone marrow-derived MSCs (BM-MSCs) were obtained from human fetal spinal vertebrae at 12–15 weeks of gestation with an amphotropic, replication-incompetent retroviral vector containing v-myc (Nagai et al., 2007). The BM-MSCs (BM3.B10, provided by Dr. Seung U. Kim, Professor Emeritus of Neurology, University of British Columbia, Vancouver) were cultured in alpha-minimum essential medium (α-MEM) (Invitrogen-Gibco, USA) supplemented with L-glutamine, deoxyribonucleosides, ribonucleosides, 10% FBS, and 1% penicillin/streptomycin for seven days in a plastic dish. MSCs were then taken from the plastic dish and continuously subcultured onto 2.5 cm × 2.5 cm pieces of AM or into plastic dishes. When the MSCs reached 90% confluence on the AM or in the plastic dish they were detached and separated into single cells by incubation in Hank's Balanced Salt Solution (HBSS) containing 0.05% trypsin and 0.53 mM EDTA for 5 min at 37 °C, followed by vigorous agitation by pipetting. MSCs achieved 90% confluence faster when grown in plastic dishes than on AM; time to 90% confluence was six days in plastic dishes compared to 15 days on AM. Following isolation to single cells, MSCs were subcultured at 1:2 on either AM or in plastic dishes in the same manner for two passages. MSCs were initially cultured for 24 h in α-MEM, after which the medium was switched to α-MEM containing 10% KCM and the concentration of KCM was gradually increased to a final concentration of 40% KCM. When the concentration of KCM was greater than 40%, the rate of MSC multiplication decreased. MSCs were cultured for 15 days on an AM or in a plastic dish for each passage, and the medium was replaced every two to three days.

2.5. Quantitative RT-PCR

As a positive control, total RNA was extracted from minced human corneal stromal tissue using the TRIzol reagent (Invitrogen-Gibco). Total RNA was similarly extracted from keratocytes and MSCs cultured on an AM or in a plastic dish. Total cDNA was synthesized with a cDNA synthesis kit (Takara Bio Inc, Otsu, Japan). qRT-PCR was performed using SYBR Premix Ex Taq (Takara). The gene-specific primers that were used for keratocan, lumican, ALDH1A1, α-SMA, and β-actin are shown in Table 1. SybrGreen fluorescence of the amplified cDNA products was quantified using the CFX96™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA) and an appropriate standard curve from autonomous qPCR assay reactions. Each sample and the positive control were analyzed by triplicate qRT-PCR reactions. The expression of each gene of interest was calculated as the average quantity from the