International Society for Cellular Therapy ISCT

Cytotherapy (2007) Vol. 9, No. 3, 252–258

informa healthcare

Mesenchymal stromal cell-like characteristics of corneal keratocytes

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Background

The unique potential of mesenchymal stromal cells (MSC) has generated much research interest recently, particularly in exploring the regenerative nature of these cells. Previously, MSC were thought to be found only in the BM. However, further studies have shown that MSC can also be isolated from umbilical cord blood, adipose tissue and amniotic fluid. In this study, we explored the possibility of MSC residing in the cornea.

Methods

Human cornea tissues were chopped to fine pieces and cultured in DMEM supplemented with 10% FBS. After a few days, the crude pieces of cornea were removed. Isolated keratocytes that were adherent to tissue culture flasks were grown until confluency before being passaged further. The immunophenotype was evaluated by flow cytometry. Assays were performed to differentiate cultured cells into adipocytes and osteocytes.

Results

Isolated corneal keratocytes exhibited a fibroblastoid morphology and expressed CD13, CD29, CD44, CD56, CD73, CD90, CD105 and CD133, but were negative for HLA-DR, CD34, CD117 and CD45. These properties are similar to those of BM-MSC (BM-MSC). In addition, corneal keratocytes were able to differentiate into adipocytes and osteocytes.

Discussion

Our results indicate that corneal keratocytes have MSC-like properties similar to those of BM-MSC. This study opens up the possibility of using BM-MSC in corneal tissue engineering and regeneration. Furthermore, discarded corneal tissue can also be used to generate MSC for tissue engineering purposes.

Keywords

adult stem cells, mesenchymal stromal cells, tissue engineering.

Introduction

BM-derived mesenchymal stromal cells (MSC) (BM-MSC) constitute a unique group of stem cells that have been studied extensively in the past. *In vivo*, MSC give rise to the stromal components of BM, such as blood vessels and connective tissues, whereas *in vitro*, isolated MSC have many potential applications in clinical medicine, such as tissue repair, immunomodulation and gene therapy [1–4]. Previous studies have shown that these multipotent stem cells can be isolated not only from human BM but also from human umbilical cord blood, adipose tissue, amniotic fluid and peripheral blood [5–11].

The cornea is the transparent structure forming the anterior part of the fibrous coat of the eye. It comprises five layers: (1) the corneal epithelium, (2) the anterior limiting layer also known as Bowman's layer, (3) the stromal layer, (4) the posterior limiting layer known as Descment's membrane, and (5) the endothelial layer. Keratocytes are found in the stromal layer of the cornea. Their main function is to maintain the turnover of collagen and proteoglycans in the extracellular matrix. They also have an important role in wound healing following injuries to the cornea. To date, no studies have successfully identified cornea stromal stem cells, and their origins and characters are ill understood. Recent studies have shown that murine stem cells from BM migrate to the cornea to repopulate the epithelia and stroma, suggesting that BM is the source of a supply of stem cells for repair purposes [12].

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DOI: 10.1080/14653240701218508

Adult stem cell research has generated considerable interest in recent years because of their multipotency and the fact that they are less controversial than embryonic stem cells [13]. Using autologous MSC for regenerative purpose has its advantages because they are less likely to be rejected or transmit an infection. In the past it was believed that MSC are unipotent, but recent studies have shown that these cells are more plastic than previously thought [14–17].

Corneal keratocytes are sometimes described as corneal fibroblasts because they are morphologically similar to fibroblasts grown from the skin. Under a different culture medium, corneal keratocytes are capable of producing striation. This has led to the term 'myofibroblast' being used to describe these cells [18]. It has been shown that, in the presence of TGF-β, corneal keratocytes are capable of transforming into myofibroblasts [19,20]. Such differentiation can be reversed using FGF-2 [21].

The finding that MSC could be isolated from sites other than BM in the body, especially from liposuction aspirates, encouraged us to determine the possibility of MSC residing in the cornea. Furthermore, the identity of corneal keratocytes has always been a subject of debate. Thus we carried out this study to determine whether human corneal keratocytes and MSC found in the BM share similar cellular characteristics.

Methods

Isolation and growth of corneal keratocytes

Corneal keratocytes were isolated from a single cornea–scleral ring, which is normally discarded following removal of the central corneal donor button during corneal transplant surgery. Consent had been given for the donor tissue to be used for transplantation and research purposes. The epithelium and the endothelium were removed by scraping with a surgical blade. The corneal stroma was cut off from the sclera and chopped into fine pieces. Small pieces of cornea were cultured in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS, 0.05 U/mL penicillin and 0.05 μg/mL streptomycin (Gibco, Grand Island, NY, USA), and incubated at 37°C in 5% humidified CO_2. The fine pieces were left for a week without medium changes. Then small pieces of cornea were removed and adherent cells were allowed to continue culture in the same culture medium until confluency. Cells were passaged for another two to three times using 0.25% trypsin/EDTA (Gibco) before the following investigations were performed on passage 3 or passage 4 cells. The morphology of the cells was observed using Wright-Giemsa staining (Sigma-Aldrich).

Immunophenotyping of corneal keratocytes

Aliquots of corneal keratocytes (1×10^5 cells/tube) were incubated with FITC or PE-conjugated MAb for 30 min in the dark. The Ab used were CD13, CD29, CD34, CD44, CD45, CD56, CD73, CD90, CD105, CD117, CD147 and HLA-DR (Becton Dickinson, San Jose, CA, USA). After incubation, the cells were washed and rediluted with sheath fluid and subjected to flow cytometry analysis using FACScan and CellQuest software (Becton Dickinson).

Differentiation studies

Adipogenic differentiation

Cells were grown to 50–70% confluency and incubated in an adipogenic medium [11], which contained DMEM supplemented with 10% FBS, 0.05 U/mL penicillin, 0.05 μg/mL streptomycin (Gibco), 1 μm dexamethasone, 10.0 μg/mL insulin, 0.5 mm methylisobutylxantine and 200 μm indomethacine (all from Sigma-Aldrich). The medium was changed every 3–4 days for 21 days. Cells were then washed with PBS, fixed in 10% paraformaldehyde for 30 min and stained with fresh Oil Red-O solution (Sigma-Aldrich) for 15 min. Stained cells were analyzed using an Axiovert 200 microscope (Carl Ziess Inc., Oberkochen, Germany).

Osteogenic differentiation

Cells grown to 50–70% confluency were incubated in osteogenic medium containing DMEM supplemented with 10% FBS, 0.05 U/mL penicillin, 0.05 μg/mL streptomycin (Gibco), 50 μg/mL ascorbate, 10 mm β-glycerophosphate and 100 nm dexamethasone (Sigma-Aldrich). The medium was changed every 3–4 days for 21 days. Culture was washed with PBS, fixed in methanol for 30 min at 4°C and stained for 1 h with 5% Alizarin Red at pH 4.0 (Sigma-Aldrich). Stained cells were analyzed using an Axiovert 200 microscope (Carl Ziess Inc.).

RT-PCR

Cells were trypsinized and centrifuged to obtain cell pellets. Total RNA from the cells was isolated using TriReagent (Molecular Research Center Inc., Ohio, CA, USA) according to the manufacturer's protocol. The RT-PCR process was performed using a BcaBest RNA PCR

Kit (Takara, Shiga, Japan) according to the manufacturer's protocol. Primers for identification of the alkaline phosphatase were forward primer 5′-GTA CTG GCG AGA CCA AGC G-3′ and reverse primer 5′-AGG GGA ACT TGT CCA TCT CC-3′. Following 30 cycles of amplification (94°C, 15 seconds; 58°C, 45 seconds; 72°C, 30 seconds) using an Eppendorf Mastercycler Gradien (Eppendorf, Hamburg, Germany), the PCR reaction was resolved on a 1.2% agarose gel. The band was observed under UV light and photographed.

Control samples

BM-MSC were used as a control. These cells were harvested as previously reported by our research group [22] and studied in a similar way.

Results

Morphology in cultures

Fibroblast-like cells were grown out successfully from chopped human cornea explants incubated in DMEM supplemented with 10% FBS after 3 days in culture. The explants were removed on day 7 and the fibroblast-like cells continued to grow until they reached 80–90% confluency in another 3–4 days (Figure 1). Adherent cells were subcultured. Even after seven passages, corneal keratocytes were observed to maintain the spindle-shaped, fibroblastic morphology seen using Wright-Giemsa staining.

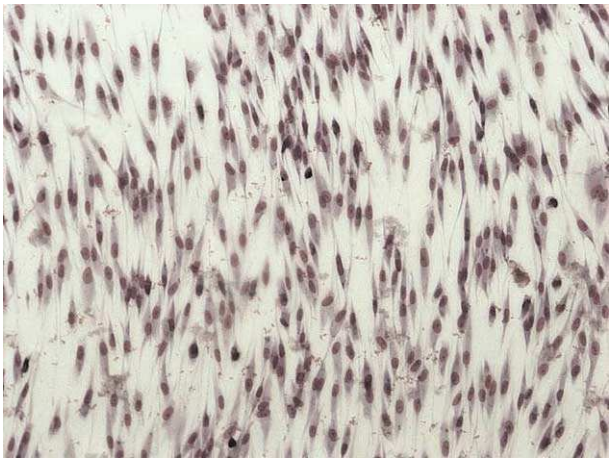

Figure 1*. Morphology of corneal keratocytes. Corneal keratocytes were cultured in DMEM supplemented with 10% FBS. Corneal keratocytes displayed a spindle-shaped and fibroblastic appearance even after seven passages (× 50 magnification, Wright-Giemsa stain).*

Immunophenotyping of corneal keratocytes

Isolated corneal keratocytes were immunophenotyped using flow cytometry. The keratocytes expressed CD13 (55%), CD29 (58%), CD44 (49%), CD56 (38%), CD73/SH 3 (51%), CD90 (66%), CD105/SH2 (55%) and CD133 (38%). They did not express CD34, CD45, CD117/c-kit and HLA-DR (Figure 2). Lack of HLA-DR and CD117 expression suggests that these cells are different from fibroblasts. In addition, morphologically the keratocytes exhibited long dendritic structures not typically seen in fibroblasts. These characteristics were similar to those of BM-MSC, summarized in Table 1.

Differentiation study

Adipogenic differentiation was induced by treating the cells with dexamethasone, insulin, methylisobutylxantine and indomethacine. Differentiation into adipose tissues was apparent by the accumulation of lipid-rich vacuoles in about 25% of the cells (Figure 3). The lipid droplets could be seen in the induction culture as early as 2 weeks and stained positively with O Red Oil. The control using uninduced corneal keratocytes did not pick up any O Red Oil staining, indicating that the cells were not differentiating into adipocytes.

After incubating with defined osteogenic differentiating media for 21 days, 15% of the induced cells formed aggregates and calcium deposits, as shown in Figure 4. RT-PCR analysis showed an increase in alkaline phosphatase expression after osteogenic induction (Figure 5). These changes were not seen in corneal keratocytes in the negative control.

Discussion

Our results show that corneal keratocytes isolated under culture conditions for BM-MSC exhibit a spindle-shaped, fibroblast-like appearance similar to that of BM-MSC [1–7,23,24]. Isolated corneal keratocytes can be distinguished from hemopoietic stem cells and fibroblasts because they are adherent to the surface of tissue culture flasks and express different cell-surface markers.

Cellular characterization using flow cytometry has demonstrated that isolated corneal keratocytes are positive for several MSC-related Ag, including CD13, CD29, CD44, CD73 (SH3), CD90 and CD105 (SH2). SH2 and SH3 Ab, originally developed by Caplan *et al.* [25], recognize CD105 and CD73, respectively, and are putative markers that readily identify MSC. Many investigators

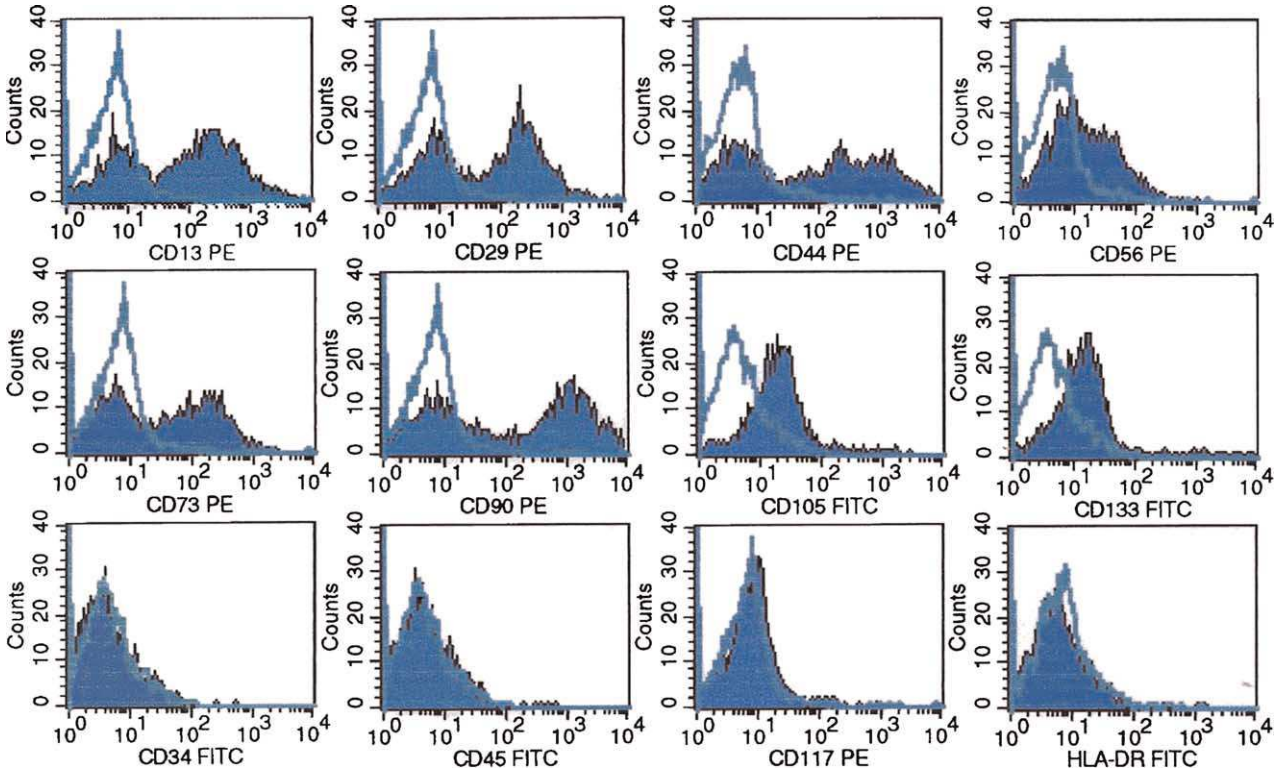

Figure 2. *Immunophenotyping of corneal keratocytes. Flow cytometry showed that the cells were positive for CD13, CD29, CD44, CD56, CD73 (SH3), CD90, CD105 (SH2) and CD133 but did not express CD34, CD45, CD117 (c-kit) and HLA-DR.*

have explored CD105 as an important antigenic determinant in the identification of MSC [7,8,10,11]. Pittenger has reported that CD29, CD44 and CD90 are important cellular markers for MSC [26]. In addition, isolated corneal keratocytes are also positive for CD56 and CD133. A recent study has demonstrated the expression of putative stem cell marker CD133 in ocular tissues [27]. However, they do not express CD34, CD45, CD117 and HLA-DR. Our results indicate that the isolated cells are not hematopoietic stem cells, which are $CD34^+$, and are not leukocytes, which are $CD45^+$ and $HLA\text{-}DR^+$. Our results are consistent with the findings by Sosnova *et al.* [28], which indicated that the corneal keratocytes are $CD34^-$. In fact, in comparison with BM-MSC, they show great similarity, as demonstrated in Table 1.

Table 1. Comparison of surface markers expression

Surface Ag	BM-MSC	Corneal MSC
CD13	+	+
CD29	+	+
CD34	−	−
CD44	+	+
CD45	−	−
CD56	+/−	+
CD73	+	+
CD90	+	+
CD105	+	+
CD117	−	−
CD133	+/−	+
HLA−DR	−	−

Data show the comparison of the surface markers expressed by BM-MSC and corneal keratocytes. Both populations of cells were cultured similarly and surface markers were detected using flow cytometry.

Under defined culture conditions, corneal keratocytes could be induced to differentiate into cells of various mesenchymal lineages, such as adipocytes and osteogenic cells. In the presence of adipogenic medium, isolated corneal keratocytes were shown to develop into adipocytes. The appearance and properties of adipocytes were similar to those differentiated from BM-MSC, as reported previously [29,30]. Control corneal keratocytes grown without adipogenic medium did not differentiate into adipocytes.

Isolated corneal keratocytes were also found to differentiate into cells of the osteogenic lineage when exposed to osteogenic induction for about 3 weeks. Such osteogenic media have been used successfully to differentiate

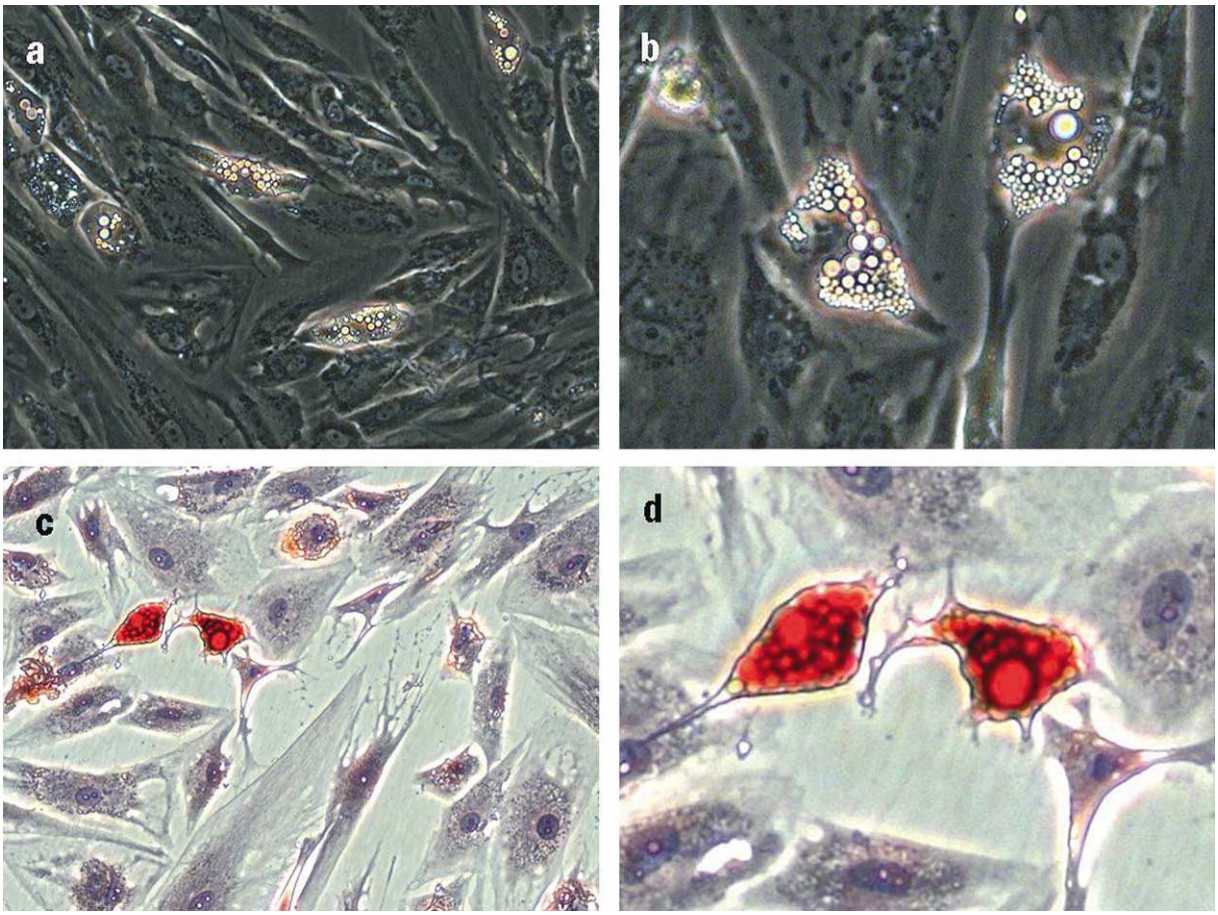

Figure 3. *Adipogenic differentiation potential of corneal keratocytes. Cultures of corneal keratocytes were exposed to adipogenic medium and adipocytes were detected by accumulation of lipid drops (a, c; × 200 magnification) that stained positively with Oil Red O (b, d, × 400 magnification).*

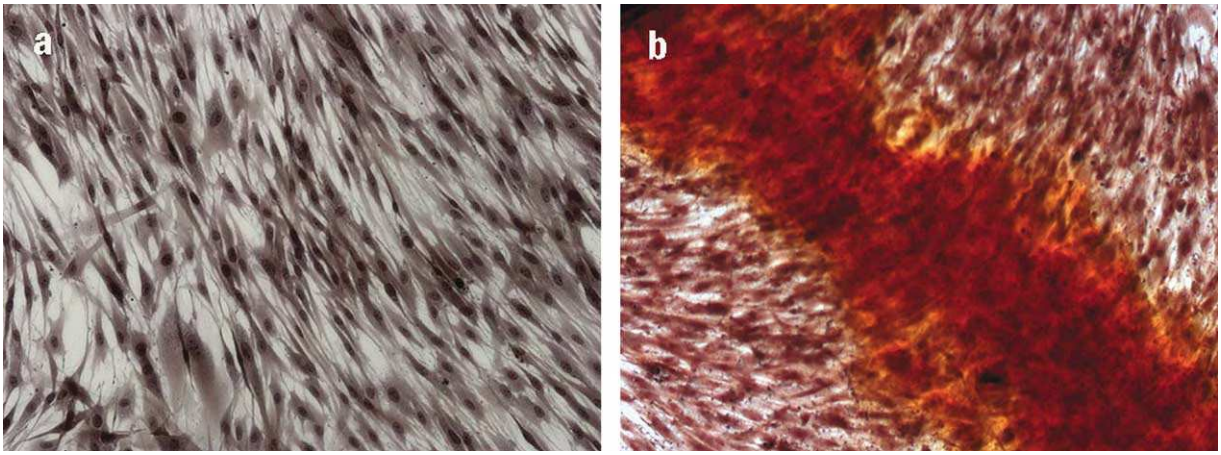

Figure 4. *Osteogenic differentiation potential of corneal keratocytes. (a) Uninduced corneal keratocytes did not pick up any Alizarin Red stain. (b) Calcium deposition that was stained by the Alizarin Red stain confirmed the differentiation of corneal keratocytes to osteogenic lineage (× 50 magnification).*

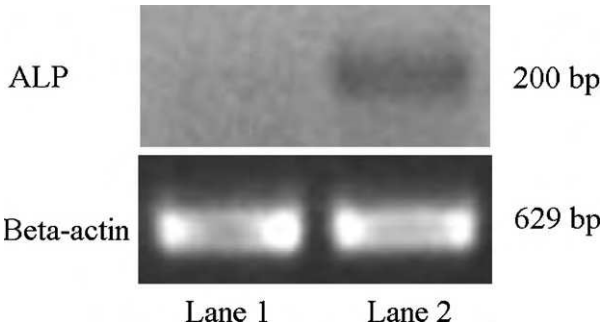

Figure 5*. Identification of alkaline phosphatase using RT-PCR. Uninduced corneal keratocytes did not express alkaline phosphatase mRNA (lane 1). Corneal keratocytes after osteogenic induction expressed alkaline phosphatase mRNA (lane 2).*

BM- MSC into osteocytes [31,32]. In our study, isolated corneal keratocytes could be transformed into a mixture of late pre-osteoblasts, osteoblasts and early osteocytes. Cell lineage identity was confirmed by positive expression of alkaline phosphatase mRNA [33] (Figure 5) and positive Alizarin Red S staining, which indicated calcification around the induced corneal keratocytes (a functional property of differentiated osteogenic cells).

Our results indicate that isolated corneal keratocytes have properties similar to those of BM-MSC, including their multipotency. It is not certain whether isolated corneal keratocytes are in fact MSC migrating into the cornea from BM or whether they are native cells with characteristics similar to MSC. A recent study has shown multipotent stem cells residing in the human corneal stroma [34]. This study demonstrated the presence of a subset cell population in human corneal stroma expressing stem cell marker ABCG2 and the potential for this population to differentiate into chondrogenic and neuron-like cells. BM-MSC are reported to exhibit these differentiation properties.

Our study has many different clinical and therapeutic implications. It is plausible that corneal keratocytes are more multipotent than traditionally thought. This may offer an explanation for one of the aetiologic mechanisms underlying lipid (lipid keratopathy) and calcium (band keratopathy) deposition in diseased corneas. Conversely, BM-MSC or indeed MSC derived from other tissues could be used to regenerate corneal tissue in the future. Ma *et al.* [35] have demonstrated that human MSC derived from BM could be used to reconstruct chemically burned rat corneal epithelium. Possible therapeutic applications for autologous MSC include (1) topical application of the MSC for threatened corneal perforations in patients with ocular surface disease, (2) *in vitro* regeneration of the cornea, which may facilitate management of corneal stromal melts and spontaneous perforations, and (3) other optical transplant procedures, negating the use of allogenic corneal transplantation. In summary, our work has shown the possibility of MSC residing at the cornea and this may provide a better understanding of the biologic repair mechanism of the eye and novel treatments for corneal tissue engineering and regeneration.

Acknowledgements

We acknowledge the funding support from MAKNA (National Cancer Council), Malaysia. We thank the staff and fellow researchers from the Stem Cell Unit, National University of Malaysia Hospital, for their support and help.

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