Research article

Effect of bone marrow and adipose tissue-derived mesenchymal stem cells on the natural course of corneal scarring after penetrating injury

Bengi Demirayak a,*, Nursen Yüksel b, Onur Sinan Çelik b, Cansu Subaşı c, Göknan Duruksu d, Z. Seda Unal d, Demir Kürsat Yıldız e, Erdal Karaöz c, f

a Department of Ophthalmology, Bakırköy Dr. Sadi Konuk Education and Research Hospital, Istanbul, Turkey
b Department of Ophthalmology, School of Medicine, Kocaeli University, Kocaeli, Turkey
c Center for Regenerative Medicine and Stem Cell Research and Manufacturing, Liv Hospital, Istanbul, Turkey
d Center for Stem Cell and Gene Therapies Research and Practice, Department of Stem Cell, Institute of Health Sciences, Kocaeli University, Kocaeli, Turkey
e Department of Pathology, School of Medicine, Kocaeli University, Kocaeli, Turkey
f Department of Histology and Embryology, Faculty of Medicine, Istanbul University, Turkey

ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 8 June 2016
Accepted in revised form 23 August 2016
Available online 25 August 2016

Keywords:
Cornea
Keratocan
Mesenchymal stem cells
Stromal wound healing

ABSTRACT

In the present study, we investigate and compare the efficacy of bone marrow- and adipose tissue-derived mesenchymal stem cells (MSCs) in corneal wound healing. A penetrating injury was created in the right corneas of Wistar rats (n = 40). Ten microliters of phosphate-buffered solution (PBS) containing 2 × 10^5 green fluorescent protein (GFP) labeled bone-marrow-derived MSCs to group 1 (n = 15), 10 μl of PBS containing 2 × 10^5 GFP-labeled adipose-tissue-derived MSCs to group 2 (n = 15), 10 μl PBS was injected into anterior chamber in group 3 (n = 10, control). Corneal opacity scoring, in vivo confocal microscopy, and histopathological evaluation were done at the end of 8 weeks. Immunofluorescence sections were evaluated to detect transplanted cells. Immune staining was performed to measure the expression levels of keratocan, aldehyde dehydrogenase (ALDH) and CD34. The gene expression levels of tumor necrosis factor (TNF-α), interleukin 6 receptor (IL-6R), interleukin 12b (IL-12b), and transforming growth factor beta (TGF-β1) was measured on corneas. The establishment of stem cells in the corneas of the transplanted groups was confirmed by immunofluorescence staining. The expression of keratocan, ALDH, and CD34 increased in the transplanted groups (p < 0.05). The density of keratocytes increased significantly in both transplanted groups according to the in vivo confocal microscopy data (p < 0.05). The expression of TNF-α, IL-6R, and IL-12b decreased significantly in the transplanted groups (p < 0.05). Based on our findings, we consider that allogeneic stem cells facilitate the regeneration of corneal stroma and can be a cell source for stromal repopulation in diseased cornea.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Corneal transplanting is the most effective way of treating corneal blindness caused by microbial infection, mechanical and chemical injuries, and congenital defects. Cell-based therapy is a promising therapeutic approach to overcome complications of corneal transplantation and an insufficient number of corneal grafts. Human corneal epithelial stem cells and human corneal endothelial cells have been cultured and transplanted into animal and human hosts successfully (Engelmann et al., 1988; Tsubota et al., 1999; Pellegrini et al., 1997; Tsai et al., 2000; Rama et al., 2001; Engelmann et al., 1999; Mimura et al., 2004, 2005). Recently, non-ocular cells have also been used to reconstruct corneal epithelium, mainly using autologous oral mucosal epithelium (Nakamura et al., 2004; Hayashida et al., 2005; Nishida et al., 2004). However, most corneal diseases primarily or secondarily involve the corneal stroma, which accounts for 90% of corneal thickness. Keratocytes, which are mesenchymal-derived cells, are the principal cells of the corneal stroma. In adult tissue, keratocytes are mitotically quiescent, and they are positive for CD34 and aldehyde-3-dehydrogenase (ALDH) (Jester et al., 1999a). They secrete collagens and keratan sulfate proteoglycans, such as keratocan, which is used as a keratocyte-specific marker (Hassell et al., 1980). The unusual proteoglycan composition of the corneal stroma...
is essential for corneal transparency (Carlson et al., 2005). During corneal wound healing, keratocytes are activated and transform into fibroblasts and/or myofibroblasts. This results in the loss of their dendritic morphology and a reduction in ALDH, keratan sulfate and corneal transparency (Jester et al., 1999b; Mahatta et al., 2006; Funderburgh et al., 2003; Funderburgh and Chandler, 1989). Mesenchymal stem cells (MSCs) are a type of multi-potent cell originally isolated from bone marrow that have subsequently been isolated from other tissues, such as adipose tissue, heart tissue, cord blood, and oral tissue (Zuk et al., 2001). Recently, the application of MSCs for the treatment of corneal chemical burns have been studied, with encouraging results (Yao et al., 2012).

In the present study, we investigated and compared the efficacy of bone marrow and adipose tissue-derived MSCs in altering the natural course of corneal scarring after a penetrating injury to the cornea in a rat model.

2. Methods
2.1. Animals

All procedures used in this study were in accordance with the principles of the Declaration of Helsinki, and they were all performed in our institute. The study was approved by the Research Ethics Committee of Kocaeli University. Six-week-old female Wistar rats weighting 180–220 g were anesthetized by an intra-peritoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). At the end of the experiment, all the rats were euthanized with an overdose of ketamine hydrochloride. The corneas of the rats were harvested, and only the right eye of each was used.

2.2. Isolation and culture of rat adipose tissue-derived MSCs (rAT-MSCs)

The rats (n = 5) were anesthetized by an injection of 10 mg/kg of xylazine and 80 mg/kg of ketamine. A total of 1–2 cm² of preperitoneal adipose tissue was removed. Tissue samples were washed several times with Hanks’ balanced salt solution, supplemented with 5% antibiotic–antimycotic solution (Gibco Life Technologies, Paisley, UK), and vascular structures were removed. The tissue was minced and enzymatically digested in Dulbecco’s modified Eagle’s medium-low glucose (L-DMEM) medium (Gibco Life Technologies) containing 0.075% collagenase (Sigma, St Louis, MO) at 37 °C for 60 min. The cell suspension was filtered with a 70-μm mesh nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ). After centrifugation at 1800 rpm for 10 min, the cells were re-suspended and seeded with L-DMEM medium with 1% penicillin/streptomycin and 10% phosphate buffered solution (PBS). After 5–7 days, the medium was replaced with fresh medium and subsequently replaced twice a week. At 70–80% confluence, the cells were harvested with 0.025% trypsin-ethylenediaminetetraacetic acid (EDTA) for 3–4 min and collected by centrifugation. The cells were counted and sub-cultured at a 1:3 ratio.

2.3. Isolation and culture of rat bone marrow-derived MSCs (rBM-MSCs)

Under sterile conditions, both femur and tibiae from each rat were excised, and 21-gauge needle was used to flush the marrow with L-DMEM medium supplemented with 10% fetal bovine syndrome (FBS), 1% penicillin/streptomycin. Marrow plug suspension was dispersed by pipetting, filtered through 70 μm mesh nylon filter, and centrifuged at 1800 rpm for 10 min. The bone marrow was diluted to 1:3 with PBS and layered over a Histopaque-1077 (1.077 g/ml, Sigma-Aldrich, St. Louis, MO, USA) for gradient centrifugation. The low-density mononuclear cells were collected, and washed twice with PBS. The cells were seeded on plastic tissue culture flasks and incubated for 3 days under standard conditions. MSCs were isolated based on their ability to adhere to culture plates. To remove unattached cells, fresh medium was added. The cells were passaged as described above. After three more passages, characterization studies were carried out.

2.4. Flow cytometry

After passage 3 (P3), the stem cells were harvested. Flow cytometry was performed using a FACS Calibur (BD Biosciences, San Jose, CA). The immunophenotyping analysis was performed against the following antigens: CD29, CD45, CD54, CD90, CD106 and major histocompatibility complex (MHC) Class II (BD Biosciences). The data were analyzed with Cell Quest software (BD Biosciences).

2.5. In vitro differentiation of the MSCs

Adipogenic and osteogenic differentiation was performed in vitro according to a published protocol (Karaoz et al., 2008). For osteogenic differentiation, the cells were incubated in the osteogenic medium minimum essential medium (MEM) (Invitrogen/GIBCO) was supplemented with 100 nM demethylasone (Sigma-Aldrich), 0.05 μM ascorbate-2-phosphate (Wako Chemicals,
Richmond, VA, USA), 10 mM b-glycerophosphate (Sigma-Aldrich), 1% antibiotic/antimycotic and 10% FBS (Invitrogen/GIBCO) for 4 weeks, and osteogenic differentiation was assessed via staining with 2% Alizarin Red S (pH 4.1–4.3; Fluka, Buchs, Switzerland). Adipogenic differentiation was performed by incubation of MSCs in the adipogenic medium MEM (Invitrogen/GIBCO) supplemented with 10% FBS (Invitrogen/GIBCO), 0.5 mM isobutyl methylxanthine (IBMX-Sigma-Aldrich), 10 μM dexamethasone (Sigma-Aldrich, Fluka Chemie AG, Buchs, Switzerland), 10 μg/ml insulin (Invitrogen/GIBCO), 200 μM indomethacin (Sigma-Aldrich), and 1% antibiotic/antimycotic (Invitrogen/GIBCO) for 4 weeks. Adipogenic differentiation was shown by staining of intracellular lipid droplets with 0.5% oil red O (Sigma-Aldrich, St. Louis, MO).

2.6. Green fluorescent protein (GFP) labeling of the MSCs

The MSCs were transfected with pGFP-N (Clontech, Palo Alto, CA) by electroporation (Neon Transfection System, Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. After the first 48 h of culture in L-DMEM-medium with 10% FBS, the transformed cells were selected with G418 (200 μg/ml) under culture conditions.

2.7. Animal model

The animals’ eyes (n = 40) were rinsed with povidone iodine and topically anesthetized with a drop of proparacaine. An experimental penetrating injury was created, as described by Matsuda et al. (1997). Under a microscope (Topcon OMS 75, Topcon Corporation, Japan), a penetrating linear incision of uniform size (2 mm) was made with a knife (Beaver-Visitec International) in the upper paracentral of the right cornea of each rat. The incision was sutured. A Hamilton micro-syringe (Fisher Hamilton, USA) with a 22-gauge needle was used to inject the material through the incision into the anterior chamber.

Forty rats were randomized into a BM-MSC group, an AT-MSC group and a control group. The rats in the BM-MSC group received an injection of 10 μl of PBS containing 2 × 10^5 GFP-labeled BM-MSCs MSC (n = 15). The rats in the AT-MSC group received an injection of 10 μl of PBS containing 2 × 10^5 GFP-labeled AT-MSCs (n = 15). The rats in the control group received an injection of 10 μl of PBS only (n = 10). For prevention of infection, antibiotic eye drops were administered 3 times daily for 5 days.

2.8. Clinical evaluation

Each eye was examined with a portable slit lamp 1, 4, and 8 weeks after the surgery to look for corneal inflammation, opacities, or any other ocular surface or anterior chamber complication. A blinded expert ophthalmologist evaluated the severity of haze on a scale of 0–4, as follows: grade 0 for a completely clear cornea; grade 1 for more prominent haze not interfering with the visibility of fine iris details; grade 2 for mild obscuration of iris details; grade 3 for moderate obscuration of the iris and lens; and grade 4 for complete opacification of the stroma in the area of penetration (Fantes et al., 1990).

2.9. In vivo confocal microscopy

In vivo analysis of the corneal stroma was performed with a Heidelberg Retinal Tomograph- (HRT3) Rostock Cornea Module (HRT3, Heidelberg Engineering Inc., Germany) under general anesthesia before euthanasia. Before the examination, a drop of Viscoatears Gel (Novartis Pharmaceuticals Corp., USA) was applied to the tip of the HRT3 objective as immersion fluid. Subsequently, a series of images were collected, encompassing the whole stromal thickness, starting from the basa layer of the corneal epithelium and ending at the corneal endothelium. The stromal thickness was determined by measuring the axial distance from the basal epithelium to endothelium of cornea.

Keratocytes classically have a dendritic (fibroblast-like) cell shape which was determined by the presence of large, flat elongated (fusiform and/or spindle shaped) cells with branched cytoplasmic projections. Keratocytes, which are clearly observed in the first section after basal epithelium, were counted and then anterior keratocyte density (AKD) was calculated using the software program in HRT3. Keratocytes that are determined clearly before endothelium were counted and posterior keratocyte density (PKD) was calculated in the same way.

2.10. Histological evaluation

One eye in the BM-MSC group and one eye in the AT-MSC group were excluded because of infection. The remaining rats were euthanized with an overdose of ketamine hydrochloride 8 weeks after the MSC injections. Ten eyeballs were randomly selected from each MSC injection group, and six eyeballs were randomly selected from the control group. The eyeballs were removed en bloc and fixed in formalin and then placed in paraffin-embedded blocks for sectioning at a thickness of 10 μm. Tissue sections were stained with hematoxylin and eosin (H&E) for histological evaluation (Sigma-Aldrich, St. Louis, MO).

All the slides were examined under blinded conditions, and the degree of inflammation and fibroblastic activity were evaluated in each slide. The degree of inflammation was scored between 0 and 3, with 0 denoting no inflammation, 1 denoting weak and focal inflammation, 2 denoting focal inflammation, and 3 denoting diffuse inflammation. The degree of fibroblastic activity was scored between 0 and 3: 0, normal; 1, slight increase in number of fibroblasts without change of normal spindle morphology; 2, moderate increase in number of fibroblasts with slight polarity loss in normal morphology; 3, a significant and diffuse increase in number of fibroblasts with lack of normal polarity.

2.11. Immunostaining of the rAT- and rBM-MSCs

Immunohistochemical staining of the corneal sections of the control and transplanted groups was performed 8 weeks after the injections. All sections were taken from wound region, 2 mm max. length to penetrating line. Slides were de-paraffinized in two cycles of xylene for 5 min each, and were rehydrated in a series of graded ethanol solutions. Antigen retrieval of the sections was performed using a steamer-citrate buffer antigen retrieval method. Endogenous peroxidase was inhibited by incubation with fresh 3% H2O2 in PBS buffer. Dual immunofluorescence assays for GFP (sc-5385, Santa Cruz Biotechnology) and kerotocan (sc-33244, Santa Cruz Biotechnology), GFP and ALDH1/2 (sc-166362, Santa Cruz Biotechnology) were performed. For immunofluorescence staining, the samples were rinsed briefly in PBS. After permeabilization with 0.025% Triton X-100 (Merck, Darmstadt, Germany), nonspecific staining was blocked with a mixture of two different sera (Santa Cruz Biotechnology, Heidelberg, Germany) (with respect to the type of antibodies used for blotting) in 1.5% PBS for 30 min at room temperature, followed by incubation overnight at 4 °C with primary antibodies. After three PBS washes, the samples were incubated with secondary antibody for 60 min. The samples were mounted with mounting medium containing DAPI (Santa Cruz Biotechnology). The GFP-, kerotocan-, ALDH- and CD34-positive cells were detected with a Leica DMI 4000 Microsystems fluorescence.
microscope. Five sections for each case were evaluated, and cells in all fields were counted for statistical analysis. Negative control stainings of GFP, Keratocan, CD34 and ALDH1/2 groups were performed in rAT, rBM and control groups without primer antibodies to avoid possible false-positive staining (shown as Supplementary Data).

2.12. Quantitative gene expression analysis by real-time PCR

The gene expression levels of TGF-β1, IL12B, IL-6r, and TNF-α were quantified in the corneal tissues to evaluate effect of MSCs on inflammation. Four corneas from each group were washed with PBS and subjected to real-time polymerized chain reaction (PCR) analysis. All sections were taken from wound region, 2 mm max. length to penetrating line. Total RNA was isolated from corneal tissue using the GeneJET RNA purification kit (Fermentas, Thermo Scientific, Lithuania), following the manufacturer’s instructions. After isolation, RNA quantification was measured with a picodrop microscope. Five sections for each case were evaluated, and cells in all fields were counted for statistical analysis. Negative control stainings of GFP, Keratocan, CD34 and ALDH1/2 groups were performed in rAT, rBM and control groups without primer antibodies to avoid possible false-positive staining (shown as Supplementary Data).
spectrophotometer (Pico100; Pico, Saffron Walden, UK). Total RNA was converted into cDNA using Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics, Switzerland). Equal amounts of cDNA were used in the amplification of the target fragments using the Universal Probe Library according to the manufacturer's recommendations. Primers and TaqMan probes were designed previously and validated by the manufacturer (Roche Diagnostics). All quantifications were estimated relative to the housekeeping genes ACTB and GAPDH, providing the basis for the normalization of sample-to-sample differences. The gene expression data normalization process was performed via the Log2 (− ΔCT) method where ΔCT = CT, target − CT, reference (here, CT, target and CT, reference are the threshold cycles for the target and reference gene amplification, respectively). All real-time PCR experiments were performed in triplicate.

2.13. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science Version 10 software (SPSS, Chicago, USA). Histopathological and in vivo confocal microscopic data are shown as the mean ± standard error. Clinical data were analyzed using the Kruskal–Wallis test. Five sections for each case were evaluated and cells in all fields were counted for immunohistochemical study and data were analyzed with a paired t-test and the Newman–Keuls method of multi-variant analysis. Each analysis was repeated three times, and p < 0.05 was considered statistically significant.

3. Results

3.1. Culture and characterization of the MSCs

Flow cytometric analyses of the MSCs revealed the existence of previously defined markers of MSCs (Dominici et al., 2006). The data indicated that the rBM-MSCs and rAT-MSCs expressed CD29, CD54, CD90 and MHC Class I but not MHC Class II and CD45 (Fig. 1A). The findings were consistent with the undifferentiated state of the cells, with both types of MSCs possessing immune phenotypic characteristics of MSCs.

During the isolation of the rBM-MSCs, most of the attached cells on the culture flasks displayed fibroblast-like, spindle-shaped formation during the early days of incubation. These cells began to proliferate after 3–4 days of incubation and gradually grew to form small colonies (Fig. 1B). After plating for 11–15 days, these primary cells reached 70–80% confluence. At later passages, the majority of these stem cells exhibited large, flattened or fibroblast-like morphology (Fig. 1C). Cells stored at −80 °C for a longer period demonstrated high vitality and a capability to quickly restart proliferation. The rAT-MSCs also displayed a fibroblast-like, spindle-shaped morphology during their early days of incubation following isolation (Fig. 1C) and showed similar morphological changes to the

---

Fig. 4. The percentage of cells that expressed ALDH, keratocan, CD34 and GFP. Mesenchymal stem cells are thought to increase the expression of these markers and the expression of these markers was slightly increased in the BM-MSC and AT-MSC groups (*p < 0.05 or **p < 0.01). GFP was not observed in control group, BM-MSC and AT-MSC were labeled with GFP.

Fig. 5. The expression of Keratocan in the paraffin sections. The number of cells that stained positive for the keratocan antibody slightly increased in the transplanted groups (The white arrow shows the intra-stromal location of GFP+ MSCs).
rBM-MSCs (Fig. 1D, E). These cells were capable of differentiating into adipocytes and osteoblasts (Fig. 1F–I).

### 3.2. Clinical evaluation

The corneal haze scores (CHSs) 1, 4, and 8 weeks after the surgery were compared in all groups. In the BM-MSC group, the CHS was decreased in two eyes, increased in one eye and not changed in 11 eyes, four weeks after procedure when compared with one week postoperatively. In the AT-MSC group, the CHS was decreased in four eyes, increased in no eye and not changed in 10 eyes after four weeks. The CHS was increased in two eyes, decreased in one eye, and not changed in seven eyes in the control group four weeks postoperatively. There was no difference in the CHS of the groups at four and eight weeks. Despite the decrease in the CHS of the eyes in the AT-MSC group, there were no significant differences between groups in the CHSs (p = 0.49).

### 3.3. In vivo confocal microscopy

The mean anterior keratocyte density (AKD) was 135.5 cells/mm² in the BM-MSC group, 135 cells/mm² in the AT-MSC group and 85.7 cells/mm² in the control group. The mean posterior keratocyte density (PKD) was 117 cells/mm² in the BM-MSC group, 139 cells/mm² in the AT-MSC group and 89.6 cells/mm² in the control group. Images from anterior and posterior stroma of samples are shown in Fig. 2. There was a statistically significant difference in the mean AKD and mean PKD values of the transplanted groups compared to those of the control group (Fig. 3). There was also a significant difference in the PKD measurements (p = 0.003) of the BM-MSC group compared to those of the AT-MSC but no significant difference with respect to the AKD (p = 0.936) (Fig. 3). The mean stromal...
thickness was 139.3 μm in the BM-MSC group, 130.1 μm in the AT-MSC group and 128.1 μm in the control group. The difference was not found significant (p = 0.8).

3.4. Histological analysis

There were no significant differences in the degree of inflammation and fibroblastic activity scores of the MSC groups compared to those of the control group (data not shown).

GFP labeling was used to localize the MSCs in the tissue after transplantation. Following the injection, both the GFP-labeled rBM- and rAT-MSCs were localized in the stroma and in epithelial and endothelial tissue according to the microscopic analysis. The number of cells that stain with GFP in AT-MSC group was higher than that in the BM-MSC group, but the difference was statistically insignificant (p = 0.267) (Fig. 4).

3.5. Immunohistochemical evaluation

The expression of ALDH, CD34 and the corneal stroma-specific proteoglycan keratocan by the BM-MSCs and AT-MSCs was studied to verify that the cells had differentiated into functional keratocytes. Five sections for each case were evaluated and cells in all fields were counted to make statistical analysis. Results are summarized as a graphic in Fig. 4. The immunofluorescence analysis demonstrated that the expression of keratocan was significantly increased in both the BM-MSCs and AT-MSCs when compared to the expression in the control group (p = 0.02 and p = 0.035, respectively). Cells that stained positive for the keratocan antibody and GFP in sections from each of groups are shown in Fig. 5. It can be seen that there are cells non-specific stained for GFP in epithelium of AT-MSC group in Fig. 5. ALDH was highly expressed in the BM-MSC and AT-MSC groups compared to its expression in the control group (p = 0.005 and p = 0.029, respectively). Cells that stained positive for the ALDH and GFP, CD34 antibody and GFP in sections from each of groups are shown in Figs. 6 and 7, respectively.

3.6. Gene expression in tissues

The gene expression levels of TGF-β, IL-6r, IL-6r and TNF-α in the control and transplanted groups was determined by real-time PCR. The expression of TGF-β was higher in the rBM-MSC transplanted group than in the control group but there was no significant difference in its expression compared to that in the rAT-MSC transplanted and control groups (p = 0.012 and p = 0.087, respectively). The gene expression of IL-6r, TNF-α and IL12b was highest in the control group (Fig. 8). There was a significant decrease in the expression of IL-6r in the rBM-MSCs and a significant decrease in the expression of the IL-12b gene in the rAT-MSCs (p = 0.046 and p = 0.036, respectively).

4. Discussion

Currently, corneal transplantation is the only available method for treating corneal blindness. An alternative to transplantation is needed because of a lack of donor corneas and complications associated with the procedure. Hence, we investigated whether MSCs might be a source of corneal stromal cells.

We demonstrated that both rBM-MSCs and rAT-MSCs can survive in tissue following injection into the anterior chamber. After 8 weeks, the gene expression pattern of these cells was similar to that of corneal keratocytes, with the cells expressing the cornea-specific proteoglycan keratocan, ALDH, and CD34. It is demonstrated that the keratocyte density had been increased in the rBM-MSC and rAT-MSC groups in vivo confocal microscopy. This study is the first to compare the efficacy of BM-MSCs versus AT-MSCs in traumatic
corneal scarring. They showed similar migration to the stroma, viability, and differentiation into functional keratocytes when injected into the anterior chamber of eyes. However, the rAT-MSCs have a number of advantages compared to rBM-MSCs. For example, they can be easily isolated in large numbers, and they can be obtained with higher safety.

MSCs were previously transplanted by intra-stromal injection into limuncan-defective corneas (Du et al., 2009; Liu et al., 2010). In another study, stem cells were transplanted under a stromal flap created by an excimer laser (Arnalich-Montiel et al., 2008). We injected MSCs into the anterior chamber of rat corneas through a penetrating wound. This is the first report of usage of this technique to transplant MSCs into the cornea.

A few studies have reported improvements in stromal thickness following MSC transplantation based on in vivo imagining (Du et al., 2009). In our study, there was no difference in the stromal thickness of any of the groups. However, the keratocyte density was increased in the rBM-MSC and rAT-MSC groups. Based on these findings, we propose that MSCs may replace keratocytes in situations that cause keratocyte loss.

We observed moderate haze in several corneas in both the BM-MSC and AT-MSC groups, as well as in the control group. Similar to the study of Arnalich-Montiel et al., we found no significant difference in corneal haze scores, despite the presence of MSCs and functional keratocytes in the cornea (Arnalich-Montiel et al., 2008). The low number of transplanted cells or the single injection might explain the inadequate corneal transparency. In addition, our wound model which has not been used for MSCs studies on cornea before, might not generate a significant scar to demonstrate the efficacy of the MSCs. Another possible explanation may be that all the transplanted cells might not have been positive for keratocan. Alternatively, there might be any other expression of keratocytes that is responsible for corneal transparency.

Mesenchymal stem cells show different properties with respect to the tissue from which they are isolated. The secretion level of different cytokines might show alterations due to differential levels and time of inflammation. This could determine different response of MSCs in the same conditions and effects of MSCs are largely paracrine in nature (William et al., 2013; Chen et al., 2008). We found that the expressions of TNF-α, IL-6r and IL-12b which are known pro-inflammatory were decreased on damaged corneas in both MSC transplanted groups when compared with the control group. There was also a significant difference in the expression level of IL-12b in the AT-MSC group compared to the BM-MSC group. These results show that MSCs may have anti-inflammatory properties when transplanted into a cornea containing a penetrating wound. Rather than simply replacing keratocytes, these cells are likely exerting a paracrine influence on repopulating the wounded region as described by Basu et al. (2014). AT-MSCs may be superior to BM-MSCs with regard to anti-inflammatory properties.

Transforming growth factor beta (TGF-β) was described as a pleiotropic modulator in the regulation of inflammation. Considering the level of IL-6, the role of the TGF-β changes in the tissue. Additionally, the level of TGF-β is related to the number of myofibroblasts and corneal scarring, and decreases in the TGF-β level cause apoptosis of myofibroblasts (Wilson, 2012; Singh et al., 2011). Recently, Singh et al. reported that TGF-β regulate corneal myofibroblast development from bone marrow-derived precursor cells and keratocyte/corneal fibroblast-derived precursor cells (Singh et al., 2014). In our study, the expression of TGF-β was decreased in the AT-MSC group when compared with that of the BM-MSC and control groups. The corneal opacity score was also reduced in more rats in the AT-MSC group, and this may be related to the TGF-β level. Studies with larger numbers of animals are needed to shed light on the association between the TGF-β level and corneal opacity scores.

5. Conclusions

Despite the lack of perfect optic transparency, we demonstrated the establishment of MSCs with functional keratocyte activities in rat corneas and increased keratocyte densities following the transplantation of the MSCs. We suggest that BM-MSCs and AT-MSCs may be used as a substitute for keratocytes in situations that cause keratocyte loss, such as traumatic loss, corneal thinning diseases, and corneal damage after infections. The approach used here (i.e., injection into the anterior chamber) is a simple surgical procedure and can be performed even under sub-optimal medical conditions in developing countries.

Further investigation is required to determine the optimal injection time, frequency, and dosing regime. The detection of functional keratocyte activities of MSCs pre-transplantation may be useful to achieve optical transparency.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

This study was supported by grants from the Kocaeli University Scientific Research Projects Unit (KOU-BAP project no: 2010-047). English editing was supported by Elsevier editing system. We thank to Ozgül Altintas for the statistical analysis.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jexer.2016.08.011.

References


