USE OF THE NANOFIBER SCAFFOLD FOR TRANSFER OF STEM CELLS ONTO THE INJURED OCULAR SURFACE IN MOUSE EXPERIMENTAL MODEL

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Abstract

Corneal damage is one of the most common causes of impaired vision or even blindness. When the injury is more extensive and the limbal region is involved, the natural regeneration of the cornea is not sufficient. Such damage can lead to the limbal stem cell deficiency (LSCD). The only option for LSCD treatment is transplantation of the limbal tissue or a transfer of limbal stem cells (LSCs) cultured from the healthy eye. The allogenic transplantation of the limbus or cultivated LSCs with a systemic administration of immunosuppressive drugs is needed in the case of bilateral LSCD. Nevertheless, the cell therapy is very promising approach for LSCD treatment. Transplantation of mesenchymal stem cells (MSCs) seeded on an appropriate scaffold turned out to be a suitable therapy of the LSCD. In our experimental model of LSCD we use nanofiber scaffold for MSC and LSC cultivation and for transplantation of these cells onto the chemically injured mouse eye. MSCs have immunosuppressive and immunomodulatory properties. We showed that MSCs have the ability to inhibit production of molecules associated with the inflammation and support epithelial regeneration in the damaged cornea. These inhibitory properties were confirmed in both *in vitro* and *in vivo* mouse model. Results thus showed beneficial effects of stem cell transplantation for murine corneal healing and for suppression of a local immune reaction which can impede the healing process. Such similarity of *in vivo* and *in vitro* results allows us further experiments to clarify mechanisms of MSC regenerative and healing properties after the transplantation onto the injured cornea.

Keywords: Cornea, nanofiber scaffold, limbal stem cell deficiency, mesenchymal stem cells

1. INTRODUCTION

1.1. Limbal stem cell deficiency

According to the World health organization there are 39 million of blind people worldwide of which 25 % cases are caused by corneal damage and 5 million of patients suffer from bilateral corneal blindness. Corneal epithelium has the ability to regenerate. This function is held by limbal stem cells (LSCs), which continuously migrate from limbal region to the center of the cornea and differentiate to corneal epithelial cells. LSCs are a small population (3-5 %) of slowly dividing cells in the limbus, the area between cornea and conjunctiva. When the corneal damage is so extensive, that it includes the limbal tissue, the limbal stem cell deficiency (LSCD) occurs and the corneal regeneration is abrogated. Consequently, neovascularization and production of pro-inflammatory cytokines occur. Corneal immune privilege is lost due to chronic inflammation and the conjunctival epithelium migrates over the damaged cornea. Corneal transparency gradually decreases and this process leads to visual impairment or even to blindness [1].

1.2. LSCD treatment

Corneal transplantation is the most successful transplantation of a solid tissue because of its immune privilege. However, in the case of LSCD, such transplantation alone is not a successful treatment. The healthy limbus with sufficient amount of LSCs is needed for further corneal regeneration. Transplantation of the limbal

(keratolimbal) tissue or cultured LSCs from patient´s or related donor´s healthy eye represents the only treatment of unilateral LSCD. Clinical studies have shown therapeutic efficacy of cultured LSC transplantation onto the ocular surface. The use of LSCs is the only approved stem cell (SCs) treatment of the corneal injury in European union [2]. In the case of bilateral LSCD, it is necessary to use the allogeneic limbal tissue or LSCs. The limbus is highly vascularized tissue and such allogeneic transplantation is associated with the administration of high doses of immunosuppressive medication with side effects. Transplantation of autologous LSCs has better outcomes than allogeneic LSC transplantation [3]. Although it is possible to obtain larger grafts of the limbal tissue from cadaver donors for allogeneic transplantation, the use of limbal grafts from healthy donors has better results [4]. However, such treatment is associated with a risk of damaging the healthy eye [5].

1.3. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) represent a suitable autologous cell substitution for the cell therapy of LSCD. MSCs are multipotent SCs, which can be isolated from the most of tissues in our body. They are capable of differentiation to many cell types, migration to the site of injury, immunomodulation and inhibition of inflammation or apoptosis [6,7]. MSCs have been used in numerous experimental studies of LSCD treatment in murine [8,9] or rabbit [10,11] models. The regeneration of the corneal epithelium and inhibition of the inflammation were observed. The differentiation of MSCs into corneal epithelial cells were observed in some of experiments [12]. Despite the large number of experimental works on SC therapy of LSCD, the mechanism of MSC inhibitory properties on the ocular surface is still not well recognized.

1.4. Scaffolds for stem cell transplantation onto the injured cornea

SCs cannot be seeded directly onto the cornea because of tears continuously wash the ocular surface. Therefore, it is necessary to use the scaffold which is suitable for seeding, cultivating and for a transfer of SCs onto the damaged cornea.

Amniotic membrane (AM) is used in various corneal injury therapies, for example after the corneal transplantation, for its capability to promote the epithelialization and the healing of wounds [13]. Such properties of AM can be used for SC transplantation as well. AM was successfully used as the scaffold in both allogeneic and autologous LSC transplantation [14]. MSCs seeded on AM transferred onto the animal ocular surface supported corneal healing [15].

Fibrin glue is used in various chirurgical procedures and it has been also used in successful clinical studies of autologous LSC transplantation onto the cornea with LSCD [16].

The first autologous transplantation of LSCs was performed with special contact lenses [17]. LSCs cultivated on contact lenses produced factors important for corneal epithelium regeneration [18].

Nanofiber scaffold (NS) represents one of promising alternatives of SC carrier. The three-dimensional structure of NS imitates extracellular matrix and natural niche, in which SCs can survive and retain their abilities. Such carriers can be prepared from natural polymers like collagen and chitosan or from synthetic polymer (e.g. polyamide). NS can be manufactured with an addition of pharmacological substance like immunosuppressive drugs or antibiotics, which may have beneficial effect during SC therapy [19,20]. In our laboratory, we have used in experimental models LSCs seeded on the NS to treat mechanically injured cornea. LSCs migrated from NS to the injured ocular surface and there inhibited the local inflammation and supported a corneal epithelium restoration [8]. MSCs transferred on the chemically burned rabbit corneas also inhibited inflammation [10]. MSCs and LSCs transplanted onto the chemically burned cornea with NS had comparable immunomodulatory and regenerative properties [11].

2. METHODS

2.1. *In vivo* mouse model of LSCD

BALB/c mice were anesthetized by an intramuscular injection of 1:1 mixture of xylazinum hydrochloridum 2 % (0.175 ml; Rometar; Spofa) and ketaminum hydrochloridum 5 % (0.175 ml; Narkamon; Spofa). Right corneas of anesthetized mice were injured by 0.25N sodium hydroxide (NaOH) on the corneal surface (8 µl of NaOH on cornea-size filter paper attached on the cornea). These eyes were rinsed with an excess of phosphate buffered saline (PBS).

2.2. Isolation and cultivation of MSCs

MSCs were isolated from the femurs of mice. The bone marrow was flushed out and a single-cell suspension was prepared by homogenization. The cells were seeded in DMEM (Sigma-Aldrich) containing 10 % fetal calf serum (FCS), antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin), and 10 mM HEPES buffer in 75 cm2 tissue culture flasks (TPP). After 48 hours of incubation, nonadherent cells were washed out and the adherent cells were cultured with a regular exchange of the medium and passaging of the cells to maintain their optimal concentration.

2.3. *In vivo* model of MSC treatment of LSCD

Nanofiber scaffolds were prepared from the biocompatible polymer poly(L-lactic) acid (PLA) by a needleless electrospinning procedure. Scaffolds were cut into squares (1.5 x 1.5 cm) and fixed into CellCrown TM24 inserts (Scaffdex Ltd.). The inserts with nanofibers were transferred into 24-well tissue culture plates (TPP). SCs (3 x 10^5 cells per well) in 700 ml of DMEM with 10 % FCS were transferred into each well. The plates were incubated for 24 hours to allow the cells to adhere to the scaffold. For SC transfer, NS seeded with SCs were transplanted immediately after the injury with the cell side facing down on the damaged ocular surface. The scaffolds were sutured to the limbal tissue region with four sutures using 11.0 Ethilon (Ethicon, Johnson & Johnson). The eyelids were sutured together using Resolon 7.0 (Resorba Medical GmbH) for 72 hours. An ophthalmic ointment compound (Ophthalmo-Framykoin; Zentiva Group) was applied on the ocular surface after the stitching. The NS was removed from the ocular surface on day 3 after the operation. Corneas were excised 7 days after the procedure and immediately transferred into 500 µl of TRI Reagent (Molecular Research Centre Inc.) and stored in -80 °C. The expression of genes for inflammatory cytokines, inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF) as a marker of neovascularization in corneas was detected using real-time PCR. The expression of gene for cytokeratin 12 (K12), as a marker of corneal epithelium, was similarly detected.

2.4. *In vitro* model of MSC treatment of the inflammation

For our *in vitro* experiments, SCs were cultured in 48-well tissue culture plates (TPP) with 500 µl of DMEM with 10 % FCS for 24 hours in concentration 5 x 10^4 cells per well. Excised corneas were added to the culture and stimulated by proinflammatory cytokines interleukin-1β (IL-1β), interferon-γ (IFN-γ) and IL-17. After a 48-hour co-cultivation, the expression of genes for inflammatory cytokines, iNOS and VEGF in corneas was detected using real-time PCR.

2.5. Real time PCR

The details of RNA isolation, transcription, and the PCR parameters have been described previously [13]. The relative quantification model with efficiency correction was applied to calculate the expression of the target gene in comparison with GAPDH used as the housekeeping gene. Primers used for amplification were

GAPDH: 5´-AGAACATCATCCCTGCATCC-3´ (sense), 5´-AGAA-CATCATCCCTGCATCC-3´ (antisense), K12: 5´-ATCGAGGACCTGAAGAGCAA-3´ (sense), 5´-TCGA-TCTGCAGGAGGACATT-3´ (antisense), IL-1β: 5´-TTGGGTTAAATGACCGCAACA-3´ (sense), 5´-GA-GCGCTCACGAACAGTTG-3´ (antisense), IL-6: 5´-GCTACCAAACTGGATATAATCAGGA-3 (sense), 5´-CCAGGTAGCTATGGTACTCCAGAA-3´ (antisense), iNOS: 5´-CTTTGCCACGGACGAGAC-3´ (sense), 5´-TCATTGTACTCTGAGGGCTGAC-3´ (antisense), VEGF: 5´-AAAAACGAAAGCGCAA-GAAA-3´ (sense), 5´-TTTCTCCGCCTCTGAACAAGG-3´ (antisense).

3. RESULTS AND DISCUSSION

3.1. A local cytokine, iNOS and K12 expression after corneal damage and treatment with MSCs seeded on the nanofiber scaffold

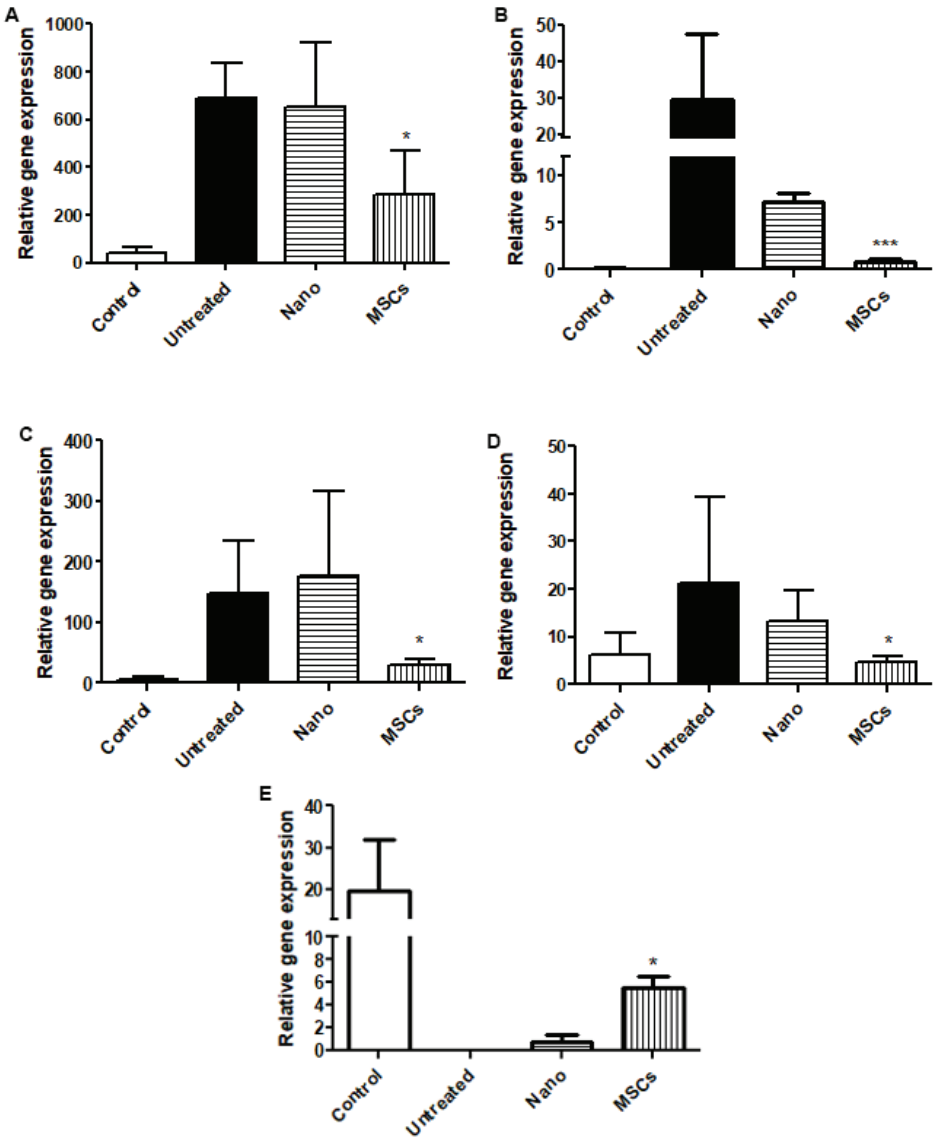

Figure 1 Relative expression of genes for pro-inflammatory molecules IL-1β (A), IL-6 (B), iNOS (C), VEGF (D) and for corneal marker K12 (E). Values with asterisks represent statistically significant (*P < 0.05, ***P < 0.001) difference from injured untreated corneas

Mouse corneas were burned by 0.25M NaOH and rinsed with PBS. Healthy control corneas were left without burning (Control). Mice in the positive control group were injured and left without treatment (Untreated). Mice

in the other control group was injured and treated with NS (Nano) without cells. In the experimental group of mice, MSCs were seeded onto the NS and transplanted on injured corneas (MSCs). Relative gene expression of IL-1β (**Figure 1A**), IL-6 (**Figure 1B**), iNOS (**Figure 1C**) and VEGF (**Figure 1D**) was significantly decreased in MSC-treated corneas in comparison to injured control corneas untreated or treated with NS alone. The regeneration of corneal epithelium was observed as increased expression of K12 in MSC-treated corneas (**Figure 1E**). These results are comparable to previous experiments with rabbit or rat models [10,11,15]. Regenerative and immunomodulatory properties of transplanted MSCs are also comparable with transplanted LSCs [11].

3.2. The effect of MSCs on cytokine and iNOS production in corneas *in vitro*

The establishing of a new *in vitro* model is crucial for further investigation of MSC regenerative mechanisms after the transplantation onto the burned ocular surface. Corneas were stimulated with IL-1β, IFN-γ and IL-17 (+cyt) for simulation of inflammatory environment in our *in vitro* model of corneal inflammation. Treated corneas were cultured alone in complete DMEM (control) and with MSCs. Relative gene expression of IL-1β (**Figure 2A**), IL-6 (**Figure 2B**), iNOS (**Figure 2C**) and VEGF (**Figure 2D**) in corneas co-cultured with MSCs were significantly lower than in control corneas. There are similarities between our *in vivo* and *in vitro* models in inhibitory properties of MSCs. These findings and the ability to change inflammatory environment according to results from *in vivo* experiments will be used for further understanding of MSC therapeutic mechanisms after transplantation onto the injured ocular surface.

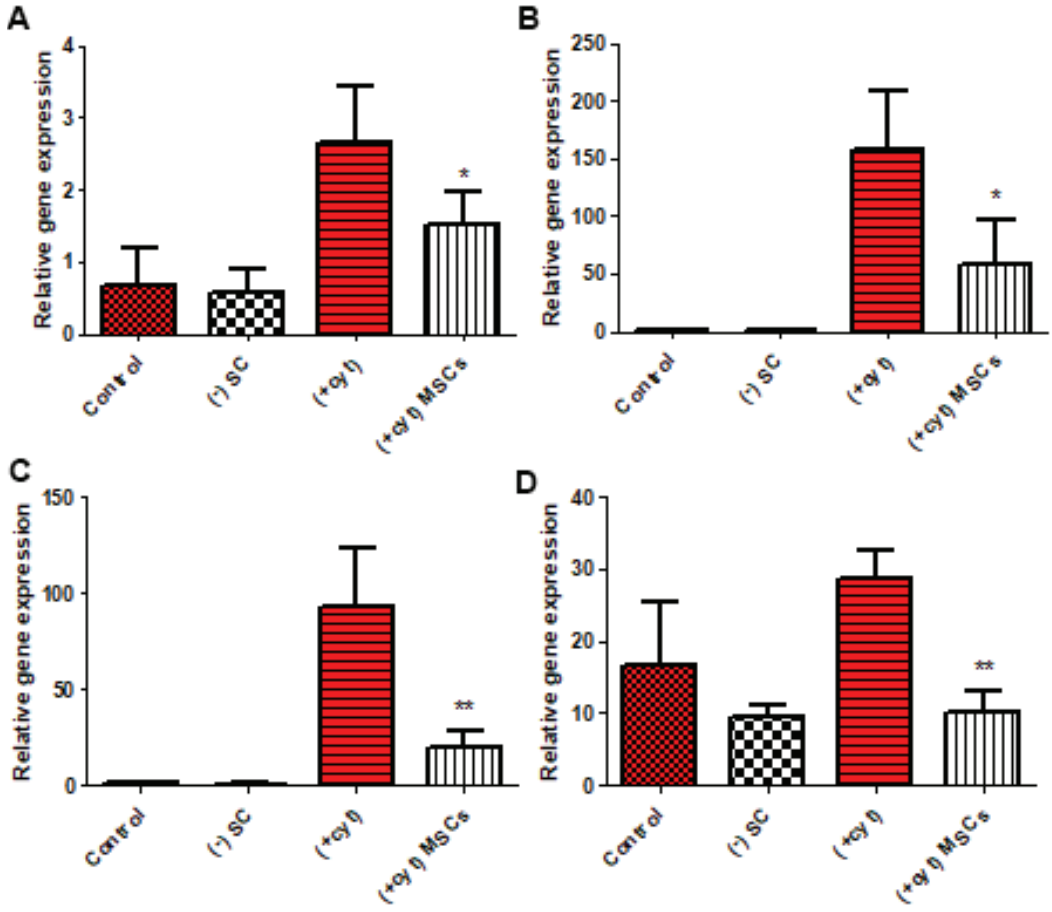

Figure 2 Relative gene expression of IL-1β (A), IL-6 (B), iNOS (C) and VEGF (D) in in vitro model of corneal inflammation. Values with asterisks represent statistically significant (*P < 0.05, **P < 0.01) difference from injured untreated corneas

4. CONCLUSION

Autologous MSCs seeded on nanofiber scaffold represent a suitable source of SCs for the therapy of LSCD, where is no possibility to use LSCs. MSCs inhibit a local production of pro-inflammatory molecules, inhibit neovascularization and support corneal regeneration as previously described in rabbit model. These effects *in vivo* are similar in our new *in vitro* model, which has not been described previously. The establishing of this *in vitro* mouse model should help us to describe therapeutic mechanisms of transplanted MSCs onto the injured ocular surface in the future as well as to describe the fate of transplanted MSCs.

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