Histological Study on the Role of Mesenchymal Stem Cells in Corneal Wound Healing in Acute Chemical Injury in Rats

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Abstract

Background and Aim of Work: Corneal diseases are a major cause of blindness in the world; complications are the result of exaggerated inflammatory reaction which is beyond the physiological response occurring during corneal wound healing. Limiting inflammation and controlling the angiogenic response together with enhancing epithelial covering are critical aspects of therapy. Therefore, this study was performed to investigate the role of MSCs in the alkali-induced acute corneal injury in rats by immunohistochemical methods.

Methods and Results: Twenty five healthy adult albino rats were divided into: Group I Control Group (n=5) had irrigation with normal saline in their left eyes. Group II (injured) had a corneal alkali burn in their left eyes and were sacrificed after one week (gIIa n=5) and after 2 weeks (gIIb n=5) following the burn. Group III rats were injured and subconjunctivally injected with 0.1ml Phosphate Buffer Saline (PBS) containing 2 X 10^6 labeled MSCs (treated). (gIIIa n=5) received single injection of the MSCs immediately at the time of injury and (gIIIb n=5) received double injections of the MSCs one immediately at the time of injury and the 2nd on the 3rd day of the burn and were sacrificed 1 week after injury. Left corneas were harvested and CD44, PCNA and VEGF immunoreactivity were studied by immunohistochemical methods. Statistical analyses were performed using the ANOVA test. MSCs proved to have a therapeutic role in corneal wound healing which may be through an anti-angiogenic role.

Conclusion: Subconjunctival injection of MSCs may have a positive beneficial effect in treating corneal injury. MSCs can exert a positive influence on the native corneal cell proliferation. Its action may be through an anti-angiogenic role.

Key Words: MSCs – Cornea – Immunohistochemical – Acute chemical – Angiogenesis.

Introduction

CORNEAL diseases are a major cause of blindness in the world. Although great progress has been achieved in the treatment of corneal diseases, wound healing after severe corneal damage and immunosuppressive therapy after corneal transplantation remains problematic [1].

The disadvantages or the complications that may result from limbal grafts as limbal sampling in auto-transplantation bears the potential risk of causing Limbal Stem Cell Deficiency (LSCD) in the healthy contralateral eye [2] and the risk of significant side effects from long-term immunosuppression in limbal allo-graft are major drawbacks of these techniques [3].

The commonest complications of corneal burn are slow epithelialization, neovascularization, epithelial ulcers which may become chronic, and corneal perforation. Such complications are the result of exaggerated inflammatory reaction which is beyond the physiological response occurring during corneal wound healing [4].

Limiting inflammation and controlling the angiogenic response together with enhancing epithelial covering are critical aspects of therapy [8]. Mesenchymal Stem Cells (MSCs) are multipotent cells originally isolated from bone marrow [6]. It possesses multifunctional properties from tissue repair/regeneration to immunomodulatory/anti-inflammatory functions [7], it is assumed that these cells may be capable of inducing healing in the injured cornea while limiting the complications.

Because the cornea is an accessible organ, subconjunctival (S.C) injection of stem-cells is apparently more convenient to use than the intravenous route to ensure adequate concentration of stem cells and/or their products. S.C injection is considered an easy route of administration can be easily performed [8].
The aim of this study is to investigate the effect of S.C injection of MSCs in the alkali-induced acute corneal injury in rats, monitored by immunohistochemical and morphometric methods.

Material and Methods

In this experimental study, a total number of 25 healthy adult rats (20 females and 5 males) about 3 months old, ranging in weight between (170-200gm) each (mean 185 ± S.D. 7.57) were used.

The rats were bred at the Animal House of Faculty of Medicine, Cairo University, this was done during 2012. The animals were purchased and housed in metal cages with mesh and wood chips for bedding. They were maintained in room temperature with a 12-hour light/12-hour dark cycle, fed with standard laboratory chow and had free access to tap water. The animals were divided into the following groups, which were kept in separate cages:

Group I: (Control group): This group comprised 5 rats which had undergone irrigation with normal saline in their left eyes. It was further subdivided into two subgroups:

Subgroup IA: Consisted of 3 rats which were sacrificed 1 week following the injury.

Subgroup IB: Consisted of 2 rats which were sacrificed 2 weeks following the injury.

Group II: (Alkali burn group): This group comprised 10 rats. A corneal alkali burn was generated in the left eye of each rat. It was further subdivided into two subgroups:

Subgroup IIA: Consisted of 5 rats which were sacrificed 1 week following the burn.

Subgroup IIB: Consisted of 5 rats which were sacrificed 2 weeks following the burn, a follow-up group.

Group III: (MSCs treated group): This group comprised 10 rats. Corneal alkali burns were performed in their left eyes. The group was subdivided into two subgroups 5 rats each.

Subgroup IIIA: Rats received single S.C. injection of MSCs immediately at the time of injury and were sacrificed 1 week after the burn.

Subgroup IIIB: Rats received one injection immediately at the time of injury and the 2nd injection on the 3rd day of burn and the animals were sacrificed 1 week after injury.

Each injection consisted of 0.1ml Phosphate Buffer Saline (PBS) containing $2 \times 10^6$ labeled MSCs.

Induction of corneal alkali burn:

The rats were anaesthetized by intraperitoneal injection of 4ml/kg of 10% chloral hydrate. A disc of whatman #3 filter paper (4mm diameter) soaked in 4ml NaOH (1mol/L) was applied to the center of the cornea for 40 seconds, and then the cornea was rinsed with 60ml of saline for 1 minute [9].

Preparation of Bone Marrow-derived mesenchymal stem cells from rats:

A- Isolation of Bone Marrow-derived MSCs from rats: Bone Marrow (BM) was harvested by flushing the tibiae and femurs of 6 weeks old male white albino rats with Dulbecco’s modified Eagle’s medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine medium (GIBCO/BRL). Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). Cells were incubated at 37°C in 5% humidified CO₂ for 12-14 days as primary culture or upon formation of large colonies. When large colonies developed (80-90% confluence), cultures were washed twice with (PBS) and cells were trypsinized with 0.25% trypsin in 1ml EDTA (GIBCO/BRL) for 5 minutes at 37°C. After centrifugation (at 2400rpm for 20 minutes), cells were re-suspended with serum-supplemented medium and incubated in 50cm² culture flask Falcon). The resulting cultures were referred to as first-passage cultures [10].

MSCs in culture were characterized by their adhesiveness and fusiform shape and by detection of CD 29 one of surface markers of rat mesenchymal stem cell [11].

B- Labeling of stem cells with PKH26 dye: MSCs cells were harvested during the 4th passage and were labeled with PKH26 fluorescent linker dye. Corneal tissue was examined with a fluorescence microscope. The cells were examined using fluorescence microscopy (Sigma-Aldrich, Saint Louis, USA) to detect and trace the cells stained with PKH26.

This was done at the Biochemistry Department, Faculty of Medicine, Cairo University.
Histological study:
The animals were sacrificed with an overdose of 10% chloral hydrate. Left eye from each animal were immediately dissected, fixed in 10% formol saline, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Paraffin blocks were cut at 5-7 µm thickness, using Leica rotator microtome (Germany) and for immunohistochemical studies, sections were mounted on poly L-lysine coated slides.

Immunohistochemical study: Using the avidin-biotin peroxidase complex technique. Sections were counterstained with Meyer's hematoxylin [12] for detection of:

1- CD44: CD44 is a multistructural and multifunctional cell surface molecule involved in cell proliferation and cell differentiation. It is mouse monoclonal antibody (for detection of MSCs) both endogenous and exogenous MSCs [13,14]. This antibody targets CD44 in applications and shows reactivity with rat samples. It was supplied in a liquid form in concentration of 0.1mg/ml. The cellular staining pattern for CD44 is membranous.

Positive tissue control for CD44: Human kidney carcinoma biopsies showed +ve immunostaining in the form of brown membranous reaction for CD44.

Negative control for CD44: Additional specimens of the cornea were processed in the same sequence but the primary antibody was not added and instead, PBS was added in this step. Omission of the primary antibody gave no staining reaction.

2- Proliferating Cell Nuclear Antigen (PCNA): PCNA is a protein of DNA-polymerase enzymes, necessary for DNA synthesis and is used as a standard marker in proliferating cells mouse monoclonal antibody (for detection of nuclear DNA) [15]. This antibody reacts in human, mouse, rat and chicken and has been successfully used in ELIZA. It was supplied in a liquid form in concentration of 1mg/ml, TBS (Tris Buffered Saline). The cellular staining pattern for PCNA is nuclear.

Positive tissue control for PCNA: Human breast cancer biopsies showed +ve immunostaining in the form of brown nuclear reaction for PCNA.

Negative control for PCNA: Additional specimens of the cornea were processed in the same sequence but the primary antibody was not added and instead, PBS was added in this step. Omission of the primary antibody gave no staining reaction.

3- Vascular Endothelial Growth Factor (VEGF): VEGF is a secreted polypeptide that was initially identified by its ability to increase permeability of the vasculature [16]. It is a rabbit polyclonal antibody and was used for the detection of proliferating blood vessels. It was supplied in a liquid from in a concentration of 1mg/ml. The cellular staining pattern for VEGF is cytoplasmic.

Positive tissue control for VEGF: Human heart biopsies showed +ve immunostaining in the form of brown nuclear reaction for VEGF.

Negative control for VEGF: Additional specimens of the cornea were processed in the same sequence but the primary antibody was not added and instead, PBS was added in this step. Omission of the primary antibody gave no staining reaction.

Morphometric study:
Using Leica Qwin 500 LTD computer assisted image analysis system (Cambridge, United Kingdom). For each group, ten slides of ten different specimens were examined. From each slide, ten non-overlapping fields were measured.

The following parameter was measured:
The mean area % occupied by, strong brown membranous reaction in CD44 immunostained sections, by strong brown nuclear staining in PCNA immunostained sections and by strong brown cytoplasmic reaction in VEGF immunostained sections. This was done using the color detect menu at a magnification of x400, in relation to a standard measuring frame. The areas of positive immunoreactivity were masked by a blue binary color.

The optical density of the brown membranous reaction of CD44 immunostainin was measured using the grey detect menu at a magnification of x400.

Statistical analysis:
Quantitative data were summarized as means and standard deviations and compared using one-way analysis-of variance (ANOVA). All statistical analyses were done on an IBM personal computer using the (SPSS). Results were considered significant when probability (p) was 0.05, and highly significant when p < 0.01 [17].
Results

Immunohistochemical results:

Anti-CD44 stained corneal sections:
Few cells exhibited mild +ve membranous reaction for CD44 in corneal sections from control rats Fig. (1A). Subgroup IIA showed moderate positive membranous reaction in many epithelial cells Fig. (1B) which was only detected in few epithelial cells in subgroup IIB Fig. (1C). Subgroups IIIA and IIIB showed many cells exhibiting strong +ve immunostaining in the epithelium Fig. (1D) and Fig. (1E).

Anti-PCNA stained corneal sections:
Positive PCNA immunostaining was demonstrated in the basal epithelial cells as well as the endothelium of corneal sections from control rats Fig. (2A). In subgroup IIA positive PCNA reaction was detected in some cells in the basal layer of the epithelium Fig. (2B). In subgroup IIB, few cells exhibited +ve nuclear reaction in the basal layer of the epithelium Fig. (2C). However, in subgroup IIIA many cells exhibited +ve nuclear reaction in the basal layer of the epithelium Fig. (2D). In subgroup IIIB, many cells exhibited +ve nuclear reaction in the basal layer of the epithelium as well as in the stroma Fig. (2E).

Anti-VEGF stained corneal sections:
Control cornea showed +ve reaction for VEGF Fig. (3A). Subgroups IIA and IIB, however, showed positive cytoplasmic reaction in the endothelial cells of many newly formed blood vessels Fig. (3B,C). In subgroup IIIA, +ve reaction was detected only in the peripheral parts of the cornea in some specimens Fig. (3D), while also in the central part of the cornea in others Fig. (3E). In subgroup IIIB the epithelium and stroma showed –ve immunoreactivity for VEGF Fig. (3F).

Quantitative morphometric results:

Mean area percent of CD44 immunoreactivity (±SD) in the studied groups:
There was significant (*p*<0.05) increase in the mean area % of CD44 in Group IIA when compared to Group IIB. At the same time, Groups (IIA & IIB) showed significant (*p*<0.05) increase in the mean area % of CD44 when compared to the control groups. There was no significant difference between the 2 subgroups of Group III which showed significant (*p*<0.05) increase in the mean area % of CD44 when compared to other groups (Table 1).

Mean optical density of CD44 immunoreactivity (±SD) in the studied groups:
A significant (*p*<0.05) increase in the mean optical density of CD44 +ve immunostaining was estimated in subgroups IIA and IIB compared to the control group. In addition, a significant (*p*<0.05) increase was found in subgroups IIIA and IIIB when compared to other groups (Table 1).

Mean area percent of PCNA immunoreactivity (±SD) in the studied groups:
A significant (*p*<0.05) increase in the mean area % of PCNA +ve immunostaining was detected in subgroups IIA, IIIA and IIIB when compared to Group I and subgroup IIB. In addition, a significant (*p*<0.05) increase was found in subgroups IIIA and IIIB compared to IIA (Table 1).

Mean area percent of VEGF immunoreactivity (±SD) in the studied groups:
A significant (*p*<0.05) increase in the mean area % of VEGF +ve immunostaining was detected in subgroups IIA, IIB and IIIA compared to subgroups I and IIB. At the same time there was no significant difference in the mean area % of VEGF between control group and group (IIIB) (Table 1).

Table (1): Mean epithelial height, mean area% of CD44 +ve reaction, mean optical density of CD44 +ve reaction, mean area% of PCNA +ve reaction and mean area% of VEGF +ve reaction in control and experimental subgroups.

<table>
<thead>
<tr>
<th>Groups and subgroups</th>
<th>Area % of CD44</th>
<th>Optical density of CD44</th>
<th>Area % of PCNA</th>
<th>Area % of VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group I</td>
<td>0.06±0.01 a</td>
<td>0.23±0.05a</td>
<td>1.07±0.15a</td>
<td>0.06±0.01 a</td>
</tr>
<tr>
<td>Subgroup IIA</td>
<td>2.31±0.67b</td>
<td>0.33±0.07b</td>
<td>1.58±0.32b</td>
<td>1.03±0.27b</td>
</tr>
<tr>
<td>Subgroup IIB</td>
<td>0.62±0.18c</td>
<td>0.33±0.05b</td>
<td>1.15±0.26a</td>
<td>1.28±0.23b</td>
</tr>
<tr>
<td>Subgroup IIIA</td>
<td>3.28±0.73 d</td>
<td>0.51±0.07c</td>
<td>2.08±0.34c</td>
<td>0.91±0.17b</td>
</tr>
<tr>
<td>Subgroup IIIB</td>
<td>3.59±0.62d</td>
<td>0.73±0.06d</td>
<td>2.31±0.43c</td>
<td>0.15±0.03a</td>
</tr>
</tbody>
</table>
Fig. (1): A photomicrograph of a corneal section of control rat showing (A) Few cells exhibiting mild +ve membranous reaction in the epithelium for CD44 (arrows) (CD44 immunostaining, X1000). (B) Corneal section of subgroup IIA showed moderate positive membranous reaction in some epithelial cells (arrows) (CD44 immunostaining, X1000). (C) Corneal section of subgroup IIB showed moderate positive reaction in few epithelial cells (arrows) (CD44 immunostaining, X1000). (D) Corneal sections of subgroup IIIA showed many cells exhibiting strong +ve membranous reaction in the epithelium (arrows) (CD44 immunostaining, X1000). (E) Corneal section of subgroup IIIB showed many cells exhibiting strong +ve membranous reaction in the epithelium (arrows) (CD44 immunostaining, X400).

Fig. (2): A photomicrograph of a corneal section of a control rat showing (A) Positive nuclear reaction in the cells of the basal layer of the epithelium and in the endothelium (arrows) (PCNA X1000). (B) Corneal section of subgroup IIA showing positive nuclear reaction in some cells in the basal layer of the epithelium (arrows) (PCNA X400). (C) Corneal section of subgroup IIB showing few cells exhibiting +ve nuclear reaction in the basal layer of the epithelium (arrows) (PCNA X400). (D) Corneal section of subgroups IIIA showing many cells exhibiting +ve nuclear reaction in the basal layer of the epithelium (arrows) (PCNA X400). (E) Corneal section of subgroups IIIB showing many cells exhibiting +ve nuclear reaction in the basal layer of the epithelium and in the stroma (arrows) (PCNA X1000).
Histological Study on the Role of MSCs in Corneal Wound Healing

Discussion

Corneal diseases are a major cause of blindness in the world. Although great progress has been achieved in the treatment of corneal diseases, wound healing after severe corneal damage and immunosuppressive therapy after corneal transplantation remains problematic [1].

Numerous studies have reported that CD44 expression is one of the characteristics of MSCs in both humans and mice [18,19]. On examination of CD44 immuno-stained sections of the control group, few cells showed positive reaction in the basal layer of the epithelium. In injury Group (IIA), more cells exhibited the positive reaction in the epithelium. This was matching with [20] who detected increased CD44 expression early during corneal epithelial wound healing. The reaction in the epithelium was down-regulated in Group (IIB) as few cells exhibited the positive reaction. After injection of MSCs, many cells exhibited +ve reaction in the epithelium in both subgroups (single and double injections) as well as, some spindle shaped cells exhibited the reaction in the stroma.

To verify these findings, the mean area % of CD44 immuno-reactivity in the epithelial cells was assessed by morphometric measurements using the image analysis system. Morphometric analysis proved significant increase in the mean area % in the injured Group (IIA) when compared to the control group and also when compared to subgroup (IIB) and both showed significant increase in the mean area % of CD44 immuno-reactivity when compared to the control group. A significant increase in the mean area % of CD44 immuno-reactivity was reported in the MSCs-injected groups when compared to injured and control groups.

Such up regulation of CD44 immuno-reactivity in the injured Group (IIA) indicated that injury stimulated the undifferentiated stem cells of the epithelium, which was significantly decreased in injured subgroup (IIB) indicating a decreased proliferation of the undifferentiated cells after the

![Fig. (3): A photomicrograph of a corneal section showing (A) Negative reaction for VEGF in the control rat (VEGF X400). (B) Corneal section of subgroup IIA showing positive cytoplasmic reaction in the endothelial cells of many newly formed blood vessels (arrows) (VEGF X1000). (C) Corneal section of subgroup IIIB showing positive cytoplasmic reaction in the endothelial cells of many newly formed blood vessels (arrows) (VEGF X400). (D) Corneal section of subgroup IIIA showing +ve cytoplasmic reaction in the endothelial cells of blood vessels in the peripheral part of the cornea (arrows) (VEGF X400). (E) Corneal section of subgroup IIIA showing +ve cytoplasmic reaction in the subepithelial blood vessels in the central part of the cornea (arrows) (VEGF X1000). (F) Corneal section of subgroup IIIB showing –ve reaction in the epithelium and stroma (VEGF X400).]
healing of epithelium has took place. The up-regulation detected in the treated groups indicated that the exogenously injected MSCs have stimulated the proliferative activity of these undifferentiated cells which might be through cell to cell contact or through cytokines and other mediators; which helped in accelerating the healing process. This was matching with [20] who stated that the expression of CD44 increased in the basal epithelial cell layers surrounding the wound margin 3 hours after wounding and peaked at 18 hours and the density of CD44 mRNA label declined as the cells began proliferation after wounding and healing.

To examine the strength of CD44 cell marker immuno-reactivity, optical density of the reaction was assessed in different groups. Groups (IIA) and (IIB) showed significant increase in the mean optical density of CD44, when compared to the control group. Injected Group (IIIB) showed significant increase, when compared to subgroup (IIIA). Injected Groups (IIIA & IIIB) showed highly significant increase in the mean optical density of CD44, when compared to other groups. This was matching with the findings of the area % of all groups.

The present work attempted to determine the kinetics of cell proliferation during wound healing by demonstrating PCNA in the different groups. Some nuclei of the control epithelium exhibited positive immune-reactivity. In the injured Group IIA, more cells showed positive nuclear reaction. Group IIB showed decreased reactivity to show some cells exhibiting positive reaction. After MSCs injection (in both groups), many epithelial cells exhibited positive PCNA nuclear reaction. Some cells exhibited positive reaction in the stroma which might be dividing fibroblasts.

To verify these findings, the mean area % of PCNA immuno-reactivity in the epithelial cells was assessed by morphometric measurements using the image analysis system. Morphometric analysis clarified significant increase in the mean area % in the injured Group (IIA) when compared to the control group. This was followed by decrease in the rate of cell proliferation in Group IIB to reach a rate similar to that of the control group. PCNA reached its highest level in the injected groups which showed highly significant increase in the mean area % of PCNA immuno-reactivity when compared to the (control) and (IIB) groups and when compared to Group IIA.

Such up-regulation of PCNA in the injured Group IIA indicated high rate of cell proliferation in the beginning of healing process and probably explained the restoration of the epithelium which appeared almost well-formed in the injured Group IIB which was accompanied by down-regulation of the PCNA area % expression, but under the healed epithelium, there was still inflammatory cellular infiltration and vascularization. The highly significant increase of PCNA area % in the injected groups indicated accelerated rate of cell proliferation and consequently accelerated rate of epithelial healing. This indicated that MSCs stimulates proliferation of epithelial cells which is crucial to avoid the complications. This was matching with [15] who stated that, the presence of engrafted MSCs seems to stimulate the proliferation of limbal stem cells, as evidenced by increased PCNA expression.

In the present study, the presence of high reactivity of PCNA in the MSCs-injected groups together with high CD44 immuno-reactivity and with absence of MSCs migration indicated that MSCs act most probably through exerting a positive influence on the native corneal cell proliferation rather than differentiation, which was augmented by inhibition of inflammation and vascularization. MSCs can promote wound healing by stimulating and synergizing the native cells [15].

As neovascularization is an important complication that clearly affects the acuity of vision and the process of healing; the current study investigated the expression of VEGF. On examination of VEGF immuno-stained sections, no cells showed +ve cytoplasmic reaction in the control group. Almost the same was found in the MSCs-injected subgroup (IIIB) as the reaction was found only in the limbal blood vessels. While the other three Groups (IIA, IIB and IIIA) showed up-regulation of VEGF and many cells exhibited +ve cytoplasmic reaction.

To verify these findings, the mean area % of VEGF immuno-reactivity in the endothelial cells of the blood vessels was assessed by morphometric measurements. Morphometric analysis proved a highly significant increase in the mean area % in the injured Groups (IIA & IIB) and the treated Group (IIIA) when compared to the control group and treated Group IIIB. No significant difference was detected between the control group and double-injected Group IIIB.

Such up regulation of VEGF immuno-reactivity in the injury group after 14 days (IIB) indicated that vascularization persisted after being a part of the process of healing. This was significantly down-
regulated in the (double injections) MSCs-treated Group (IIIB) both in the central and peripheral parts of the cornea indicating a significant effective anti-angiogenic role of the MSCs. This may be one of its mechanisms of action in accelerating corneal wound healing in the acute stage. Subgroup (IIIA) which received single injection of MSCs showed a contradictory data where there was a high expression of the VEGF reactivity. VEGF was down-regulated in the specimens with restored epithelium and decreased inflammatory cellular infiltration, and was up-regulated in the specimens with improper epithelial healing and massive cellular infiltration.

Increased expression of VEGF reactivity was detected in inflamed and vascularized human corneas by [21]. This was in agreement with many studies which reported strong angiogenic activities of MSCs in various organs other than the cornea [1,22].

Conclusion:
Subconjunctival injection of MSCs may have a positive beneficial effect in treating corneal injury. MSCs can exert a positive influence on the native corneal cell proliferation. Its action may be through an anti-angiogenic role.

References
