·Basic Research·

Effects of amniotic membrane transplantation on cytokines expression in chemically burned rat corneas

Hong-Wei Gu, Dan-Mei Bian, Nan Hu, Jun-Fang Zhang

Foundation item: Science and Technology Project for Social Development of Nantong, China (No. S2009029)

Department of Ophthalmology, the Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China

Correspondence to: Nan Hu. Department of Ophthalmology, the Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu Province, China. hunaneye@hotmail.com

Received:2010-12-24 Accepted:2011-01-15

Abstract

• AIM: To investigate the effect of amniotic membrane transplantation (AMT) on the expressions of inflammatory-related, angiogenic-related and growth-related cytokines in rat corneas after chemical injury.

• METHODS: Alkali wounds were inflicted on the central corneas of rats by applying a round filter paper soaked in 1mol/L NaOH for 40 seconds. One week after alkali burn, 12 rats were randomly divided into 2 groups: the AMT group and the control group, and AMT was performed on the rats in the AMT group. Corneal opacity and neovascularization were observed by slit-lamp microscopy. The protein levels of interleukin (IL)-2, interferon (IFN)-γ , IL-10 and transforming growth factor (TGF)-β were determined by enzyme-linked immunosorbent assay 2 weeks after AMT. The mRNA levels of matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were evaluated by real-time quantitative PCR.

• RESULTS: In the AMT group, the corneal opacity was improved (P=0.011) and the area of corneal neovascularization was significantly decreased (P=0.005) compared with the control group. The amount of IL-2 and IFN-γ secreted by Th1 cells were decreased after AMT, whereas the amount of IL-10 and TGF-β secreted by Th2 cells were increased (P<0.05). The level of MMP-2 was significantly down-regulated (P=0.013) at the mRNA level in the AMT group, while the expression of EGF was significantly higher (P= 0.022) compared with controls.

• CONCLUSION: AMT may suppress corneal neovascularization after chemical injury by modulating the expressions of soluble factors.

• KEYWORDS: amniotic membrane transplantation; chemical injury; cornea; cytokine
DOI:10.3980/j.issn.2222-3959.2011.01.07

Gu HW, Bian DM, Hu N, Zhang JF. Effects of amniotic membrane transplantation on cytokines expression in chemically burned rat corneas. *Int J Ophthalmol* 2011;4(1):33-36

INTRODUCTION

Chemical burn represents between 7% and 10% of eye injuries and typically causes severe injury of corneal tissues. It induces a strong inflammatory reaction characterized by cell infiltration and production of proteolytic enzymes and cytokines. Progression of the injury often leads to recurrent epithelium erosion, ulceration, stromal edema and corneal neovascularization (CNV). A number of strategies have been introduced to treat the disease, among which amniotic membrane transplantation (AMT) is increasingly used for restoration the normal ocular surface anatomy and corneal transparency. The amniotic membrane (AM), consisting of a thick basement membrane and an avascular stromal matrix, is the innermost layer of the placenta. It exhibits many biological properties that may be helpful in treating ocular surface diseases, including the prevention of inflammation[1, 2], scarring, neovascularization[3] and improving wound healing [4]. In a retrospective case review of patients with alkali injuries undergoing AMT either in the acute or chronic setting after the initial injury, Tejwani *et al* [5] reported a 92.9% success in healing epithelial defects, 84.6% success in symptomatic relief, 63.5% success in ocular surface reconstruction and 63.3% success in improving limbal stem cell function. However, the mechanisms that determine how the implanted AM promotes ocular surface reconstruction remain controversial and unclear. In this study, we focus on investigating the influences of AMT on the expressions of inflammatory-related, angiogenic-related and growth-related cytokines in rat corneas after chemical injury.

Table 1 Oligonucleotide primers used in real-time quantitative PCR

	Sense	Anti-sense
VEGF	5'-CTCACCAAAGCCAGCACATA-3'	5'-AAATGCTTTCTCCGCTCTGA-3'
bFGF	5'-AAGCAGAAGAGAGAGGAGTTGT-3'	5'-TTAGCAGACATTGGAAGAAAC-3'
EGF	5'-ATGGTGGCGTGTGCATGTAT-3'	5'-ATCGTTCTCCAATATAGCCAATGAC-3'
MMP-2	5'-TGGGGGAGATTCTCACTTTG-3'	5'-CCATCAGCGTTCCCATACTT-3'
GAPDH	5'-GAGGACCAGGTTGTCTCCTG-3'	5'-GGATGGAATTGTGAGGGAGA-3'

MATERIALS AND METHODS

Materials Adult Sprague-Dawley rats(n=19) of both sexes, weighing 200-250g, were obtained from Experimental Animal Center of Nantong University. The study was conducted in accordance with the ARVO Statement for the use of animals in ophthalmic and vision research. Human amniotic membranes (AM) were obtained from normal cesarean sections with approval of the Affiliated Hospital of Nantong University. All placentas were serologically negative for human immunodeficiency, hepatitis B and C viruses and syphilis. AM were separated from the remaining chorion *via* blunt dissection and washed with normal saline (50g/L penicillin, 50g/L streptomycin, 100g/L neomycin, and 2.5g/L amphotericin B). AM samples were flattened onto a nitrocellulose membrane with the epithelium surface up and stored at -80℃ in Dulbecco modified eagle medium/glycerol 1:1 (Vol/Vol).

Methods The corneal alkali burn was made by placing a 3mm diameter circular piece of filter paper, soaked in 1mol/L NaOH, in contact with the central cornea for 40 seconds. Immediately after alkali exposure, the wounded cornea was washed with 9g/L NaCl. One week after alkali burn, 7 rats with severe hypema, hypopyon, or even corneal fistula were dropped out and 12 rats were selected for the experiment. The rats were randomly divided into 2 groups: the AMT group and the control group. After carefully keratectomized the damaged corneal surfaces under anesthesia, AM was sutured onto the corneal surface with the basement membrane facing up. The rats simply received corneal surfaces keratectomy without AMT in the control group. After surgery, the rat eyelids were sutured to avoid grasping. One week after AMT, the eyelids were opened and the rats were examined under a slit-lamp microscopy. The severities of corneal opacity were graded and the area of CNV was measured and calculated using previously described methods at 2 weeks after AMT.

Enzyme –linked Immunosorbent assay Rats were sacrificed at 2 weeks after AMT. The corneas were excised and stored at -80℃ until assayed. Samples were weighed, thawed, minced and placed in phosphate buffered solution. After mechanical homogenization, the lysate was clarified by centrifugation at 2500r/min for 20 minutes and the supernatant was colleted. The levels of IL-2, IFN-γ, IL-10 and TGF-β were determined by ELISA according to the manufacturer's instructions (R&D Systems).

Real –time Quantitative PCR Total RNA from the corneas was extracted using Trizol reagent (Sigma, USA) and reverse- transcripted to cDNA using Omniscript RT kit (Qiagen, Valencia, CA) following manufacturer's instruction. For detecting the mRNA levels of matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), the real-time quantitative PCR was conducted with an ABI 7500 real-time PCR system (Applied Biosystems, USA). Each cDNA sample underwent the reaction in a 50μL volume prepared according to the manufacturer's instruction of Power Sybr Green Mix. The primer sequences of MMP-2, VEGF, bFGF, EGF and GADPH (serving as internal control) were shown in Table 1. PCR procedures were as follows: incubation at 95℃ for 10 minutes, followed by 40 cycles of 95℃ for 15 seconds and 60℃ for 60 seconds.

Statistical Analysis The data were analyzed by ABI Sequence Detection Systems version 1.4 software (Applied Biosystems, USA). The mean C_t value for triplicate measurements was used to calculate the expression of target gene with normalization to GAPDH according to the $2^{-\Delta\Delta Ct}$ formula. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) with a Stata 10.0 software package. P <0.05 was considered statistically significant.

RESULTS

Slit –lamp Examination One week after alkali burn, all injured eyes showed mild moderate CNV with epithelial defects and opacity. There were no significant differences between the two groups on the degree of corneal opacity and the area of CNV. Two weeks after AMT, the rats in the control group suffered with severe corneal vascularization, epithelial defect and stromal opacity. In the AMT group, the corneal opacity was improved (P =0.011). Although the CNV did not show obvious recovery, the area of CNV was significantly decreased compared with the control group

Table 2 Corneal opacity, CNV area and cytokines after AMT Mean±SD

Group	Pre-AMT		Post- AMT					
	Opacity	CNV(mm^2)	Opacity	CNV(mm^2)	IL-2(pg/mL)	IL-10(pg/mL)	IFN-γ (pg/mL)	TGF-β(pg/mL)
Control	3.7±0.5	19.4±2.7	3.9±0.4	28.6±1.1	102.0±12.4	79.3±13.2	11.8±3.1	63.4±7.4
AMT	3.6±0.5	19.5±4.6	3.1±0.5[a]	24.7±2.4[a]	74.8±10.3[b]	294.8±52.9[b]	8.23±1.8[a]	257.1±24.6

[a]P<0.05, [b]P<0.01 *vs* control group

(P=0.005, Table 2).

Cytokines Expression Corneas collected from rats two weeks after surgery were analyzed for their content of IL-2, IL-10 IFN-γ and TGF-β proteins by ELISA. The amount of IL-2 and IFN-γ secreted by Th1 cells were decreased after AMT, whereas the amount of IL-10 and TGF-β secreted by Th2 cells were increased (Table 2).

MMP -2 and VEGF Expression The expressions of MMP-2 and VEGF, known to be involved in chemicalburn-induced corneal angiogenesis, were evaluated by real-time PCR. The level of MMP-2 was significantly down-regulated at the mRNA level in the AMT group, compared to the control (P=0.013). The expression of VEGF was high in both groups, showing no significant differences between the two groups (P=0.397). After AMT, the corneas showed a significantly higher expression of EGF compared with controls (P=0.022), but no significant difference on the expression of bFGF was shown between the two groups (P= 0.072, Figure 1).

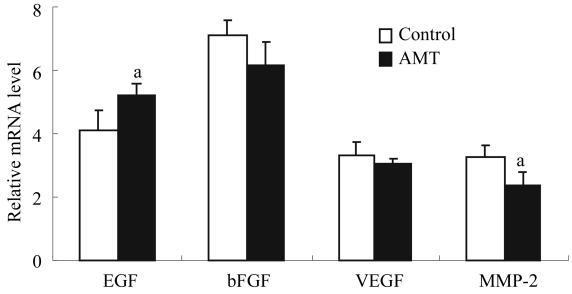

Figure 1 Effects of AMT on the expressions of EGF, bFGF, VEGF and MPP-2 in corneas of rats [a]P<0.05 *vs* control group

DISCUSSION

AMT has been reported to suppress inflammation and promote wound healing in chemical burns, infectious keratitis and autoimmune diseases. These studies generally noted that ocular surface inflammation was markedly reduced in the area covered by the AM. Although the anti-inflammatory and immunosuppressive effect of the AM has been observed clinically, the nature and the exact mechanisms of this effect remain unclear. In the present study, we examined the expressions of IL-2, IFN-γ, IL-10 and TGF-β in the alkali injured corneas two weeks after AMT. IL-2 and IFN-γ are Th1-type cytokines involved with cell-mediated or innate immunity and tend to produce the proinflammatory responses. Excessive proinflammatory responses can lead to uncontrolled tissue damage. IL-10 and TGF-β are Th2-type cytokines involved in humoral or antibody-mediated immunity and may produce an anti-inflammatory response. The results of our study showed an increased IL-10/TGF-β secretion and decreased IL-2/IFN-γ secretion in the AMT treated corneas. This suggested that AMT is able to modulate the action of inflammatory cells and cause an immmunomodulation away from Th1 cytokine to the Th2 cytokine secretion, which may be responsible for the observed improving clinical outcomes.

CNV is a common potentially vision-threatening condition found in chemical burned eyes. We found the area of CNV in the eyes received AMT was significantly decreased compared with the control group. To determine the antiangiogenic mechanism of AM in the cornea, we analyzed the expression of molecules known to be involved in corneal angiogenesis. As a result, we found that MMP-2, an inflammation-related proangiogenic factor[6] was significantly down-regulated after AMT. MMP-2, belonging to extracellular endopeptidases, is produced by activated keratocytes or myofibroblasts after corneal injury. Takahashi *et al*[7] also showed suppressive effects on the expression of MMP-2 by AM, which supports our results. In addition to its effects in the process of angiogenesis, MMP-2 is responsible for extracellular matrix degradation. Failure of cornea re-epithelization is related to an increase in MMP activity. The treatment of alkali-burned corneas with MMP inhibitors improves basement membrane integrity and promotes corneal wound healing with decreased inflammation[8]. Thus, the suppression of MMP-2 may partly explain the improved corneal transparency after AMT.

AM-induced reepithelialization after ocular surface damage is believed to occur *via* a dual action mechanism, involving both a mechanical effect and a biochemical effect. The importance of the mechanical functions of AM has been observed in previous studies. AM provides a basement membrane-type substrate to the injured corneal epithelium [3-5]. In addition, the hydration of the epithelium and the protection of the corneal epithelium from upper-lid irritation may play a role. In the present study, we focus on the biochemical effects of AM on corneal epithelium healing. EGF, one of the most important materials in

epithelialization, was checked and the result showed the content of EGF was increased after AMT. The results were consistent with the previous *in vitro* findings that AM may express soluble biochemical factors including EGF[9].

In conclusion, the results of the present study reveal that after corneal chemical injury, AMT promotes epithelial wound healing, suppresses CNV and improves corneal transparency. Modulating the expressions of soluble factors, involving IL-2, TGF-β, IL-10, IFN-γ, MMP-2 and EGF, may be one of the mechanisms by which AM display such activities.

REFERENCES

1 Bauer D, Wasmuth S, Hennig M, Baehler H, Steuhl KP, Heiligenhaus A. Amniotic membrane transplantation induces apoptosis in T lymphocytes in murine corneas with experimental herpetic stromal keratitis. *Invest Ophthalmol Vis Sci* 2009;50(7):3188-3198

2 Hori J, Wang M, Kamiya K, Takahashi H, Sakuragawa N. Immunological characteristics of amniotic epithelium. *Cornea* 2006;25(10)Suppl:S53-58

3 Shao C, Sima J, Zhang SX, Jin J, Reinach P, Wang Z, Ma JX. Suppression of corneal neovascularization by PEDF release from human amniotic membranes. *Invest Ophthalmol Vis Sci* 2004;45(6):1758-1762

4 Choi JA, Jin HJ, Jung S, Yang E, Choi JS, Chung SH, Joo CK. Effects of amniotic membrane suspension in human corneal wound healing *in vitro*. *Mol Vis* 2009;15(6):2230-2238

5 Tejwani S, Kolari RS, Sangwan VS, Rao GN. Role of amniotic membrane graft for ocular chemical and thermal injuries. *Cornea* 2007;26(1):21-26

6 Cursiefen C. Immune privilege and angiogenic privilege of the cornea. *Chem Immunol Allergy* 2007;92:50-57

7 Takahashi H, Igarashi T, Fujimoto C, Ozaki N, Ishizaki M. Immunohistochemical observation of amniotic membrane patching on a corneal alkali burn *in vivo*. *Jpn J Ophthalmol* 2007;51(1):3-9

8 Barros LF, Barros PS, Röpke CD, Silva VV, Sawada TC, Barros SB, Belfort Jr R. Dose-dependent *in vitro* inhibition of rabbit corneal matrix metalloproteinases by an extract of Pothomorphe umbellata after alkali injury. *Braz J Med Biol Res* 2007;40(8):1129-113

9 Choi JA, Jin HJ, Jung S, Yang E, Choi JS, Chung SH, Joo CK. Effects of amniotic membrane suspension in human corneal wound healing *in vitro*. *Mol Vis* 2009;15(5):2230-2238