· Original article ·

MMP and TIMP in cornea alkaline burn after amniotic membrane transplantation in mice

Jing Zhu, Xu-Kang Cheng

Department of Ophthalmology, the First Hospital of Wuhan, Wuhan 430033, Hubei Province, China

Correspondence to: Jing Zhu. Department of Ophthalmology, the First Hospital of Wuhan, Wuhan 430033, Hubei Province, China. anitta777@163. com

Received:2011-07-27 Accepted:2011-09-05

Abstract

• AIM: To study metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) expresion in cornea of mice with alkaline burns treated with amniotic membrane transplantation (AMT), and to evaluate the effect of AMT in treatment of alkaline burns.

• METHODS: Forty Balb/c mice were divided into two groups (experimental group and control group) and their right eye corneas were burned with alkali (NaOH). The cornea was treated with AMT and secured with a tarsorrhaphy in experimental group, while the control group underwent tarsorrhaphy alone. At different time points(0, 2, 7, 14 days) after AMT, mice were killed and the expressions of MMP and TIMP in cornea were measured by the Western blot technique, and the results were analyzed by enhanced chemiluminescent(ECL).

• RESULTS: In control group, MMP -2, 8, 9 expressed in the stromal cells and epithelial inflammatory cells. The level of MMP -2, 8, 9 elevated from the 2nd day, peaked on the 14th day. TIMP-1 expression was only slightly upregulated after alkaline burn at day 2, and expressed on the 7th day, peaked on the 14th day. TIMP-2 expression was increased at 2nd day, and significantly increased at 14th day. At the same time point, the level of MMP -2, 8, 9 in AMT group were greatly lower than that in control group(all $P<0.01$), while the level of TIMP-1, 2 in AMT group were greatly higher than that in control group(all $P<0.01$).

• CONCLUSION: AMT may inhibit MMP expression and promote TIMP expression in cornea, lower rapid resolution of corneal inflammation and ulceration, and play a vital role in corneal remodeling.

• KEYWORDS: alkaline burn; amniotic membrane transplantation; metalloproteinases; tissue inhibitors of matrix metalloproteinases

DOI:10. 3969/j. issn. 1672-5123. 2011. 10. 001

Zhu J, Cheng XK. MMP and TIMP in cornea alkaline burn after amniotic membrane transplantation in mice. *Guoji Yanke Zazhi(Int J Ophthalmol)* 2011;11(10):1683-1686

INTRODUCTION

Alkaline burn of cornea is a commom disease that may eventually result in loss of vision due to corneal melting, neovascularization, ulcer perforating and scarrimg. At present, the pathogenesis of corneal alkaline burn is still not clear. Recently, it has been shown that proteolvtic enzymes lead to rapid degradation of corneal stroma and ulcer perforation[1]. Metalloproteinases (MMP) are a family of proteolytic enzymes that are capable of degrading the extracellular matrix and basement membrane components. Tissue inhibitors of metalloproteinases (TIMP) are the most important endogenous regulators of MMP activity. MMP participate in tissue remodeling and also play an important role in corneal scarring[2]. Along with amniotic membrane transplantation (AMT) has been shown to be effective in the reconstruction of the conjunctival ocular surface[3], we suggest that use of human AMT in mice with alkaline burn may be a useful model for studying the amniotic membranes possess anti-inflammatory effects.

MATERIALS AND METHODS

Materials Totally 6 to 8 weeks old female inbred Balb/c mice were obtained from the animal center of Wuhan University. All animals were detected without any ophthalmic diseases and were handled according to the Association for Research in Vision and Ophthalmology(ARVO) guidelines for animal care. Goat anti-mouse MMP-2, goat anti-mouse MMP-8, goat anti-mouse MMP-9, goat anti-mouse TIMP-1, goat anti-mouse TIMP-2 were purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China); BCA™ protein assay reagent and enhanced chemiluminescence(Pierce Co., CA, USA). The human placenta was selected after elective negatived for syphilis, HIV, HBV, HCV. Under sterile conditions, the amniotic membrane (AM) was removed by blunt dissection and washed three times in phosphate-buffered saline (PBS) containing antibiotics(50mg/L penicillin,50mg/L streptomycin, 100mg/L neomycin, and 2.5mg/L amphotericin B). The AM was flattened onto nitrocellulose paper with 0.45μm microporous, with the epithelium facing away from the paper. The membranes were then stored at -80℃ in 500mL/L glycerol in 500g/L dulbecco's modified eagle medium (DMEM).

Cornea Alkaline Burn Briefly, after general anesthesia with an intraperitoneal injection of a combination of mepivacaine hydrochloride (400μg) and ketamine hydrochloride (2mg), and topical anesthesia with a drop of 0.5% proparacaine hydrochloride, a filter paper ring with a diameter of 2mm saturated with 1 mol/L NaOH, was placed on the right cornea

Table 1 Corneal pathology of mice ($\bar{x} \pm s$, score)

Pathology	2d		7d		14d	
	Control	AMT	Control	AMT	Control	AMT
Epithelial default	2.6 ± 0.6	1.0 ± 0.6[b]	3.1 ± 0.5	1.2 ± 0.4[b]	3.4 ± 0.5	1.9 ± 0.4[b]
Neovascularization	0.3 ± 0.2	0.2 ± 0.1	1.0 ± 0.6	0.4 ± 0.3[b]	2.5 ± 0.4	0.8 ± 0.4[b]

[b] $P < 0.01$ *vs*Control.

of forty Balb/c mice for 10 seconds, followed by rinsing with PBS for 1 minute. Antibiotic drops were applied to the injured eyes three times a day. Then, forty mice were divided into two groups (experimental group and control group). In experimental group, the cornea of the right eye were covered with amniotic membrane with the epithelium facing up and secured by tarsorrhaphy with eight interrupted 10-0 nylon sutures[4]. Tarsorrhaphy only was performed in the control group. After 0, 2, 7 and 14 days the clinical signs of alkaline burn were assessed. The mice were then killed (five mice at each time point), and the corneas were removed and then stored at -80℃. Mice were followed up with an operation microscope for the development of epithelial defect and corneal neovascularization before they were killed at different time points. The area of epithelial defect and the severity of neovascularization were graded as a scale. The score for the area of epithelial default: 0, not present; 1 +, less than 25%; 2 +, less than 50%; 3 +, less than 75%; 4 +, between 75% and 100%. The score for severity of neovascularization: 0, not present; 1 +, concentric growing, not to reach the pupil center or not more than the corneal radius; 2 +, reach the pupil center or exceed the corneal radius; 3 +, full of cornea[5].

MMP and TIMP Expression Detection Soluble proteins were extracted from each cornea by homogenizing the tissue in lysis buffer, after centrifugation at 10000g, the supernatants were removed and protein concentrations were assayed with BCA™ protein assay reagent. At each time point, the same quantity of protein (15 μg) from each corneal specimen was electrophorized at 4℃ under unreducing conditions in SDS-PAGE 100mL/L crosslinked polyacrylamide gels for 2 hours at 120 V. Proteins were electrophoretically transferred to nitrocellulose membranes for 2 hours at 110mA. After blocking of nonspecific binding sites with 50mL/L non-fat dry milk and 50mL/L BSA in TTBS buffer at RT for 1 hour, membranes were incubated with the primary antibodies [all overnight at 4℃ with agitation: goat anti-mouse MMP-2 (1∶1000 in PBS), goat anti-mouse MMP-8 (1∶1000 in PBS), goat anti-mouse MMP-9 (1∶1000 in PBS), goat anti-mouse TIMP-1 (1∶1000 in PBS) and goat anti-mouse TIMP-2 (1∶1000 in PBS)]. After three washing steps with TTBS, each membrane was transferred to a 1∶1000 diluted solution of horseradish peroxidase (HRP) conjugated secondary bovine anti-goat or bovine anti-rabbit antibody in TTBS and was incubated at RT for 1 hour. After three to four washes with the same solution, proteins were visualized by enhanced chemiluminescence (ECL) detection. Image acquisition and analysis software were applied to analyze the band to get the gray intensity.

Statistical Analysis Data are presented as the mean ± SEM. Student's *t*-test was applied to compare all experimental numbers between two groups (all analysis were performed with SPSS software). $P < 0.01$ was considered statistically significant.

RESULTS

Corneal Alkaline Burn Following corneal injure with alkaline burn, corneal opacity were observed immediately in accordance with diameter of filter paper ring. In control group, the corneas have epithelial edema and default, stromal edema at day 2. The stromal necrosis, infiltration, local ulcer and neovascularization progressed until day 7. Significant stromal inflammation, severe ulceration and neovascularization developed in all mice after 14 days, 2 mice were observed corneal perforation. In all corneas, the score for the area of epithelial default progressed from 3.1 ± 0.5 at day 7 to 3.4 ± 0.5 at day 14, and the score for the severity of neovascularization progressed from 1.0 ± 0.6 at day 7 to 2.5 ± 0.4 at day 14. In experimental group, the corneal inflammation showed released at day 2 after AMT. In contrast to the control eyes, the epithelial default and neovascularization rapid decreased at day 7 and 14 after AMT. The score for the area of epithelial default decreased from 1.2 ± 0.4 at day 7 to 1.9 ± 0.4 at day 14, and the score for the severity of neovascularization progressed from 0.4 ± 0.3 at day 7 to 0.8 ± 0.4 at day 14. Both of which were significant different from scores in control group ($P < 0.05$, Table 1).

Expression of MMP and TIMP Our results demonstrated MMP-2 was found slightly expressed in normal cornea, and increased at day 2, then peaked on the 14th day. MMP-8 expression was very similar to MMP-2, which strong expressing was detected in the neutrophils at day 14. MMP-9 was found at day 0, but it rapidly increased at day 2, then the expression again elevated and reached a maximum at day 14. MMP-9 expression was significantly increased the cornea, especially in the area of necrosis and close to the ulcer. The bands identified as MMP-2, 8, 9 showed a high level of expression in the corneas harvested on day 14 (Figure 1, Table 2). TIMP-1 expression was only slightly upregulated after alkaline burn at day 2, and expressed on the 7th day, peaked on the 14th day. TIMP-2 expression was increased at day 2, and increased to a peak at day 14. Compared with the control group, the TIMP-1 expression in the cornea was not altered profoundly after AMT 2 days, enhanced expressing was found in the 7 days after AMT, but TIMP-2 expression significantly

Table 2 Expression of MMP-2,8,9 secreted by Western blot ($\bar{x} \pm s$, Grey intensity)

		0d	2d	7d	14d
MMP-2	Control	182.1 ±7.2	154.3 ±6.7	121.5 ±5.2	106.6 ±5.1
	AMT	191.9 ±5.6	180.2 ±4.2[b]	149.5 ±4.1[b]	138.3 ±4.2[b]
MMP-8	Control	201.4 ±3.2	195.6 ±6.9	154.6 ±5.2	126.2 ±5.3
	AMT	197.3 ±2.2	174.2 ±3.2[b]	171.2 ±4.3[b]	144.5 ±3.3[b]
MMP-9	Control	208.7 ±2.3	117.9 ±2.8	101.6 ±5.7	86.7 ±4.8
	AMT	201.9 ±4.6	128.2 ±5.7[b]	127.4 ±4.6[b]	136.5 ±3.6[b]
TIMP-1	Control		209.7 ±2.9	183.2 ±3.7	148.0 ±2.7
	AMT		141.9 ±3.2[b]	126.5 ±3.2[b]	83.32 ±4.1[b]
TIMP-2	Control		174.0 ±2.6	137.6 ±2.5	94.31 ±5.2
	AMT		131.2 ±3.9[b]	78.2 ±2.5[b]	63.31 ±2.2[b]

[b]$P < 0.01$ *vs*Control.

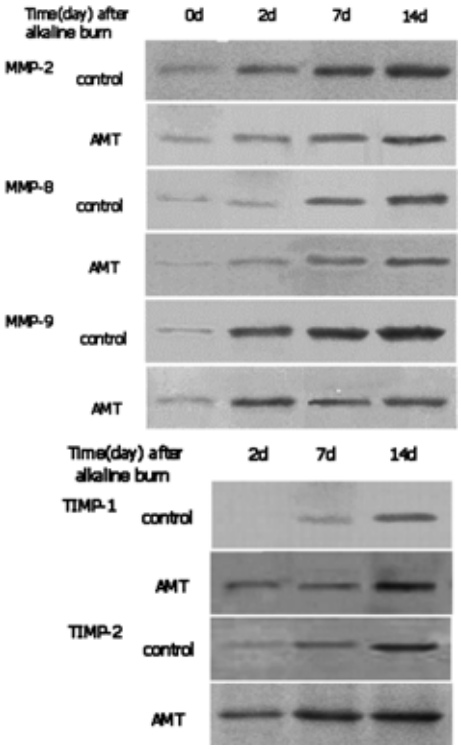

Figure 1 Western blot analysis of the expression of MMP-2,8, 9 and secreted

at day 2 and sustained in the cornea (Figure 1, Table 2). Briefly, at the same time point (2,7,14 days), the level of MMP-2, 8, 9 in AMT group were greatly lower than that in control group ($P < 0.01$), while the level of TIMP-1, 2 in AMT group were greatly higher than that in control group ($P < 0.01$, the setting range of gray intensity is from 0 to 255, 0 represented color white, 255 represented color black).

DISCUSSION

Chemical injury is one of the most common and severe ocular emergencies in our daily life, especially injured with alkaline burns. The prognosis for an injured eye depends not only on the severity of injury, but also on the rapidity and mode of treatment. Recently, the treatment of alkaline burn is focused on promoting epithelialization and reducing inflammation and to prevent progressive corneal melting[6]. The amniotic membrane has also been used as a temporary patch or biological bandage for acute alkaline burns[7]. The purpose of this study was to determine the expression of MMP and TIMP during the healing of alkaline burn mice corneas, and to investigated the mechanism of how to reduce inflammation and to prevent progressive corneal melting and neovascularization after AMT. The matrix MMP are a family of protein-cleaving enzymes that degrade extracellular matrix (ECM) and basement membrane components[8]. They can be divided into four classes on the basis of their preferred substrate: collagenases, gelatinases, stromelysins, and membrane type. MMP-2 and MMP-9 belong to gelatinases, they can degrade gelatins, native type IV-, V-collagens and elastin. MMP-8 belong to collagenases, which come from the polymorphonuclear cells (PMN). It has been found that MMP are not only produced by the corneal epithelium and stormal cells, but also regulated by the numerous cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor a (TNF-a)[9]. Recent experiments suggest that the proinflammatory cytokines IL-1 and TNF-a play major roles in the pathogenesis of inflammation[10]. TIMP are the major endogenous regulators of MMP activity in tissue, the expression of TIMP in tissue is controlled during tissue remodeling and physiological conditions in order to maintain a balance in the metabolism of the extracellular matrix. It has been reported to specific inhibit all-known MMP activities in tissues, the balance may play a vital role in many biological and pathological processes, such as wound repairing, tissue remodeling, tumor transferring, vascular remodeling[11-12].

Our results indicated that MMP-2 and MMP-8 have slightly expressed in normal corneas, and MMP-2, MMP-9 increased in the epithelium and the superficial stroma at day 2 after alkaline burn, especially at the areas of corneal ulceration. Many of the inflammatory cells, particularly the PMN, secreted positively for MMP-8, therefore the MMP-8 expression markly sustained increased in the control group. On the contrary, the TIMP expressions were not detected at the early time, which may not be enough to restrict the activities of MMP, we suggest that MMP actively participate in corneal melting and destruction through disintegrating some components of the ECM released after early alkaline burn,

which may be involved in the ECM remodeling. There is profound evidence that resident corneal cells and infiltrating PMN and macrophages could produce proteolytic enzymes after alkaline burn, the level of IL-1 and TNF-a were increased significantly which may stimulate production of MMP, therefore, the expressions and activities of MMP sustained increased in the corneas injured by alkaline burn[13]. Our research confirmed that the expression of MMP-2, 8, 9 were remarkbly reduced in corneal specimens obtained from mice in the AMT group ($P<0.01$), whereas the expression of their inhibitors TIMP-1 and TIMP-2 in mice corneas were increased significantly after AMT ($P<0.01$). These data collectively indicate that AMT may have an effection for suppressing the expression of MMP in the epithelium and inflammatory cells. Furthermore, natural inhibitors of various MMP have been found in amniotic membrane[14], which exert an inhibitory effect on various proteinases, and as a result it may decrease inflammation and corneal ulcerating destruction. It has been suggested that the amniotic membrane induces neutrophils to undergo programmed cell death and prevent their contributing to tissue destruction[15]. This is another potential reason for the MMP expressed downregulation.

We suggest that applying with the AMT for alkaline burn at the early time have a direct anti-inflammatory effect and promote wound healing and prevent corneal remodeling by suppressing the expression of MMP and upregulating the expression of TIMP, but also may have an indirect effect through corneal epithelial cells by promoting rapid epithelial healing. Future studies are defined the specific mechanism of the balance between MMP and TIMP.

REFERENCES

1 O'Brien TP, Li QJ, Sauerburger F, Reviglio VE, Rana T, Ashraf MF. The role of matrix metalloproteinases in ulcerative keratolysis associated with perioperative diclofenac use. *Ophthalmology* 2001;108(4):656-659

2 Sivak JM, Fini ME. MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology. *Prog Retin Eye Res* 2002;21(1):1-14

3 Femandes M, Sridhar MS, Sangwan VS, Rao GN. Amniotic membrane transplantation for ocular surface reconstruction. *Cornea* 2005;24(6):643-653

4 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

5 Heiligenhaus A, Bauer D, Meller D, Steuhl KP, Tseng SC. Improvement of HSV-1 necrotizing keratitis with amniotic membrane transplantation. *Invest Ophthalmol Vis Sci* 2001;42(9):1969-1974

6 He J, Bazan NG, Bazan HE. Alkali-induced corneal stromal melting prevention by a novel platelet-activating factor receptor antagonist. *Arch Ophthalmol* 2006;124(1):70-78

7 Shahriari HA, Tokhmehchi F, Reza M, Hashemi NF. Comparison of the effect of amniotic membrane suspension and autologous serum on alkaline corneal epithelial wound healing in the rabbit model. *Cornea* 2008;27(10):1148-1150

8 Wong TT, Sethi C, Daniels JT, Limb GA, Murphy G, Khaw PT. Matrix metalloproteinases in disease and repair processes in the anterior segment. *Surv Ophthalmol* 2002;47(3):239-256

9 Li DQ, Zhou N, Zhang L, Ma P, Pflugfelder SC. Suppressive effects of azithromycin on zymosan-induced production of proinflammatory mediators by human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2010;51(11):5623-5629

10 Lu Y, Fukuda K, Li Q, Kumagai N, Nishida T. Role of nuclear factor-kappaB in interleukin-1-induced collagen degradation by corneal fibroblasts. *Exp Eye Res* 2006;83(3):560-568

11 Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases structure, function, and biochemistry. *Circ Res* 2003;92(8):827-839

12 Ikema K, Matsumoto K, Inomata Y, Komohara Y, Miyajima S, Takeya M, Tanihara H. Induction of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs correlates with outcome of acute experimental pseudomonal keratitis. *Exp Eye Res* 2006;83(6):1396-1404

13 Sosne G, Christopherson PL, Barrett RP, Fridman R. Thymosin-beta4 modulates corneal matrix metalloproteinase levels and polymorphonuclear cell infiltration after alkali injury. *Invest Ophthalmol Vis Sci* 2005;46(7):2388-2395

14 Bouchard CS, John T. Amniotic membrane transplantation in the management of severe ocular surface disease: indications and outcomes. *Ocul Surf* 2004;2(3):201-211

15 Dua HS, Gomes JA, King AJ, Maharajan VS. The amniotic membrane in ophthalmology. *Surv Ophthalmol* 2004;49(1):51-77

碱烧伤小鼠行羊膜移植后 MMP 及 TIMP 表达的变化

朱 晶,程旭康
(作者单位:430033 中国湖北省武汉市第一医院眼科)
作者简介:朱晶,硕士,研究方向:眼表及角膜病。
通讯作者:朱晶. anitta777@163.com

摘要

目的:通过检测羊膜移植对小鼠角膜碱烧伤后不同时间点基质金属蛋白酶(metalloproteinases, MMP-2,8,9)及金属蛋白酶组织抑制剂(tissue inhibitor of metalloproteinases, TIMP-1,2)的表达,探讨羊膜移植在碱烧伤病程中所起的作用。

方法:将 40 只 Balb/c 小鼠随机分为实验组和对照组,采用 1mol/L 氢氧化钠溶液烧伤小鼠角膜;实验组小鼠右眼行羊膜移植加睑裂缝合术,对照组仅行睑裂缝合术。分别在羊膜移植后的第 0,2,7,14d 处死小鼠,应用 Western blot 检测不同时间点 MMP-2,8,9 及 TIMP-1,2 的表达,增强化学发光法(enhanced chemiluminescent, ECL)对结果进行分析。

结果:对照组角膜中 MMP-2,8,9 在第 2d 出现表达,第 14d 达到峰值,且表达主要位于基质层及上皮下的炎性细胞中。碱烧伤后第 2d TIMP-1 仅微弱表达,第 7d 可见表达增加,第 14d 到达峰值。TIMP-2 第 2d 即可见表达增加,后持续增强。实验组各时间点 MMP-2,8,9 表达均低于对照组($P<0.01$),TIMP-1,2 的表达均高于对照组($P<0.01$)。

结论:行羊膜移植可通过抑制 MMP 的表达,促进 TIMP 表达,从而抑制和延迟碱烧伤后角膜炎性浸润及溃疡的发生和发展,对碱烧伤后角膜的重塑起着重要作用。

关键词:碱烧伤;羊膜移植;基质金属蛋白酶;金属蛋白酶组织抑制剂