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【实验研究】

# MEK/ERK 参与大鼠脉络膜新生血管基质金属蛋白酶-2 和基质金属蛋白酶-9 的表达调控△

杨秀梅 王雨生

作者简介:杨秀梅,女,1979 年 10 月出生,北京人,博士,主治医师。研究方向:眼底病的基础与临床研究。联系电话:15810302633;E-mail:yangxm108@163.com

About YANG Xiu-Mei: Female, born in October, 1979. Doctor degree, attending doctor. Tel: 15810302633; E-mail: yangxm108@163.com

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作者单位:100700 北京市,北京军区总医院眼科(杨秀梅);710032 陕西省西安市,第四军医大学西京医院眼科,全军眼科研究所(杨秀梅,王雨生)
通讯作者:王雨生,E-mail:wangys003@126.com

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From the *Department of Ophthalmology, Beijing General Command Hospital* (YANG Xiu-Mei), *Beijing* 100700, *China*; *Department of Ophthalmology, Institute of Ophthalmology of Chinese PLA* (YANG Xiu-Mei, WANG Yu-Sheng), *Xijing Hospital, Fourth Military Medical University, Xi'an* 710032, *Shaanxi Province, China*
Responsible author: WANG Yu-Sheng, E-mail: wangys003@126.com

## Contribution of MEK/ERK pathway in regulation of MMP-2 and MMP-9 expression in rat choroidal neovascularization

YANG Xiu-Mei, WANG Yu-Sheng

**【Key words】** matrix metalloproteinase-2; matrix metalloproteinase-9; choroidal neovascularization; rat

**【Abstract】** **Objective** To investigate the expression and regulation mechanism of matrix metalloproteinase-2 (MMP-2) and MMP-9 in rat choroidal neovascularizaiton (CNV). **Methods** Twenty-three adult male Brown Norway (BN) rats were divided into 2 groups: the PD98059 treated group and the simple photocoagulation group. Both eyes of each animal were induced by laser photocoagulation with 532 nm laser, the animals in PD98059 treated group were intravitreous injected the PD98059 timely immediately after photocoagulation. At 3 days, 7 days and 14 days after photocoagulation, the expression of MMP-2 and MMP-9 were examined by HE, immunohistochemistry staining and immunofluorescence staining, the effect of intravitreous injection of PD98059 on CNV formation was observed by FFA and HE staining, and the effect of intravitreous injection of PD98059 on MMP-2 and MMP-9 expression were detected by Western blotting. **Results** At 3 days after photocoagulation, the expression of MMP-2 and MMP-9 in the simple photocoagulation group were positive, and increased at 7 days and 14 days after photocoagulation. At 7 days after photocoagulation, the expression of MMP-2 and MMP-9 were obviously inhibited by PD98059, the inhibitive rate were 69% and 80%, respectively. Intravitreous injection of PD98059 dramatically decreased the level of p-ERK to 54% and 60% at 3 days and 7 days after photocoagulation, respectively, while had no effect on ERK expression. PD98059 was able to inhibit the growth of CNV to 57% by HE staining and decrease the leakage to 51% by FFA at 14 days after photocoagulation. Immunofluorescence staining had the similar result as immunohistochemistry which showed PD98059 attenuated the expression of MMP-2 and MMP-9 to 73% and 64%, respectively. **Conclusion** MMP-2 and MMP-9 are involved in the development of CNV, positively express at 3 days and increase at 14 days after photocoagulation. MEK/ERK pathway at least partly regulate the expression of MMP-2 and -9 during the development of CNV.

**【关键词】** 基质金属蛋白酶-2;基质金属蛋白酶-9;脉络膜新生血管;大鼠

**【摘要】** **目的** 探讨基质金属蛋白酶 2(matrix metalloproteinase-2,MMP-2)和 MMP-9 在大鼠脉络膜新生血管(choroidal neovascularization,CNV)内的表达及其可能的调控机制。**方法** 将 23 只成年雄性棕色挪威大鼠随机分为 2 组,一组为玻璃体内注药组,视网膜光凝后即刻玻璃体内注射 3 μL PD98059;另一组为单纯光凝组,单纯行视网膜光凝。观察时间为光凝后 3 d、7 d 和 14 d。各时间点处死大鼠后摘除眼球,免疫组织化学法和免疫荧光法观察 MMP-2 和 MMP-9 在大鼠 CNV 内不同时间点的表达特点。眼底荧光血管造影(fundus fluorescence angiography,FFA)和 HE 法观察玻璃体内注射 PD98059 对 CNV 生成的作用,Western blotting 检测观察注药对 CNV 内 MMP-2 和 MMP-9 表达的影响。**结果** 光凝后 3 d,单纯光凝组光凝区即有 MMP-2 和 MMP-9 的阳性表达;光凝后 7 d 和 14 d 二者表达均逐渐增强。光凝后 7 d,玻璃体内注药组 MMP-2 和 MMP-9 均显著被抑制,分别被抑制约 69% 和 80%。Western blotting 检测结果示玻璃体内注射组可显著抑制 ERK 的磷酸化,光凝后 3 d 及 7 d 的抑制率分别为 54% 和 60%,而对 ERK 总量的表达无明显作用。FFA 和 HE 显示,玻璃体内注药组光凝后 14 d 减少光凝局部 CNV 的荧光素渗漏约 51%,抑制 CNV 厚度达 57%。免疫荧光法检测结果示光凝后 7 d,玻璃体内注药组抑制 MMP-2 和 MMP-9 的表达分别为 73% 和 64%。**结论** MMP-2 和 MMP-9 参与了大鼠 CNV 的生成,光凝后 3 d 即有阳性表达,至光凝后 14 d 表达进一步增强,MEK/ERK 通路至少部分参与了 MMP-2 和 MMP-9 在 CNV 内的生成调控。

脉络膜新生血管(choroidal neovascularization, CNV)是发达国家老年视力丧失的主要原因之一[1]。研究显示,蛋白水解在CNV发生及CNV突破Bruch膜的过程中发挥关键作用。在众多的蛋白水解酶中,基质金属蛋白酶(matrix metalloproteinase, MMP)-2和MMP-9由于其特异性水解胶原Ⅳ活性而备受瞩目[2],胶原Ⅳ是CNV内血管基底膜、Bruch膜及细胞外基质的重要组成成分[3]。本研究主要观察MMP-2及MMP-9在CNV局部的表达特点并初步探讨二者在CNV局部表达的可能调控机制。

## 1 材料与方法

### 1.1 材料

**1.1.1 实验动物与分组** 成年雄性棕色挪威(brown norway, BN)大鼠23只(46眼),体质量200~250 g,由北京维通力华实验动物有限公司提供。实验前双眼眼前节和眼底检查均正常。23只大鼠随机分为2组,一组为玻璃体内注药组(8只),另一组为单纯光凝组(15只)。

**1.1.2 主要仪器设备** 半导体倍频532 nm激光器(美国IRIS公司);共焦激光眼底血管造影仪(德国Heidelberg公司);Leica DM LB2光学显微镜(德国Leica公司)。

**1.1.3 主要试剂** 复方托品酰胺滴眼液(北京双鹤现代医药技术有限责任公司),速眠新注射液(长春军医大学兽医研究所);爱维(山东正大福瑞达制药有限公司);200 g·L$^{-1}$荧光素钠注射液(广西梧州制药股份有限公司产品);小鼠抗MMP-2单克隆抗体、小鼠抗MMP-9单克隆抗体、兔抗ERK-1/-2抗体、鼠抗pERK-1/-2抗体、鼠抗β-actin抗体、FITC标记的山羊抗小鼠IgG、罗丹明标记山羊抗鼠IgG、免疫组织化学染色SP试剂盒及AEC显色试剂盒(中杉金桥,北京);PD98059(cell signaling公司,美国)。

### 1.2 方法

**1.2.1 动物模型的建立** 腹腔内注射速眠新注射液(1 mL·kg$^{-1}$)麻醉大鼠,双眼复方托品酰胺滴眼液散瞳。实验眼滴用爱维,眼前放置-53.00 D角膜接触镜,经裂隙灯用532 nm激光(功率140 mW,光斑直径75 μm,曝光时间100 ms),围绕视盘、距离视盘1.0~1.5 PD等距离光凝8点。光凝后有气泡产生或伴有轻度出血(有时伴有轻响)标志击破Bruch膜,记为有效点,提示模型建立成功[4],每只鼠双眼均光凝。

**1.2.2 玻璃体内注射** 玻璃体内注药组光凝后即刻行玻璃体内注射,手术显微镜下沿角膜缘剪开颞上象限球结膜,钝性分离,暴露巩膜,30 G针头于角膜缘后2.0~2.5 mm处穿刺,沿穿刺口插入微量注射器,注入PD98059(5 mmol·L$^{-1}$,3 μL),7 d后重复注射一次[5-6]。单纯光凝组光凝后不作处理。

**1.2.3 眼底荧光血管造影** 光凝后14 d,2组各取2只大鼠麻醉并散瞳,经尾静脉注射200 g·L$^{-1}$荧光素钠(1.0 mL·kg$^{-1}$),注入后即刻计时,于注射后5 min采集造影图像。眼底荧光血管造影(fundus fluorescence angiography, FFA)图像结果采用Image-pro plus6.0(IPP6.0)软件分析。利用该软件计算造影后5 min CNV渗漏面积。

**1.2.4 HE染色** 光凝后14 d,取单纯光凝组3只、玻璃体内注药组2只大鼠过量麻醉处死,立即摘取眼球,40 g·L$^{-1}$多聚甲醛固定,去除眼前节,制作眼杯。经二甲苯、乙醇梯度脱水,透明、浸蜡、石蜡包埋后连续切片,切片厚4 μm。常规HE染色,行CNV厚度测量,利用IPP 6.0观察连续切片中CNV最大中央厚度(视网膜色素上皮层至视网膜内最高点的距离)。

**1.2.5 免疫组织化学和免疫荧光法检测** 单纯光凝组于光凝后3 d、7 d和14 d分别取大鼠3只、3只和2只,玻璃体内注药组光凝后7 d取2只大鼠,过量麻醉后经心脏灌注40 g·L$^{-1}$冰多聚甲醛100 mL,摘除眼球,去除球周组织,置于40 g·L$^{-1}$多聚甲醛中后固定2 h,300 g·L$^{-1}$蔗糖溶液4 ℃过夜,OCT包埋剂包埋,切片,切片厚10 μm。按试剂盒说明书进行,采用SP法染色,AEC显色,MMP-2和MMP-9的工作浓度均为1∶50。0.01 mol·L$^{-1}$ PBS取代一抗孵育,作为空白对照。阳性染色为棕红色显色。免疫组织化学染色结果采用IPP 6.0图像处理系统测量。

免疫荧光法检测:切片室温下干燥2 h,PBS洗涤10 min×2次,50 g·L$^{-1}$牛血清白蛋白+体积分数5%正常山羊血清+体积分数1% Triton X-100封闭液室温封闭3 h,洗涤后加入MMP-2和MMP-9一抗工作液(1∶50),4 ℃过夜;洗涤后分别加入FITC标记的山羊抗小鼠IgG(1∶100)和罗丹明标记的山羊抗兔IgG(1∶100),室温2 h;缓冲甘油封片,激光扫描共聚焦显微镜观察。0.01 mol·L$^{-1}$ PBS替代一抗,并行平行染色。免疫荧光染色结果利用IPP 6.0软件分别计算CNV区域内MMP-2和MMP-9的相对荧光密度单位(relative fluorescence unit, RFU)。

**1.2.6 Western blotting检测** 光凝后3 d和7 d各组分别取2只大鼠麻醉后经心脏灌注冰生理盐水100 mL,立即摘除眼球,冰上去除球周组织及眼前节组织,制作眼杯,液氮保存,各组取1个眼杯,加入300 μL冰蛋白裂解液,冰上裂解,12 000 r·min$^{-1}$,4 ℃离心30 min;取上清,蛋白定量后加入8×loading buffer,沸水煮沸5 min,12 000 r·min$^{-1}$,4 ℃离心5 min;100 g·L$^{-1}$ SDS-PAGE胶电泳,PVDF膜电转移,50 g·L$^{-1}$脱脂奶粉4 ℃过夜;洗涤后加入一抗工作液4 ℃过夜,分别为:抗ERK-1/2(1∶200)、抗pERK-1/2(1∶200)、抗β-actin(1∶500);洗涤后加入相应二抗工作液,室温孵育1 h,洗涤后加入发光液,暗室中X光曝光显影。实验重复3次。Western blotting结果采用Gel-pro Analyzer 4.5图像分析软件对结果进行灰度值分析,以目的条带与β-actin条带的比值代表蛋白的相对表达水平。

**1.3 统计学分析** 采用SPSS 19.0统计软件对数据进行统计分析。各组间均数的比较采用 $t$ 检验，$P<0.05$ 为差异有统计学意义。

## 2 结果

**2.1 免疫组织化学检测结果** 单纯光凝组光凝后3 d，光凝局部可见MMP-2和MMP-9阳性表达细胞，光凝损伤区上方移行细胞也可见阳性表达细胞；之后表达逐渐增强，光凝后7 d约是3 d时表达量的2倍（$P<0.05$），可见二者在视网膜神经纤维层、神经节细胞层、内网状层及光凝区细胞、光凝区内新生血管管腔周围细胞阳性表达；至光凝后14 d表达继续升高（$P<0.05$），在光凝区新生血管细胞外基质及视网膜内基质可见强阳性表达（图1）。玻璃体内注射MEK抑制剂PD98059可显著抑制光凝局部MMP-2和MMP-9的表达，免疫组织化学结果显示光凝后7 d，PD98059抑制MMP-2及MMP-9在光凝局部的表达，抑制率分别约为69%和80%（图2）。

**Figure 1** Expression of MMP-2 and MMP-9 in rat CNV at different time after photocoagulation in simple photocoagulation group. A－C：Expression of MMP-2 at 3 days，7 days and 14 days after photocoagulation；D：Statistical graph of MMP-2 expression in rat CNV at different time after photocoagulation；E－G：Expression of MMP-9 at 3 days，7 days and 14 days after photocoagulation；H：Statistical graph of MMP-9 expression in rat CNV at different time after photocoagulation（Compared with 3 days，$^{*}P<0.05$；Bar＝50 μm；×40） 单纯光凝组光凝后不同时间MMP-2和MMP-9在大鼠CNV内的表达。A－C分别为光凝后3 d、7 d和14 d MMP-2的表达；D为光凝后不同时间MMP-2在大鼠CNV局部表达差异统计图；E－G分别为光凝后3 d、7 d和14 d MMP-9的表达；H为光凝后不同时间MMP-9在大鼠CNV局部表达差异统计图（Compared with 3 days，$^{*}P<0.05$；Bar＝50 μm；×40）

**Figure 2** Effects of PD98059 on expression of MMP-2 and MMP-9 by immunohistochemistry staining. A－C：Effects of PD98059 on expression of MMP-2 in rat CNV at 7 days after photocoagulation；A：Simple photocoagulation group；B：PD98059 treated group；C：Statistical graph of effect of PD98059 on the expression of MMP-2 in rat CNV at 7 days after photocoagulation；D－F：Effects of PD98059 on the expression of MMP-9 in rat CNV at 7 days after photocoagulation；D：Simple photocoagulation group；E：PD98059 treated group；F：Statistical graph of effect of PD98059 on the expression of MMP-2 in rat CNV at 7 days after photocoagulation（$^{*}P<0.05$；Bar＝50 μm；×40） 免疫组织化学法观察PD98059对大鼠CNV MMP-2及MMP-9表达的作用。A－C为光凝后7 d PD98059对大鼠CNV内MMP-2表达的作用，其中A为单纯光凝组，B为玻璃体内注药组，C为PD98059对大鼠CNV内MMP-2表达作用的统计图；D－F为光凝后7 d PD98059对大鼠CNV内MMP-9表达的作用，其中D为单纯光凝组，E为玻璃体内注药组，F为PD98059对大鼠CNV内MMP-9表达作用的统计图（$^{*}P<0.05$；Bar＝50 μm；×40）

**2.2 Western blotting 检测结果** 光凝后立即行玻璃体内注射 PD98059 可显著抑制 ERK 的磷酸化，光凝后 3 d 及 7 d 的抑制率分别为 54% 和 60%，而对 ERK 总量的表达无显著影响（图 3）。

**2.3 FFA 结果** 玻璃体内注药组光凝局部 CNV 的荧光素渗漏明显减少约 51%（图 4A－C）。

**2.4 HE 染色结果** 玻璃体内注药组 CNV 的生成被阻止或延缓，光凝后 14 d 抑制 CNV 厚度达 57%，由（119.193 ± 15.029）μm 减少至（51.331 ± 9.290）μm（图 4D－F）。

**2.5 免疫荧光法检测结果** 玻璃体内注药组光凝局部 MMP-2 和 MMP-9 的表达被显著抑制，免疫荧光结果显示光凝后 7 d，玻璃体内注药组抑制 MMP-2 及 MMP-9 在光凝局部的表达约达 73% 和 64%（图 5）。

**Figure 3** Effects of PD98059 on expression of p-ERK/ERK in rat retina by Western blotting（$^{*}P < 0.05$） Western blotting 法观察 PD98059 对大鼠视网膜 p-ERK/ERK 表达的影响（$^{*}P < 0.05$）

**Figure 4** Role of PD98059 on growth of rat CNV. A－C: Effects of PD98059 on rat CNV fluorescence leakage at 14 days after photocoagulation by FFA; A: Simple photocoagulation group; B: PD98059 treated group; C: Statistical graph of effect of PD98059 on rat CNV fluorescence leakage at 14 days after photocoagulation by FFA; D－F: Effects of PD98059 on rat CNV thickness at 14 days after photocoagulation by HE staining; D: Simple photocoagulation group; E: PD98059 treated group; F: Statistical graph of effect of PD98059 on rat CNV thickness at 14 days after photocoagulation by HE staining（$^{*}P < 0.05$; Bar = 50 μm; ×40） PD98059 对大鼠 CNV 生成的作用。A－C 为 FFA 法观察光凝后 14 d PD98059 对大鼠 CNV 荧光素渗漏的影响，其中 A 为单纯光凝组，B 为玻璃体内注药组，C 为 PD98059 对大鼠 CNV 荧光素渗漏面积作用的统计图；D－F 为 HE 法观察光凝后 14 d PD98059 对大鼠 CNV 生成厚度的作用，其中 D 为单纯光凝组，E 为玻璃体内注药组，F 为 PD98059 对大鼠 CNV 厚度作用的统计图（$^{*}P < 0.05$; Bar = 50 μm; ×40）

## 3 讨论

本研究结果显示，MMP-2 及 MMP-9 在 CNV 局部高表达，于光凝后 3 d 即有表达，之后表达持续升高至光凝后 14 d，光凝早期主要表达于 CNV 局部的细胞成分中，而在晚期（光凝后 14 d）则主要表达于 CNV 内的基质成分中。我们的前期研究结果提示光凝后 3～7 d 为大鼠 CNV 的生成阶段，即相当于早期 CNV（活动期 CNV），而光凝后 14 d 为 CNV 形成的稳定阶段，可持续至光凝后 110 d[5]，该期相当于稳定期 CNV。Batimastat 及 Marimastat 是 MMPs 抑制剂，其应用于新生血管生成早期则具有抑制作用，而在血管生成阶段之后应用则又具有稳定新生血管及抑制新生血管回退的作用[6]。因而推测本实验 MMP-2 和 MMP-9 早期表达升高（光凝后 3～7 d）可能是参与了早期新生血管形成过程，而晚期表达的持续升高可能参与了新生血管晚期回退[7-11]。其早期参与 CNV 生成的机制可能为：MMPs 通过将内皮细胞从基底膜上释放出来、降解血管周围细胞外基质及形成细胞外基质降解产物趋化促使内皮细胞移行。另外 MMPs 还可通过将血管内皮生长因子等生长因子肽段自细胞外基质蛋白中水解下来以增加其生物利用度[12]。而其参与新生血管晚期回退的机制可能是由于回退的血管内皮细胞自细胞外基质

**Figure 5** Effects of PD98059 on expression of MMP-2 and MMP-9 by immunofluorescence staining. A – C: Effects of PD98059 on expression of MMP-2 in rat CNV at 7 days after photocoagulation; A: Simple photocoagulation group; B: PD98059 treated group; C: Statistical graph of effect of PD98059 on expression of MMP-2 in rat CNV at 7 days after photocoagulation; D – F: Effects of PD98059 on expression of MMP-9 in rat CNV at 7 days after photocoagulation; D: Simple photocoagulation group; B: PD98059 treated group; C: Statistical graph of effect of PD98059 on the expression of MMP-9 in rat CNV at 7 days after photocoagulation( $^{*}P<0.05$; Bar = 50 μm; ×40) 免疫荧光法观察 PD98059 对 MMP-2 及 MMP-9 表达的作用。A – B 为光凝后 7 d PD98059 对大鼠 CNV 内 MMP-2 表达的作用,其中 A 为单纯光凝组,B 为玻璃体内注药组,C 为 PD98059 对大鼠 CNV 内 MMP-2 表达作用的统计图;D – E 为光凝后 7 d PD98059 对大鼠 CNV 内 MMP-9 表达的作用,其中 D 为单纯光凝组,E 为玻璃体内注药组,F 为 PD98059 对大鼠 CNV 内 MMP-9 表达作用的统计图( $^{*}P<0.05$; Bar = 50 μm; ×40)

脱离,降解新生血管网及降解围绕内皮细胞的细胞外基质支架等[13-14]。在人 CNV 内 MMP-2 主要表达于内皮细胞,视网膜色素上皮细胞也有一定表达,除了视网膜色素上皮细胞外,MMP-2 还表达于浸润至 CNV 局部的巨噬细胞[14],而 MMP-9 则特异表达于 Bruch 膜附近及视网膜色素上皮细胞、内皮细胞和基质细胞[15-16]。浸润至局部的炎症细胞也是 MMP-9 的又一重要来源[17]。说明 MMP-2 和 MMP-9 可通过调控多种参与细胞的功能而参与 CNV 的生成。进一步分析其在 CNV 内对参与细胞的作用对于深入了解 CNV 的发生是十分必要的。

基因敲除 MMP-2-/-或 MMP-9-/-或联合敲除 MMP-2-/-和 MMP-9-/-的小鼠,光凝诱导的 CNV 生成受到显著抑制。选择性 MMP 抑制剂亦对 CNV 的生成具有抑制作用[18]。我们的研究结果提示玻璃体内注射 MEK1 抑制剂(PD98059)可显著抑制光凝局部 ERK 的磷酸化水平,同时抑制 CNV 的发生,免疫组织化学及免疫荧光结果示 PD98059 可抑制 CNV 局部 MMP-2 及 MMP-9 的表达水平,说明 MEK/ERK 通路通过调控 CNV 局部 MMP-2 及 MMP-9 的表达而参与 CNV 的发生,阻断 MEK 通路,抑制 MMP-2 及 MMP-9 的表达可部分抑制 CNV 的生成。

综上所述,本研究结果提示,MMP-2 和 MMP-9 参与了大鼠 CNV 的生成,MEK/ERK 通路至少部分参与了 MMP-2 和 MMP-9 在 CNV 内的生成调控。本实验尚需进一步探讨 MMP-2 和 MMP-9 参与 CNV 生成的机制。

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【实验研究】

# TLR2/MyD88 信号系统在大鼠角膜移植术后排斥反应中的作用△

郑艳华 白浪 梁伟怡 于健 杨娟 谭青

作者简介:郑艳华,女,1989 年 3 月出生,湖北潜江人,硕士。E-mail:ophzyh@foxmail.com

About ZHENG Yan-Hua: Female, born in March, 1989. Master degree. E-mail: ophzyh@foxmail.com

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作者单位:510515 广东省广州市,南方医科大学研究生院(郑艳华,梁伟怡);510515 广东省广州市,南方医科大学南方医院眼科(白浪,于健,杨娟,谭青)
通讯作者:白浪,E-mail:bailangsfy@126.com
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From the *Graduate School of Southern Medical University* (ZHENG Yan-Hua, LIANG Wei-Yi), *Guangzhou* 510515, *Guangdong Province*, *China*; *Department of Ophthalmology*, *Nanfang Hospital of Southern Medical University* (BAI Lang, YU Jian, YANG Juan, TAN Qing), *Guangzhou* 510515, *Guangdong Province*, *China*
Responsible author: BAI Lang, E-mail: bailangsfy@126.com

## Role of TLR2/MyD88 signaling system in immune rejection after corneal transplantation in rat

ZHENG Yan-Hua, BAI Lang, LIANG Wei-Yi, YU Jian, YANG Juan, TAN Qing

【Key words】 TLR2 monoclonal antibody; TLR2/MyD88 signaling system; corneal transplantation; immune rejection

【Abstract】 **Objective** To explore the role of Toll like receptor (TLR2)/MyD88 signaling system in immune rejection after penetrating keratoplasty (PKP) in rats. **Methods** Fourteen SD rats were chosen as the donators, and 28 Wistar rats as receptor, 28 allograft corneal transplantation models were established. The models were divided into 14 allograft corneal transplantation group (6 cases were used to observe the postoperative rejection) and 14 allograft corneal transplantation treated with TLR2 monoclonal antibody group (6 cases were used to observe the postoperative rejection). Another 16 Wistar rats were chosen and divided into isograft corneal transplantation group (8 cases) and normal control group (8 cases). PKP was performed in three corneal transplantation groups. The isograft and allograft groups were treated with saline, while TLR2 monoclonal antibody group with equal numbers of 0.5 g·L$^{-1}$ TLR2 monoclonal antibody, they were injected at 0 day, 2 days, 4 days, 6 days and 8 days from the bulbar conjunctiva, respectively. The corneal opacity and neovascularization were observed by slit-lamp microscope and scored according to the rejection index, with normal cornea serving as the control. The corneal tissues were sampled at 9 days after the transplantation for HE and immunohistochemistry staining. The expression of TLR2 and MyD88 mRNA were detected by qPCR. **Results** With the passage of time, edema, opacities and neovascularization of the corneal graft occurred after the operation in three corneal transplantation groups, especially in allograft group. HE staining showed that severe corneal edema, a lots of inflammatory cells infiltration and new vessels in stroma were seen in allograft group, while mild inflammatory response was found in isograft group and TLR2 monoclonal antibody group. Immunohistochemistry staining result display that TLR2 and MyD88 were weakly detected in normal group, isograft group and TLR2 monoclonal antibody group, they were increased in epithelium and stroma in allograft group, particularly in stroma. The expression of TLR2 mRNA and MyD88 mRNA in allograft group were significantly higher than those in isograft group ($P$ = 0.000, 0.004) and normal control group ($P$ = 0.000, 0.000), in addition, the expression in TLR2 monoclonal antibody group were significantly decreased in comparison with those in allograft group ($P$ = 0.000, 0.003). **Conclusion** TLR2 monoclonal antibody inhibits the expression

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