学校编码：10384　　分类号＿＿＿密级＿＿
学号：24520131153533　　UDC＿＿＿＿＿＿＿＿

厦门大学

硕 士 学 位 论 文

人脐带间充质干细胞角膜缘注射后的命运及其对局部微环境的影响

The fate of umbilical cord derived mesenchymal stem cells and their effect on local microenvironment after limbal injection

李 娟

指导教师姓名：李　炜 教授
专 业 名 称 ：眼　科　学
论文提交日期：2016 年 05 月
论文答辩时间：2016 年 05 月
学位授予日期：2016 年　　月

2016 年 05 月

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果，均在文中以适当方式明确标明，并符合法律规范和《厦门大学研究生学术活动规范（试行）》。

另外，该学位论文为（李炜）课题（组）的研究成果，获得（李炜）课题（组）经费或实验室的资助，在（厦门大学眼科研究所）实验室完成。（请在以上括号内填写课题或课题组负责人或实验室名称，未有此项声明内容的，可以不作特别声明。）

声明人（签名）：

年　　月　　日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

（ ）1.经厦门大学保密委员会审查核定的保密学位论文，于

年　月　　日解密，解密后适用上述授权。

（ ）2.不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：

年　　月

中文摘要

目的：近年来，间充质干细胞（mesenchymal stem cell, MSC）结膜下注射被用来治疗眼表化学伤，在动物实验中表现了良好的效果。但结膜下注射后 MSC 的命运及其对局部微环境的影响还不清楚。我们通过在大鼠角膜缘注射人脐带间充质干细胞来探究 MSC 在角膜缘球结膜下的存活、迁移、增殖、分化及对角膜缘微环境的影响，同时观察 MSC 对眼表上皮增殖及分化的影响，以及对角膜缘干细胞损伤的修复作用。

方法：采用改良的胶原酶及透明质酸酶联合消化法分离培养获得人脐带间充质干细胞，并进行流式细胞学鉴定。SD 大鼠右眼颞侧角膜缘球结膜下注射 2×10^5 个 MSC 的 DMEM 混悬液，左眼相同部位注射同样体积的 DMEM 培养基作为对照组。注射后第 3、6、9 及 12 天，进行裂隙灯照相观察眼表，然后处死实验动物，进行 HE 染色、双重免疫荧光染色（Vimentin、Ki67、α-SMA）、TUNEL 染色、流式细胞学检测（CD45、CD11b）、Real-Time PCR（生长因子、炎症因子、胶原及信号通路相关分子）、ELISA 检测(SCF、HGF、IL-1β、IL-10)及蛋白印迹检测（VEGF、III 型胶原、Notch1）。为了探究不同免疫状态对 MSC 命运及对周围组织的影响，选取经 4GyX 线照射大鼠及裸大鼠进行 MSC 角膜缘注射，注射方法及细胞量同前，注射后第 3、6、9 及 12 天，进行裂隙灯照相观察结膜充血水肿情况，然后处死实验动物并进行以上检测。为验证 MSC 对损伤的修复作用，选取雄性健康 SD 大鼠建立角膜缘损伤模型，然后于角膜缘球结膜下注射 50μl 含 2×10^5 个 MSC 的 DMEM 混悬液，左眼注射同样体积的 DMEM 培养基做为对照组。注射后 6 天内每天观察角膜上皮修复情况、新生血管形成及结膜充血情况。

结果：采用改良的酶消化法对脐带进行分离培养可得到形态良好、增殖能力强的 MSC，流式细胞学鉴定符合现有标准。MSC 注射后第 6 天角膜缘球结膜可见轻度充血；HE 染色显示结膜下细胞团逐渐缩小，伴有少量炎症细胞浸润，注射区域角膜缘结膜上皮轻度增厚，以注射后第 6 天较为明显；人 Vimentin 免疫荧光染色显示 MSC 注射后阳性细胞逐渐减少，第 9 天完全消失，且并未转移到其他部位，Vimentin 阳性细胞中 α-SMA 表达阴性且仅见少量 Ki67 阳性细

胞，但其周围结膜基质及角膜缘上皮基底层可见多量 Ki67 阳性表达，角膜缘上皮基底层细胞 K14 染色增强，均以第 3 天及第 6 天较明显；TUNEL 与 Vimentin 双重染色可见，Vimentin 阳性细胞中可见多量 TUNEL 阳性细胞，随着观察时间的延长逐渐减少；流式细胞学检测结膜中 CD45 及 CD11b 比例轻度升高，第 9 天达到高峰，而后逐渐降低至正常；Real-Time PCR 结果显示促炎因子及抑炎因子的表达均有升高，且幅度相当，多种生长因子及 I、II、III 型胶原均在第 3 天及第 6 天明显升高；ELISA 及蛋白印迹结果与 PCR 基本一致。MSC 注射到 X 线照射大鼠及裸大鼠角膜缘球结膜下，未引起明显炎症反应，Vimentin 与 Ki67 染色结果显示，MSC 在 X 线照射大鼠角膜缘结膜下可存活 9 天，在裸大鼠中可存活 12 天，Vimentin 阳性细胞的 Ki67 表达阴性，但 Vimentin 阳性细胞周围结膜中仍可见多量 Ki67 阳性细胞，Real-Time PCR 显示 X 线照射大鼠结膜中的炎症因子轻度升高，而裸大鼠的持续正常水平，但两者结膜中多种生长因子及胶原的表达均明显增加。将 MSC 角膜缘球结膜下注射于角膜缘损伤大鼠，第 2 天即可观察到 MSC 组角膜上皮缺损面积明显较对照组小，角膜缘处可见长度约 1~2mm 新生血管侵入角膜，到第 4 天所有大鼠的角膜上皮恢复完整，MSC 组新生血管已全部消退，整个过程中大鼠球结膜并未出现明显充血。

结论：MSC 角膜缘球结膜下注射后能存活约 9~12 天，并未发生增殖、转移及分化为肌成纤维细胞。MSC 可通过旁分泌作用、轻度炎症刺激及 Notch 与 BMP 信号通路的激活影响角膜缘微环境，从而促进角膜缘干细胞的增殖及角膜上皮的修复。

ABSTRACT

Purpose: Mesenchymal stem cell (MSC)-based therapy has been proposed as a therapeutic strategy for ocular surface diseases over a decade. Previous studies mainly focused on their therapeutic effects on corneal wound healing, while the fate of MSCs and their effect on the local microenvironment remains unknown. In this study, we used rat models to investigate the survival, migration, proliferation, differentiation and paracrine functions of human umbilical cord mesenchymal stem cells, as well as their impact on the limbal microenvironment after limbal injection. Further more, we observed the effect of MSCs injection on limbal wound healing.

Material and methods: MSCs were isolated from neonatal umbilical cord by collagenase and hyaluronidase digestion and characterized by flow cytometry. Sprague-Dawley rats were subjected to MSCs subconjunctival injection close to the limbal area. For each rat, the right eye was injected with $2X10^5$ MSCs suspended in DMEM, and the left eye was administered an equal amount of DMEM as control. On the 3rd, 6th 9th and 12th day after injection, the rats were examined under slit-lamp microscope to evaluate the conjunctival edema and hyperemia. After that the rats were sacrificed, double-immunofluorescence was performed to investigate the expression of Vimentin, α-SMA and Ki-67. TUNEL assay was performed to evaluate cell apoptosis. CD11b or CD45 positive cells in conjunctiva were quantified by flow cytometry. Furthermore, Real-Time PCR, ELISA or Western blot was applied to detect cytokines, collagens and Notch and BMP signaling pathway related proteins. In order to investigate the fate of MSCs and their injection related changes in the local microenvironment in rats with different immune status, the X-ray exposed rats and nude rats were subjected to MSCs limbal injection. On the 3rd, 6th 9th and 12th day after injection, the rats were examined under slit-lamp microscope. After that, the rat were sacrificed, and immunostaining, TUNEL and Real-Time PCR was examined as above. In order to confirm the therapeutic effect of MSCs for corneal wound healing, limbal stem cell deficiency

model was established and subjected to MSCs limbal injection. On the 3rd, 6th, 9th and 12th day after injection, the corneal epithelium defect, conjunctival hyperemia and neovascularization was evaluated.

Results: MSCs were isolated according to their ability to adhere to cell culture plastic plates. Flow cytometry analysis results met the basic criterion. At the injection site, rat conjunctiva showed mild hyperemia on day 6 and vanished gradually. HE staining showed that limbal cell mass gradually reduced, with a small amount of immune cell infiltration and conjunctival epithelial hyperplasia of the injection area. All the injected cells remained in the same region as they had originally been engrafted, and none of them could be detected in the injured cornea and other tissues, suggesting no cell migration. The majority of MSCs survived till day 6 after injection. However, the cell mass gradually decreased, and disappeared on day 9 after injection. Using human specific vimentin as a marker, we could observe quite a few Ki67 positive cells surrounding the vimentin+ cell mass, while the Ki67 positive cells were undetectable within the injected cell mass. Immuno-staining against α-SMA was negative from day 3 to day 12. In addition, K14 expression in limbal basal epithelium was stronger on day 3 to day 6 than control group. TUNEL positive cells were present in the injected cell mass and reduced gradually. Compared with the control eyes, the quantities of CD11b+ and CD45+ cells were increased slightly in the injected eyes, which gradually reduced to normal basal levels on day 12. The mRNA expression levels of pro-inflammatory and anti-inflammatory factors were both up-regulated. The expression of growth factors such as KGF, EGF, SDF-1α, collagen, Notch1, HES-1 and BMP4 increased on day 3 and day 6 after injection. ELISA and Western blot results also supported the Real- Time PCR results. For the X-ray exposed rat and nude rat, the conjunctiva showed no hyperemia at the injection site. Double immunofluorescent staining against vimentin and Ki67 showed that there were Ki67 positive cells surrounding the vimentin positive cell mass, though no Ki67 positive cells inside the cell mass. Vimentin positive cells vanished on day 9 in X-ray exposed rat and day12 in nude rat. The mRNA expression level of inflammatory factors increased slightly in X-ray exposed rats with MSCs

injection, while no difference between nude rats with or without MSCs injection. The expression of growth factors and collagen is up-regulated dramatically in X-ray exposed rats and nude rats with MSCs injection. For limbal injury rats, the recovery of the corneal surface was significantly faster in the MSC group than control group on day 2 after limbal injection. Compared to the control group, there was 1~2mm corneal neovascularization after MSCs injection. On day 4, corneal epithelium was completely healed and the new blood vessels were regressed.

Conclusions: MSCs could survive for 9 to 12 days after limbal injection and gradually eliminated from the injection site without cell migration, proliferation and differentiation. The microenvironment was altered through paracrine function, slight inflammatory stimulus and the activation of Notch and BMP signaling pathways. MSCs may promote corneal epithelial wound healing through modulation of limbal niche.

目录

Table of Contents

第一章 前言

1.1 间充质干细胞概述

1.1.1 MSC 的概念及来源

间充质干细胞（mesenchymal stem cells，MSC）是一种来源于中胚层的具有自我更新能力及较高可塑性的成体干细胞[1]。间充质干细胞广泛的存在于各种间充质组织中，其中最常见的，也是研究最多的为骨髓[2]、脐带华通氏胶[3]及脂肪组织[4]，除此之外研究学者还在脐带血[5]、外周血[6]、经血[7]、输卵管[8]、人角膜基质、牙髓[9, 10]、巩膜[11]及结膜[12]中分离出间充质干细胞。

1.1.2 MSC 的分化潜能

MSC 具有干细胞多向分化的潜能，由于其来自于中胚层，在体外及体内可以诱导分化成多种间充质组织细胞[13-16]，如骨髓基质、成骨、软骨、脂肪及肌肉组织。另有研究表明，在一定的条件下，间充质干细胞还能向其他胚层发育的组织转分化，如来源于外胚层的角膜上皮，皮肤组织，神经组织等，来源于内胚层的肝脏组织，肺泡组织，小肠组织等[14, 17]。

1.1.3 MSC 的免疫特性

MSC 具有低免疫原性，以往认为是由于 MSC 在无任何刺激情况下细胞表面低表达主要组织相容性抗原 II 类分子（MHC II），不表达一些共刺激分子，如 CD40，CD40L，B71，B72[16]。但有体外实验表明，将 MSC 混合在淋巴细胞中共同培养，即使加入干扰素 γ 诱导 MSC 表达完整的 MHCII 分子，仍未见免疫应答，且在混合细胞反应中提供 CD40，CD40L，B71，B72 等共刺激因子后，未能引起免疫应答。提示 MSC 的低免疫原性可能与 MHCs 分子及共刺激分子表达无关，而与 MSC 对免疫细胞的直接抑制作用及分泌相关因子形成免疫抑制微环境有关。也有动物实验将经体内扩增的 HLA 相合供者或第三者来源的 MSC 输入动物体内后，未发生免疫排斥反应，且能显著延长移植器官存活时间，提示 MSC 植入体内也具有低免疫原性，且能发挥免疫抑制效应[18]。另外 MSC 分化成其他类型如软骨脂肪成骨等细胞后，仍然具有低免疫原性[19]。综上所述，无论何种 MSC 都不会诱发宿主强烈的免疫排斥反应，因此 MSC 可作为一种安