

体外培养人角膜缘上皮细胞抑制激活态角膜基质细胞的生长*★

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Growth of actived keratocytes inhibited by limbal epithelial cells cultured *in vitro*

Yu Xiao-fei¹, Xu Zhong-zhong², Du Lian-xin², Wang Li-ya¹

Abstract

BACKGROUND: When cornea injured, it can induce the phenotypic transition of keratocyte into the fibroblast, which causes the formation of the scar in the stroma and the loss of the vision.

OBJECTIVE: To observe the interaction between the corneal epithelial cell in different regions and the actived keratocyte and to explore that whether the limbal epithelial cell can inhibit the growth of the actived keratocyte.

METHODS: Enzyme digestion and mechanical dissociation were adopted to collect corneal epithelial cells and the keratocyte from central cornea, paramedian cornea, limbus, and then cultured *in vitro*. The changes of cell morphology and growth were recorded by phase contrast microscopy. The phenotype and proliferation capacity of the cultured cells were identified by immunofluorescent staining with antibodies for p63 and proliferating cell nuclear antigen (PCNA).

RESULTS AND CONCLUSION: When the contact inhibition occurred between corneal epithelial cells and fibroblasts, there was a define boundaries to divide them into two sections. In the limbal group, the positively expressing rates in the cultured corneal epithelial cells were higher for PCNA and p63, in the paramedian group the expression of PCNA is also higher and the expression of p63 was negative. In the central group, the expression of PCNA is lower and p63 was negative expression. Only the limbal group has a certain proportion of limbal epithelial stem cells. In limbal group the growth of fibroblasts was inhibited by limbal epithelial cells, after interaction four weeks, fibroblasts were degenerated into dead cell clusters. Because of the limbal stem cells deficiency in central group and paramedian group, the area of fibroblasts was increasing obviously, but the growth of corneal epithelial cells was inhibited and some were displaced by fibroblasts. It is indicated that limbal epithelial cells can inhibit the growth of actived keratocytes and degenerate them *in vitro* culture.

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摘要

背景: 角膜受到损伤后, 角膜基质细胞激活转变为成纤维细胞, 引起角膜基质瘢痕化, 导致视力下降甚至丧失。

目的: 观察角膜不同部位上皮细胞与角膜基质细胞的相互作用, 探索角膜缘上皮细胞群能否抑制激活态角膜基质细胞的生长。

方法: 采用酶消化及机械外力相结合的方法获取人角膜中央、角膜旁中央及角膜缘处角膜上皮细胞与浅层角膜基质细胞, 进行体外培养。相差显微镜下观察细胞形态及生长变化。待培养角膜上皮细胞与基质细胞发生接触抑制时, 记作“0周”, 采用免疫荧光染色技术检测培养细胞中 PCNA 及 p63 蛋白的表达。

结果与结论: 培养的角膜上皮细胞与成纤维细胞发生接触抑制时, 两种细胞间有明显分界线。角膜缘组上皮细胞中 PCNA 及 p63 蛋白均有较高的表达; 角膜旁中央组 PCNA 有较高的表达, p63 蛋白阴性表达; 角膜中央组 PCNA 表达较低, p63 蛋白阴性表达; 从鉴定结果中可以得出只有角膜缘组中存在一定比例的角膜缘上皮干细胞。角膜缘组上皮细胞逐渐包围并化解成纤维细胞, 在相互作用 4 周后, 成纤维细胞聚集成死细胞团, 缺乏角膜缘干细胞的中央组及旁中央组中成纤维细胞生长面积增加, 上皮细胞生长受到抑制甚至死亡。说明体外培养的角膜缘上皮细胞群可以抑制激活态角膜基质细胞的生长。

关键词: 角膜上皮; 角膜缘; 干细胞; 角膜基质细胞; 成纤维细胞; 抑制

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0 引言

角膜上皮细胞位于角膜表层, 其更新源于角膜缘上皮基底部的上皮干细胞^[1]。角膜缘干细胞缺乏可以导致角膜上皮结膜化及新生血管的长入, 因此具有重要的生理屏障功能^[2]。角膜基质细胞在生理情况下处于静止状态; 当角膜上皮损伤后, 角膜基质细胞受到刺激, 细胞发生凋亡或从静止状态转变为激活态, 转化为修复表型^[3]。

体外培养时, 在血清刺激的条件下角膜基质细胞可以由静止状态转化为成纤维细胞表型的激活状态^[4]。本实验在体外培养条件下, 观察不同角膜部位上皮细胞与激活态角膜基质细胞的相互作用, 探索角膜缘上皮细胞群能否抑制激活态角膜基质细胞生长。

1 材料和方法

设计: 细胞学体外实验。

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材料:

标本来源: 正常人角膜和角膜缘组织取自河南省眼库, 为30~50岁男性捐献者。

纳入标准: ①捐献者无角膜炎及角膜营养不良等角膜疾病。②无角膜接触镜配戴史者。③无眼外伤、眼部激光治疗史及手术史者。④裂隙灯检查示角膜组织透明, 无角膜新生血管者。⑤乙型、丙型肝炎病毒, 梅毒和HIV病毒检查结果均为阴性者。

主要试剂:

试剂	来源
DMEM/F12、Alexa-fluor 488 及 546 山羊抗小鼠抗体	美国 Invitrogen 公司
胰岛素、转铁蛋白、亚硒酸钠、Dispasell、EDTA、fluoromount-G 系列封固剂	美国 Sigma 公司
胰蛋白酶、胎牛血清	美国 Gibco 公司
小鼠抗人 p63	美国 Lab Vision 公司
小鼠抗人 PCNA 抗体	美国 Santa Cruz 公司
4',6-二脒基-2-苯基吲哚 (4',6-diamidino-2-phenylindole, DAPI)	德国 Roche 公司
Tritonx-100、L-谷氨酸	中国 Solarbio 公司

主要仪器:

仪器	来源
6孔培养板	美国 Corning 公司
细胞刮	美国 BD 公司
4 mm 及 8 mm 环钻	中国明仁医疗器械有限公司
荧光显微镜 型号 80i	日本 Nikon 公司
相差显微镜 型号 IX70	日本 Olympus 公司

实验方法:

培养液的配制: 基础培养基DMEM与F12进行1:1混合, 添加相关因子, 包括5 mg/L胰岛素、5 mg/L转铁蛋白、5 μg/L亚硒酸钠、0.5 g/L谷氨酰胺、体积分数10%胎牛血清、0.1 g/L青霉素和0.1 g/L链霉素。

角膜组织的处理与培养: 正常供者眼的角膜及角膜缘组织经灭菌D-Hank's液冲洗, 去除结膜组织、筋膜组织、内皮细胞及虹膜组织; 用含0.4 g/L青霉素和0.4 g/L链霉素的D-Hank's液浸泡30 min; 分别采用4 mm和 8 mm环钻获取中央角膜组织及旁中央环形角膜组织, 剩余组织为角膜缘组织。不同部位角膜组织获取后, 分别进行以下实验。

人角膜上皮细胞的获取: 5 g/L Dispase II 消化液中作用1 h, 37 °C^[5]; 使用细胞刮刮去上皮层, 分别收集到标记的离心管中, 终止消化。

角膜基质细胞的获取: 刮去上皮层后继续刮去浅层角膜基质, 获取浅基质层的角膜基质细胞, 分别收集到标记的离心管中, 终止消化。

角膜上皮细胞与角膜基质细胞的共培养: 将收集到的两种细胞混合, 离心1 000 r/min, 5 min; 吸出冲洗液, 加入0.7 mL 0.25% 胰蛋白酶/0.02% EDTA作用5 min, 37 °C; 终止消化, 离心1 000 r/min, 5 min×2次。分别滴加适量培养液, 制成细胞悬液, 以2 000个/cm²的细胞密度接种于6孔培养板中, 标记组别; 于37 °C、体积分数5% CO₂饱和湿度条件下进行培养, 每2 d更换培养液1次。逐日观察细胞生长情况, 待培养角膜上皮细胞与基质细胞发生接触抑制时, 记作“0周”, 在板底部用5号针头刻痕做标记; 以所作标记为指示点, 在固定和相同区域内每组每个观察点拍照6幅, 进行拼图, 角膜中央组为S_A、角膜旁中央组为S_B、角膜缘组为S_C, 观察两种细胞相互作用情况, 并记录数据进行分析。

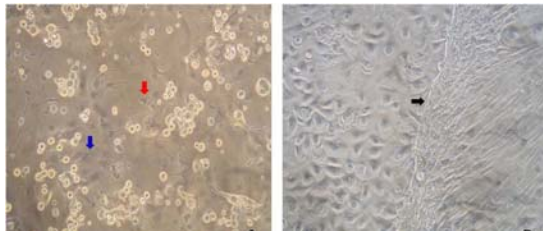
培养细胞中PCNA及p63蛋白表达的检测: 采用免疫荧光技术检测培养细胞中PCNA及p63蛋白表达。主要步骤如下^[6]: 取出培养“0周”的细胞, 20 g/L多聚甲醛溶液固定10 min, 4 °C; PBS冲洗5 min×3次; 滴加山羊血清封闭液, 于37 °C作用15 min, 倾去, 加入1:200稀释的一抗(PCNA、p63), 4 °C过夜; PBS缓冲液冲洗5 min×3次; 滴加二抗, 37 °C恒温箱中作用1 h; PBS冲洗5 min×4次; 滴加DAPI复染, Fluoromount封固荧光显微镜下观察并记录图像, 阳性细胞比例的计算方法为: 在3组实验中, 选择面积相等的观察视野, 共取10个; 计数每个视野下阳性细胞数与DAPI复染的细胞总数, 进行比例计算。

主要观察指标: 角膜缘组织、中央角膜组织和旁中央环形角膜组织角膜上皮细胞与角膜基质细胞共培养后形态变化及角膜上皮细胞PCNA、p63蛋白的表达。

统计学分析: 采用SPSS 17.0统计学软件进行统计分析, 图片拼接、处理及计算采用Nikon公司AR3.1软件进行; 表达PCNA及p63蛋白阳性细胞比例数据采用 $\bar{x} \pm s$ 表示。3组中细胞增殖能力及干细胞所占比例的比较采用各组间的多重比较分割 χ^2 法。 $P < 0.05$ 为差异有显著性意义。

2 结果

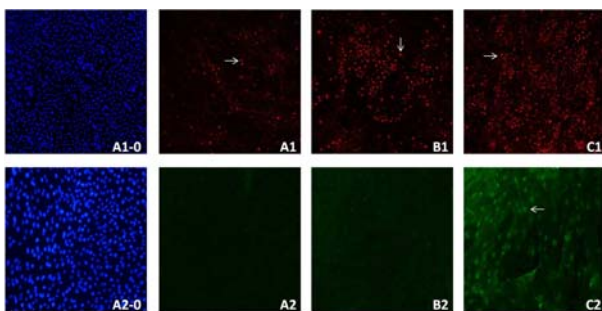
2.1 角膜上皮细胞与基质细胞的形态学观察 24 h内细胞充分贴壁, 角膜基质细胞呈树突状, 角膜上皮细胞呈圆形或椭圆形, 见图1a; 在血清的刺激下角膜基质细胞由树突状转变为长梭形。0周时, 两种细胞相互作用, 其间有明显的分界线, 见图1b; S_{A0w}区域内上皮细胞数量为1 531个, S_{B0w}为2443个, S_{C0w}为14 786个。



a: After 24 h primary culture, the keratocytes attached and spreaded out with dendritic morphology (red arrow), and corneal epithelium attached firmly and spreaded out with round or oval shape (blue arrow)
b: At the point of 0 wk, the contact inhibition occurred between corneal epithelium and the fibroblasts, and there was a define boundary to divide them into two sections (black arrow). The keratocytes were transformed into fibroblasts and showing long fusiform under continual serum stimulation

Figure 1 Morphology of the corneal epithelial cells and the keratocytes under phase contrast microscopy (x200)
图1 相差显微镜下观察角膜上皮细胞与基质细胞的形态 (x200)

2.2 各组培养角膜上皮细胞中PCNA及p63蛋白表达的检测 见图2。



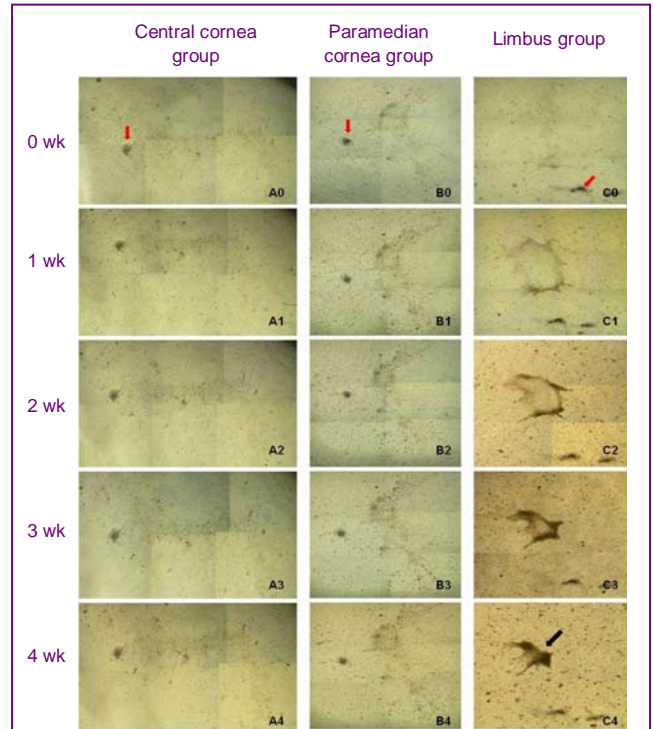
A1-C1 (x100): Detection of nucleoprotein PCNA in culture epithelial cells of central cornea group, paramedian cornea group, limbus group, and the nuclei were red (white arrow); A2-C2 (x200): Figure A2 and B2 was the detection of nucleoprotein p63 in culture epithelial cells of central cornea group and paramedian cornea group, and positive cells were not found, figure C2 was the detection of nucleoprotein p63 in limbus group, and the nuclei were green (white arrow); Figure A1-0 and Figure A2-0: Staining of DAPI, and the nuclei were showing blue colour

Figure 2 Protein expression of proliferating cell nuclear antigen (PCNA) and p63 in corneal epithelial cells
图2 各组角膜上皮细胞中 PCNA 及 p63 蛋白表达的检测

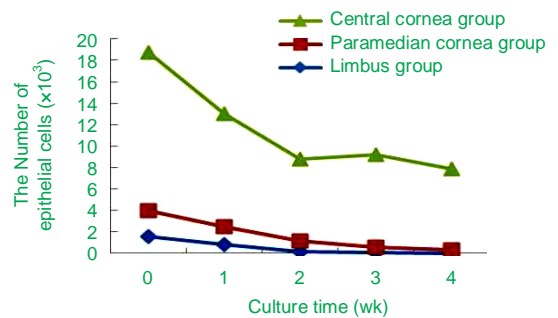
角膜上皮细胞与成纤维细胞发生接触抑制, 上皮细胞中表达PCNA阳性细胞比例分别为: 中央组为(9.56 ±

0.29)%, 旁中央组为(22.79±0.35)%, 角膜缘组(30.91±2.11)%; 3组之差异有显著性意义 ($\chi^2=113.526$, $P=0.000 < 0.01$), 角膜缘组明显高于中央组及旁中央组。角膜缘组中上皮细胞中表达p63蛋白的阳性细胞比例为(22.45±3.61)%, 而中央组及旁中央组上皮细胞中未检测到p63蛋白表达, 3组间阳性细胞比例差异有非常显著性意义, 角膜缘组明显高于中央组及旁中央组, 见图2。

2.3 各组角膜上皮细胞与成纤维细胞的相互作用 见图3。



A0-A4: There were a significant proliferation of fibroblast cells and their area was increasing obviously, but the number of epithelial cells were remarkable decrease, even disappear; Figure B0-B4: Proliferation of fibroblasts were not more significant than the central cornea group, and their area was increasing slightly; C0-C4: Epithelial cells had a significant proliferation, the growth of fibroblasts was inhibited by limbal epithelial cells, and the fibroblasts were degenerated into dead cell clusters (black arrow), and red arrow show the mark was made by No. 5 pinhead at the bottom of culture plate



C: In limbus group the number of epithelial cells was higher than the other groups, and in each group epithelial cells were decreased in amount as a whole

Figure 3 Interaction between corneal epithelial cells and fibroblasts under phase contrast microscopy (x40)
图3 相差显微镜下各组角膜上皮细胞与成纤维细胞的相互作用(x40)

角膜缘组中角膜上皮细胞逐渐包围并化解成纤维细胞, 在相互作用4周后, 成纤维细胞聚集成死细胞团, 但上皮细胞数量较0周时明显减少; 旁中央组中成纤维细胞生长面积略有增加, 中央组中成纤维细胞生长面积增加明显, 二者上皮细胞数量较0周时明显减少。3组实验中固定和相同区域内上皮细胞数量 S_{A4w} 为16个, S_{B4w} 为306个, S_{C4w} 为7547个, 见图3。

3 讨论

自从1986年Schermmer等^[7]证明角膜上皮干细胞位于角膜缘基底层, 并由此提出角膜缘干细胞的概念以来, 研究学者相继对角膜缘干细胞进行了更深入的研究^[8-9]。许多研究学者发现如果角膜缘干细胞缺乏会导致角膜上皮结膜化及大量新生血管长入, 导致严重眼表疾病甚至失明, 因此, 角膜缘干细胞具有重要的生理屏障功能^[10-11]。角膜基质细胞位于角膜基质层, 当角膜受到损伤刺激后, 其激活转变为成纤维细胞或发生凋亡, 引起角膜基质瘢痕化, 导致视力下降甚至丧失^[12-13]。本实验采用体外血清刺激条件诱导静止态角膜基质细胞向成纤维细胞方向转化; 为了证实角膜缘干细胞的存在对在成纤维细胞的生长抑制及化解起重要作用, 将角膜分为3个区域: 中央区、旁中央区及角膜缘部位, 观察不同区域培养的上皮细胞与激活态角膜基质细胞相互作用情况。

当培养角膜上皮细胞与激活态角膜基质细胞发生接触抑制时, 采用免疫荧光技术检测各组上皮细胞中PCNA及p63蛋白表达。p63是一种核转录因子, 近几年被认为是角膜缘上皮干细胞的相对特异性阳性标记物, 用于对角膜缘上皮细胞及培养细胞的鉴定^[14-15]。PCNA是一种相对分子质量为36 000的蛋白质, 在细胞核内合成, 能反映细胞的增殖能力^[16-17]。从鉴定结果中可以得出只有角膜缘组中p63蛋白表达阳性, 存在一定比例的角膜缘上皮干细胞; PCNA在旁中央区及角膜缘组均有较高表达。培养4周后, 角膜缘组成纤维细胞聚集成死细胞团, 可能是由于角膜缘组中含有一定比例的干细胞, 具有较强的增殖潜能, 干细胞在增殖分化过程中分泌的细胞因子及蛋白酶有效抑制并化解成纤维细胞, 阻止成纤维细胞进一步转化为肌成纤维细胞。而中央组成纤维细胞生长面积明显增加, 上皮细胞消失被成纤维细胞取代, 主要是由于培养上皮细胞中PCNA表达较低, p63蛋白表达阴性, 缺乏角膜缘干细胞, 未能抑制成纤维细胞生长; 旁中央组成纤维细胞生长面积增加不显著, 但上皮细胞数量及生长所占面积减少较大, 旁中央组中虽然有较多的上皮细胞表达PCNA, 由于角膜缘干细胞的缺乏, 仍不能有效抑制及化解成纤维细胞的生长。

本实验结果显示体外培养的角膜缘上皮细胞可以

抑制成纤维细胞生长并化解成纤维细胞。临床上角膜溃疡愈合后形成的瘢痕严重影响患者预后视力, 希望通过构建含有角膜缘干细胞的上皮片进行溃疡覆盖, 减轻角膜基质层瘢痕化程度, 提高患者预后视力。

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