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脂质体介导 VEGF 基因对成骨细胞增殖、合成骨钙素以及细胞周期的相关研究 *

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摘要 目的：研究脂质体介导血管内皮生长因子（VEGF）基因对成骨细胞增殖、合成骨钙素以及细胞周期的影响。**方法：**通过脂质体介导的基因转染方法，将携带外源性 VEGF 重组 pcDNA3-hVEGF 质粒导入体外培养的成骨细胞，酶联免疫吸附测定法 (ELISA) 检测转染后细胞中 VEGF 浓度变化，以判断转染效果；采用细胞计数法检测转染重组质粒的成骨细胞的增殖活性；流式细胞术检测转染重组质粒的成骨细胞周期的变化；ELISA 检测转染重组质粒的成骨细胞骨钙素浓度变化。**结果：**与对照组相比，转染组成骨细胞中 VEGF 的浓度显著增加，与对照组间差异具有统计学意义（P<0.05）；转染重组质粒的成骨细胞的增殖能力较对照组显著增强，差异具有统计学意义（*P<0.05*），与对照组相比，转染重组质粒的成骨细胞周期（G2/M+S）%明显增加，差异具有统计学意义（*P<0.05*）；转染重组质粒的成骨细胞合成的骨钙素浓度较对照组显著升高，差异具有统计学意义（*P<0.05*）。**结论：**脂质体介导成骨细胞增加血管内皮生长因子的水平，可促进成骨细胞增殖，增加成骨细胞骨钙素的浓度，从而提高成骨细胞的功能。

关键词：VEGF；成骨细胞；增殖；骨钙素

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Effect of Liposome Mediated VEGF Gene on Osteoblast Proliferation, Osteocalcin Synthesis and Cell Cycle*

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ABSTRACT Objective: To investigate the effects of vascular endothelial growth factor (VEGF) gene mediated by liposome on osteoblast proliferation, osteocalcin synthesis and cell cycle. **Methods:** The recombinant plasmid pcDNA3-hVEGF with exogenous VEGF was introduced into cultured osteoblasts by liposome-mediated gene transfection method. The concentration changes of VEGF were detected by enzyme linked immunosorbent assay (ELISA) in the transfected cells to determine transfection efficiency. The osteoblast proliferation were measured by Cell counting; the cell cycle were analyzed by flow cytometry; the expression of osteocalcin were detected by ELISA. **Results:** Compared with the control group, the concentration of VEGF in transfected osteoblasts increased significantly, and the difference was statistically significant (*P<0.05*). The proliferation of osteoblasts transfected with recombinant genes was significantly enhanced (*P<0.05*), Cell cycle (G2/M+S)% increased significantly, and the difference was statistically significant (*P<0.05*); the concentration of synthetic osteocalcin was significantly increased, and the difference was statistically significant (*P<0.05*). **Conclusion:** Liposome-mediated osteoblasts increase vascular endothelial growth factor level, promote osteoblast proliferation and increase osteoblast osteocalcin concentration to improve osteoblast function.

Key words: VEGF; Osteoblasts; Proliferation; Osteocalcin

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血管内皮生长因子（Vascular Endothelial Growth Factor, VEGF）是一种特异性细胞因子，对血管内皮细胞增殖具有促进作用，加速血管的形成[1,2]。胚胎的形成、机体正常组织的生长修复以及异常生长、肿瘤的生长、远端转移与血管的生成密切相关[3-7]。研究证实，骨折后骨折断端 VEGF 的表达量显著增加，并在骨折后的一段时间里维持着较高的水平[8,9]。在新骨形成过程中，成骨细胞发挥重要作用[10]。VEGF 以及其他细胞因子常被用于创伤修复的治疗中，但 VEGF 在体内容易被降解，不适宜全身持久用药[11]。因此，研究 VEGF 等细胞因子不同的给药方法，使其浓度维持持续性是目前研究的热点之一。本研究通过脂质体转染技术将携带外源性 VEGF 重组质粒导入成骨细胞中，分析其表达量对成骨细胞增殖以及周期的影响。

1 材料与方法

1.1 实验主要材料

人成骨细胞系购自中科院上海细胞研究所，DMEM 培养

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基购自美国 Gibco BRL 公司,胎牛血清购自杭州四季青生物有限公司,青 - 链霉素购自哈尔滨市哈药集团,胰蛋白酶、骨钙素酶联免疫吸附测定法 (Enzyme-linked immunosorbent assay, ELISA) 检测试剂盒购自江苏碧云天生物有限公司,VEGF ELISA 检测试剂盒购自美国 RD 公司,Lipofectamine 2000、pcDNA3-hVEGF 质粒购自美国 Sigma 公司, 流式细胞仪购自美国赛默飞世尔公司,酶标仪购自美国 Bio-Tek 公司,倒置显微镜购自日本 Olympus 公司。

1.2 方法

1.2.1 成骨细胞的培养 成骨细胞培养于含有 10%胎牛血清和 100U/mL 青 - 链霉素的 DMEM 培养基中, 置于 5% CO_2、37℃的恒温箱中。每天通过倒置显微镜观察细胞的生长状态,待细胞长成致密单层时,弃去培养基,吸取 5mL PBS 缓冲液清洗细胞 2 次,加入胰蛋白酶消化,加入 10 mL 细胞培养液制成细胞悬浮液,分装与两个培养瓶中,置于 5% CO_2、37℃的恒温箱中继续培养,每隔 2d 传代一次。

1.2.2 细胞转染 转染前 24h, 将对数期成骨细胞以每孔 1× 10^6 个接种于 6 孔板中,置于 37℃的恒温箱中继续培养 24h,待细胞融合度达到 90%以上时,弃去培养基,加入无血清的培养基,进行转染。将 pcDNA3-hVEGF 质粒、Lipofectamine 2000 与无血清培养基混合均匀,37℃静置 15 min,每孔加入 300 μL 该质粒混合物,置于 37℃恒温箱中继续培养 4h 后,更换为含胎牛血清的培养基,放入恒温箱中培养 48h 后更换培养基,用于后续实验。对照组为常规培养的成骨细胞。

1.2.3 成骨细胞中 VEGF 表达量的检测 收集转染和未转染成骨细胞培养 48h 的上清液,根据 VEGF ELISA 检测试剂盒说明书进行操作,30 min 内置于酶标仪中检测 450 nm 波长下的 OD 值。

1.2.4 成骨细胞生长活性的检测 通过 0.25%胰蛋白酶消化转染和未转染的成骨细胞, 加入新鲜培养基调整细胞密度至 3× 10^4/mL,接种于已加入 1 mL 完全培养基的 24 孔培养板中,置于 37℃恒温箱中培养 24h、48h、72h、96h,收集细胞,记录细胞数目,实验重复 3 次,取平均值。

1.2.5 成骨细胞周期的检测 收集细胞,PBS 缓冲液漂洗 2 次,加入含血清的培养基调整细胞密度为 1× 10^6/mL,吸取 100 μL 细胞悬浮液加入等量的 DNA 染液,37℃静置 30 min,置于流式细胞仪中检测细胞周期。

1.2.6 成骨细胞合成分泌骨钙素的检测 胰酶消化、收集转染和未转染成骨细胞培养 48 h 的上清液, 根据骨钙素 ELISA 检测试剂盒说明书进行操作,30 min 内读取酶标仪 450 nm 波长下的 OD 值。

1.2.7 统计分析 实验数据采用 SPSS 22.0 统计学软件进行分析,结果以平均数± 标准差表示($\bar{x}\pm s$),组间比较采用独立样本 t 检验,$P<0.05$ 表示差异具有统计学意义。

2 结果

2.1 转染后成骨细胞中 VEGF 的表达量

Lipofectamine 2000 介导 pcDNA3-hVEGF 质粒转染成骨细胞后, 对照组和转染组成骨细胞 OD450 nm 值分别为 25.368± 3.421、48.674± 5.320; 转染组成骨细胞中 VEGF 的表达量较对照组差异具有显著性(t= 6.382, P=0.003),具有统计学意义。

2.2 转染后对成骨细胞生长活性的影响

pcDNA3-hVEGF 质粒转染成骨细胞后, 对成骨细胞生长活性的影响结果如表 1 所示,在转染后的 24h,转染组成骨细胞数量与对照组差异不显著;从转染后 48h、72h、96h,转染组细胞数量明显增加, 较对照组间差异具有显著性 (t_1=69.872 , $P1$=0.000 ; t_2=61.801 , P_2=0.000; t_3=118.454, P_3=0.000), 具有统计学意义。

表 1 转染后对成骨细胞生长活性的影响($\bar{x}\pm s$)

Table 1 The effect of transfection on osteoblast growth activity($\bar{x}\pm s$)

Groups	Cell number(× 10^4/mL)			
	24h	48h	72h	96h
Control group	3.258± 0.041	4.157± 0.043	5.268± 0.053	6.536± 0.069
Transfected group	3.459± 0.038	6.879± 0.052#	8.574± 0.076#	13.258± 0.070#

Note: Compared with the control group, #$P<0.05$.

2.3 转染后对成骨细胞周期的影响

流式细胞仪测定转染重组质粒和对照组的细胞周期,结果显示对照组和转染组成骨细胞 (G2/M+S)%分别为 15.268± 1.265、28.323± 3.213;与对照组相比,转染组细胞(G2/M+S)%较对照组间差异显著(t=6.548, P=0.003),具有统计学意义。

2.4 转染后对成骨细胞合成分泌骨钙素的影响

酶联免疫吸附测定法测定成骨细胞培养液中骨钙素的浓度,结果显示,转染组和对照组细胞培养液中骨钙素的浓度分别为(3.625± 0.043)ng/mL、(2.145± 0.023)ng/mL;转染组成骨细胞合成骨钙素的能力显著高于对照组(t=52.567 , P=0.000),差异具有统计学意义。

3 讨论

目前,成骨细胞体外培养技术越来越成熟,已成为研究骨生长以及代谢过程中与体内环境相互作用的常见方法。机体中骨组织处于持续代谢和更新中,成骨细胞和破骨细胞在其代谢更新中发挥重要作用[8]。成骨细胞不仅在骨发生过程中起重要作用,而且通过合成骨基质胶原蛋白和钙化骨基质参与骨形成过程中。成骨细胞也调控机体内环境稳态、生理反应和骨生长代谢过程。成骨细胞和血管内皮细胞可合成、分泌血管内皮生长因子,调控骨细胞生长、分化、聚集、血管生成等[9,10]。血管内皮生长因子具有多种受体,均具有含有络氨酸激酶的细胞内区

域、免疫球蛋白样的细胞外区域以及跨膜区域，通过磷酸化膜转运受体发挥其生物特性，特异性作用于血管内皮细胞，促进新生血管的形成[11,12]。研究表明，成骨细胞合成的血管内皮生长因子通过作用与周围组织的内皮细胞，从而诱导其他细胞因子的分泌，直接或者间接作用于成骨细胞[13]。体外接种成骨细胞，血管内皮生长的表达量显著增加；外源性的血管内皮生长促进成骨细胞早期分泌标志物的水平[14,15]。在本实验中，采用脂质体将重组基因质粒转染入成骨细胞，ELISA 检测法结果显示，基因转染成骨细胞后血管内皮生长因子的水平显著增加，提示重组质粒转染成功，成骨细胞中血管内皮生长因子的增加可通过作用与内皮细胞，促进新血管形成，诱导成骨细胞发育，与 Hisham 等的研究结果一致，但其具体作用机制尚不完全清楚。

细胞增殖是指细胞中遗传物质进行复制，继而发生周期性的分裂，导致细胞数量增加，是一种细胞生物学特性的基本属性之一[20-23]。细胞的功能比如分泌、传导信号、吞噬等主要是在细胞分化的基础上完成的特定生物学作用。成骨细胞的增殖和分化主要完成骨骼活动，碱性磷酸酶一般作为成骨细胞早期分泌的标志物之一，骨钙素一般作为成骨细胞分化成熟的标志物[24-27]。研究表明，外源性的血管内皮生长因子可促进骨矿化结节的形成和提高成骨细胞碱性磷酸酶活性[19]。本实验发现，从第 48h 开始，基因转染组的细胞数量显著增加，与对照组相比二者差异具有显著性，提示成骨细胞增殖、分化加速，促进骨骼活动；流式细胞仪测定转染组和对照组细胞周期结果发现：转染重组质粒的成骨细胞（G2/M+S）%较对照组显著增加，二者之间比较差异具有统计学意义，提示血管内皮生长因子可能通过降低 G0/G1 细胞数量，促进成骨细胞增殖。ELISA 检测转染组和对照组成骨细胞分泌骨钙素的水平，结果显示转染组成骨细胞骨钙素的水平显著高于对照组，与对照组间差异显著，表明采用脂质体转染基因重组质粒可促进成骨细胞增殖能力和增加成骨细胞的功能。

综上所述，基因转染成骨细胞增加血管内皮生长因子的水平，可促进成骨细胞增殖，增加成骨细胞骨钙素的浓度，从而提高成骨细胞的功能，为血管内皮生长因子的进一步研究和应用提供依据。

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