骨髓干细胞诱导形成的内皮细胞在体内外环境中的稳定性

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摘要 采用异硫氰酸荧光素(fluorescein isothiocyanate, FITC)标记的 CD34 (FITC-CD34)、免疫细胞化学及透射电镜鉴定血管内皮生长因子(vascular endothelial growth factor, VEGF)诱导 Sprague-Dawley (SD)大鼠骨髓间充质干细胞(bone marrow mesenchymal stem cells, BM-MSCs)分化形成的内皮细胞(诱导组)。应用三维脱细胞真皮基质(acellular dermal matrix, ADM)支架组织, 接种诱导形成的内皮细胞, 种植于 SD 大鼠体内生长 14 天。同时采用不含 VEGF 的 DMEM (DMEM 组)和EGM-2培养基(EGM-2组), 体外培养诱导形成的内皮细胞14天, 酶联免疫吸附测定法(enzyme-linked immunosorbent assay, ELISA)检测各组细胞上清液中内皮素(endothelin-1, ET-1)和 VIII 因子相关抗原(VIII factor related antigen, F-VIIIAg)含量, 进行统计学处理。实验结果表明, VEGF 诱导 BM-MSCs 培养 14 天, 细胞呈“铺路石”样排列, FITC-CD34 呈现绿色荧光, 免疫细胞化学抗 CD31 和 CD34 表达阳性, 细胞质中含 Weibel-Palade 小体, 具有内皮细胞的特征。在 SD 大鼠体内生长 7 天和 14 天, 内皮细胞保留其特征, 对抗 CD31 和 CD34 表达阳性。内皮细胞在体外不含 VEGF 的 DMEM 培养基和 EGM-2 培养基中各培养 14 天后, 细胞回复到诱导前状态, FITC-CD34 反应阴性。ELISA 检测 DMEM 组和 EGM-2 组中 ET-1 和 F-VIIIAg 含量明显低于诱导组(P<0.05)。研究结果显示, BM-MSCs 诱导形成的内皮细胞, 在体内环境中生存能保留其特征; 但在缺乏 VEGF 诱导的体外培养基中生长, 不能维持其特征。

关键词 骨髓干细胞; 内皮细胞; 诱导; 血管内皮生长因子

骨髓间充质干细胞(bone marrow mesenchymal stem cells, BM-MSCs)具有多向分化潜能和自我增殖能力, 能够诱导分化为软骨细胞、血管平滑肌细胞、内皮细胞、脂肪细胞, 甚至神经细胞[1~5]。在组织损伤修复中具有很大的细胞分化潜力[6]。诱导骨髓干细胞向体细胞分化, 是寻求理想的组织工程种子细胞的重要来源。血管内皮细胞是组织工程化血管的重要组成成份。诱导分化的内皮细胞, 在体内外去诱导的环境下生长, 能否保留其特征成为临床理想的血管组织工程种子细胞, 目前有关报道较少。本实验采用 VEGF 诱导成年 SD 大鼠 BM-MSCs 分化成为内皮细胞, 探讨在体内外去诱导的环境中生长时, 内皮细胞表型的稳定性。

1 材料与方法

1.1 MSCs 的原代培养

取 2 只 SD 大鼠, 清洁级, 雌性, 150 g (河北医科大学实验动物中心提供, 合格证编号: 906019), 在无菌条件下分离股、胫骨, 暴露骨髓腔。用低糖无血清 DMEM 培养液(HyClone 公司)冲出骨髓, 离心弃上清液。加入等量淋巴分离液 Percoll, 离心吸取白色单核细胞层, 用含有 10% 胎牛血清、10^5 U/L 青霉素、100 mg/L 链霉素的低糖 DMEM 培养液(pH 7.2)培养于 50 ml 培养瓶中, 细胞浓度调整为 3×10^5 个/ml, 静置于 37 ℃、5% CO_2 培养箱内。每 4 天换液 1 次, 至 9~11 天细胞融合生长约 80% 以上, 以 0.25% 胰蛋白酶消化, 按 1∶3 传代。倒置显微镜下观察细胞形态变化。

1.2 细胞实验分组

将第二代(P2) BM-MSCs 消化后, 按 2×10^5 个/ml 的细胞密度接种于 50 ml 培养瓶中, 设诱导组与对照组。诱导组细胞培养于含 10% FBS (Gibco 公司)、10 μg/L血管内皮生长因子(vascular endothelial growth

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factor, VEGF) (Prospec 公司)、4 μg/L 碱性成纤维生长因子(basic fibroblast growth factor, bFGF)(Prospec 公司)的低糖 DMEM 培养液中; 对照组细胞换以新鲜的 10% FBS 不含 VEGF 的低糖 DMEM 培养液。

1.3 组织支架上的细胞培养

将三维脱细胞真皮基质(acellular dermal matrix, ADM) (北京清源伟业生物组织工程科技有限公司)浸泡在低糖的 DMEM 培养液中, 用眼科剪剪成“海绵”状。取诱导组与对照组的 14 天细胞, 按 3×10^7 个/ml 细胞浓度, 接种于 5 mm×5 mm×3 mm 的 ADM 支架上, 分别于各组的培养基上培养 7 天。

1.4 内皮细胞的鉴定

1.4.1 *免疫荧光标记* 取诱导组与对照组培养 14 天的细胞, 丙酮固定, 滴加 1∶50 FITC-CD34 (ICO115: sc-7324)多克隆抗体(Santa Cruz 公司), 37 ℃避光温育 30 min, 50 μg/ml DAPI (Roche 公司)复染细胞核, 倒置荧光显微镜下观察并计算 CD34 阳性表达率。

1.4.2 *透射电镜观察* 收集诱导组与对照组 14 天的细胞, 固定于 2.5% 戊二醛中, 丙酮逐级脱水, 1% 锇酸后固定, Epon 812 包埋, 超薄切片, 柠檬酸铅和醋酸双氧铀复染, 日立 H-7500 透射电镜观察。

1.4.3 *免疫细胞(组织)化学观察* 分别取诱导组与对照组 14 天的细胞爬片, 95% 乙醇固定; 载有诱导组与对照组细胞的ADM支架组织固定于10%福马林, 常规脱水、透明、石蜡包埋, 切片厚度 5 μm。细胞爬片(组织切片)入水后, H_2O_2封闭外源性抗原, 山羊抗鼠 CD31 (1∶50) [(PECAM-1 (M-20): sc-1506)] (Santa Cruz 公司)和山羊抗鼠 CD34 (15 μg/ml) (R&D 公司)各 37 ℃温育 2 h, 山羊二抗 PV-9003 试剂盒(北京中山金桥生物技术有限公司)中试剂 1、试剂 2 分别于 37 ℃温育 20 min, DAB 显色, 苏木素复染, 脱水、透明、封固。

1.5 内皮细胞稳定性检测

1.5.1 *体内实验* 取诱导鉴定后的内皮细胞及对照组细胞, 分别按 3×10^7 个/ml 的细胞浓度, 接种于预先处理好的 5 mm×5 mm×3 mm 的 ADM 支架上(方法同上), 培养 3 天。12 只 SD 大鼠(清洁级, 雌性, 150 g, 河北医科大学实验动物中心提供, 合格证编号: 906160)随机分 4 组, 诱导组与对照组各 2 组, 每组 3 只, 0.5 ml 10% 水合氯醛腹腔注射麻醉动物。将载有细胞的ADM支架组织折叠后, 分组种植于12只SD大鼠双侧后肢肌肉组织内。分别于种植后 7 天和 14 天, 腹腔注射 1.0 ml 10% 水合氯醛处死动物, 标本进行 HE 染色和抗 CD31、CD34 免疫组织化学染色, 方法同上。

1.5.2 *体外实验* 取诱导鉴定后的内皮细胞, 分别培养于不含 VEGF 的 DMEM 培养基(DMEM 组)和内皮细胞培养基 EGM-2 (Lonza 公司) (EGM-2 组)中 14 天; 另外继续培养诱导组和对照组细胞 14 天, 观察各组细胞形态变化; 采用 FITC-CD34 标记细胞, 方法同上。

1.5.3 *酶联免疫吸附测定法(enzyme-linked immunosorbent assay, ELISA)* 分别按 1×10^6 个/ml 细胞浓度, 离心收取诱导组、DMEM 组、EGM-2 组和对照组培养 14 天的细胞, 加入 5 倍体积的 RIPA 裂解缓冲液(普利莱基因技术有限公司), 冰浴、振荡, 离心收集上清液, −80 ℃冻存。山羊抗鼠 ET-1 (rat endothelin 1, E062-80) ELISA 试剂盒(GBD 公司)和山羊抗鼠 VIII 因子相关抗原(rat associated factor VIII antigen, F-VIIIAg, F059-80) ELISA 试剂盒(GBD 公司), 分别设标准孔、空白对照孔和四个待测样品孔, 取其平均值, 操作按说明书进行。酶标仪 450 nm 波长读取各孔光密度值(*A* 值), 录入数据, 得到标准曲线和各样品的测量值。

1.6 统计学分析

采用 SPSS10.0 统计分析软件, 数据以 $\bar{x}\pm s$ 表示, 采用单因素方差分析, 双侧检验, 检验水准为 $\alpha=0.05$。

2 结果

2.1 诱导分化细胞的形态学变化

SD 大鼠 BM-MSCs 原代培养 3 天后, 细胞开始贴壁。BM-MSCs 细胞呈梭形, 随着培养时间增加, 细胞体积增大, 细胞突起延长。VEGF 诱导培养后, 细胞突起变短, 胞体逐渐缩小, 排列较紧密。诱导培养 14 天, 细胞形态变小, 呈现三角形, 镶嵌排列呈“铺路石”样改变(图 1A)。未经 VEGF 诱导的对照组细胞胞体大、胞浆突起长而不规则, 类似“海星”样(图 1B)。

2.2 诱导形成的内皮细胞鉴定

2.2.1 *免疫荧光标记* 诱导形成的内皮细胞胞浆对 FITC-CD34 抗体呈现绿色荧光反应(图 2A), 荧光标记的阳性细胞占 90% 以上; 对照组细胞对 FITC-CD34 抗体反应阴性(图 2B)。

2.2.2 *透射电镜观察* 诱导形成的内皮细胞直径约 10~15 μm, 胞质有突起。细胞核/浆比例大, 胞质中含有溶酶体、质膜小泡(图 3A)、成束的微丝和特征性质膜包裹的短棒状结构——Weibel-Palade 小体(图 3B)。

2.2.3 *免疫细胞化学* 细胞爬片上诱导形成的内

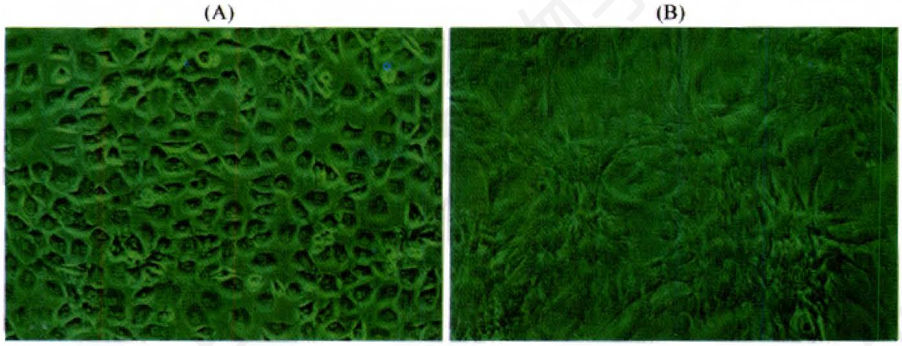

Fig.1 Differentiation of the endothelial cells which derived from BM-MSCs induced by VEGF for 14 d

A: the endothelial cells differentiated from BM-MSCs were cultured in DMEM with VEGF medium for 14 d (induced group, 200×); B: BM-MSCs were cultured in DMEM without VEGF medium (control group, 200×).

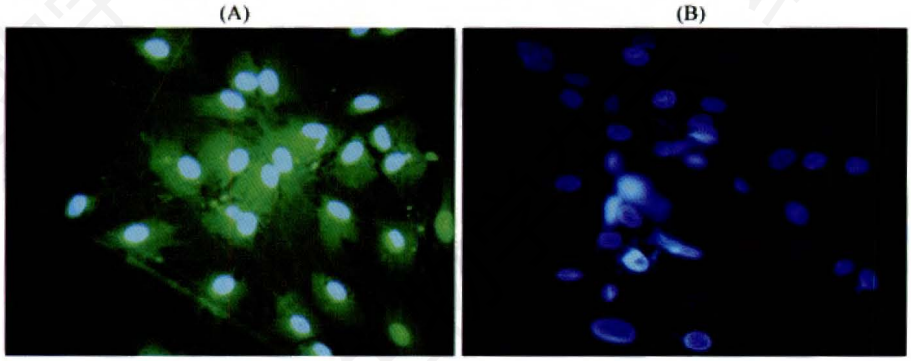

Fig.2 The endothelial cells identificated by FITC-CD34 green fluorescence

A: the endothelial cells which were differentiated from BM-MSCs induced by VEGF showed green fluorescence in the cytoplasms (induced group, 400×); B: BM-MSCs cultured in DMEM without VEGF medium did not show green fluorescence (control group, 400×).

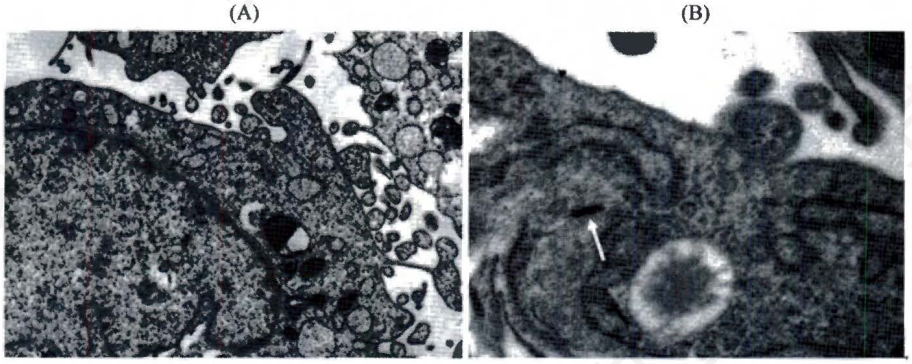

Fig.3 Observation by transmission electron microscope (TEM) of the endothelial cells which were differentiated from BM-MSCs induced by VEGF

A: the endothelial cell which were differentiated from BM-MSCs induced by VEGF showed cytoplasmic process and plasmalemmal vesiculae in the cytoplasm (12 000×); B: the endothelial cell which were differentiated from BM-MSCs induced by VEGF showed Weibel-Palade (W-P) body in the cytoplasm (arrow) (38 000×)

皮细胞呈三角形或多边形，细胞分散或连接成小片状。细胞核圆形，细胞胞浆呈现抗 CD31、CD34 阳性的棕黄色颗粒(图 4A、图 4B)；细胞爬片上对照组细胞呈多边形，分散排列，细胞胞浆对抗 CD31、CD34 反应阴性(图 4C、图 4D)。

2.2.4 免疫组织化学 在 ADM 支架组织上生长 7 天的内皮细胞呈圆形或椭圆形，核大，胞浆丰富，细胞对抗 CD31、CD34 抗体表达阳性，胞浆内可见棕

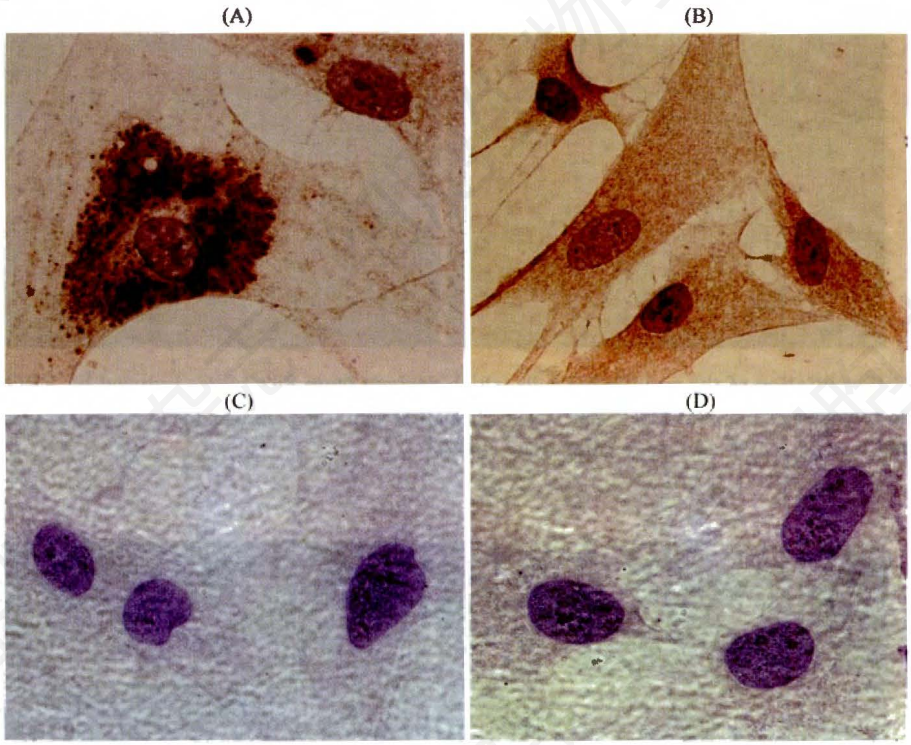

Fig.4 Observation of immunocytochemistry against CD31 and CD34 of the endothelial cells induced by VEGF

A, B: the endothelial cells which were differentiated from BM-MSCs induced by VEGF showed positive for CD31 (A, 1 000×) and CD34 (B, 1 000×); C, D: BM-MSCs of the control group cultured in DMEM without VEGF medium showed negative for CD31 (C, 1 000×) and CD34 (D, 1 000×).

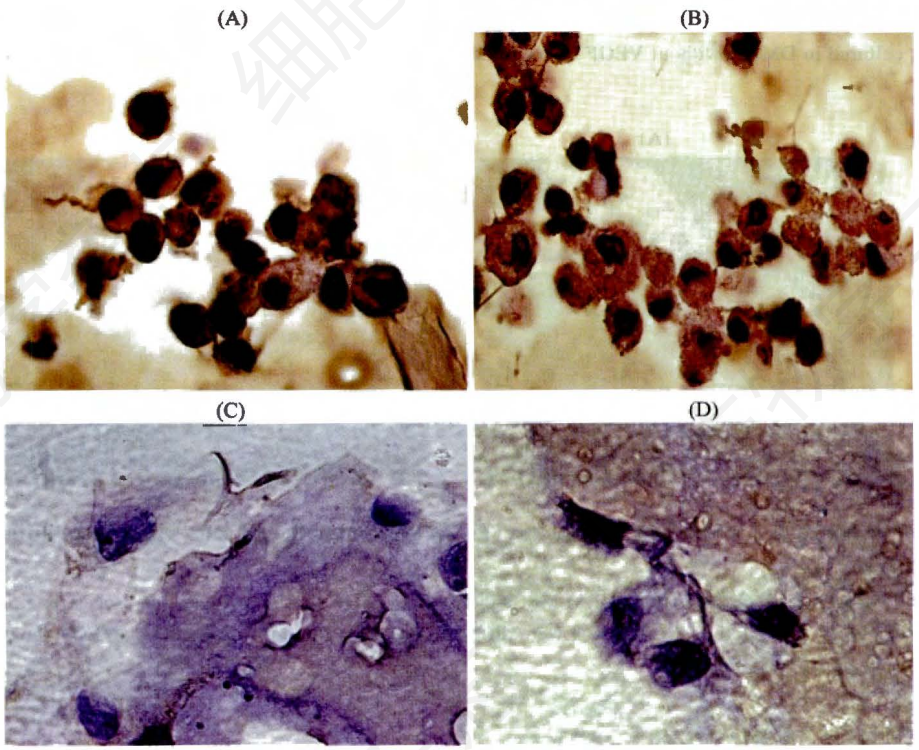

Fig.5 Observation of immunohistochemistry against CD31 and CD34 of the endothelial cells cultured on ADM scaffold for 7 d

A, B: the endothelial cells which were differentiated from BM-MSCs induced by VEGF and cultured on ADM scaffold for 7 d showed positive for CD31 (A, 1 000×) and CD34 (B, 1 000×); C, D: BM-MSCs of the control group cultured in DMEM without VEGF medium on ADM scaffold for 7 d showed negative for CD31 (C, 1 000×) and CD34 (D, 1 000×).

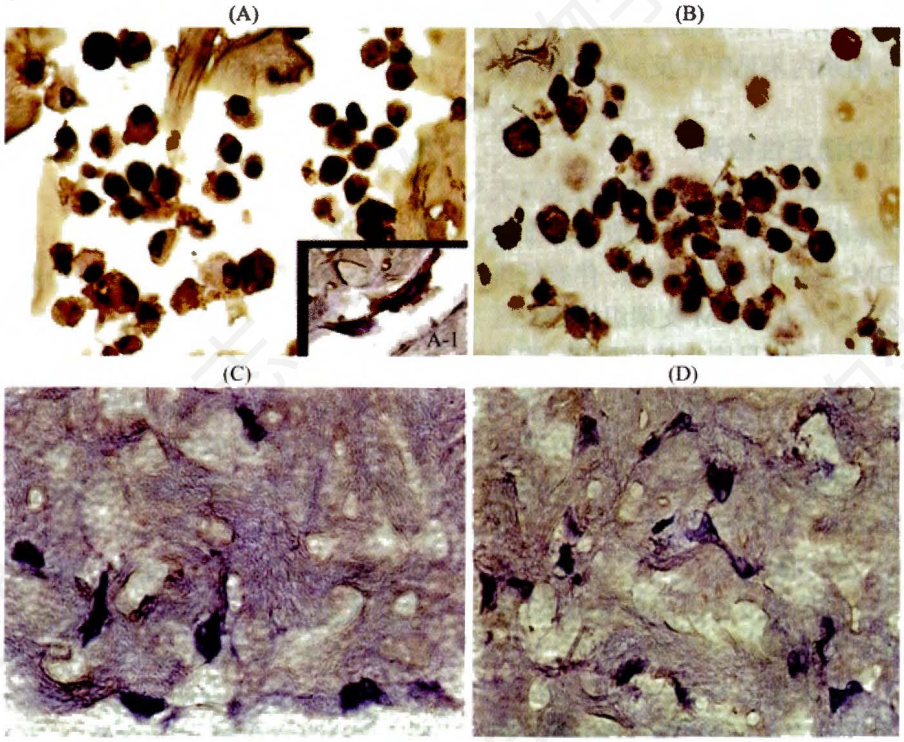

Fig.6　Observation of immunohistochemistry against CD31 and CD34 of the endothelial cells differentiated from BM-MSCs on ADM scaffold *in vivo* for 7 d and 14 d

A: the endothelial cells which were differentiated from BM-MSCs survived for 7 d on ADM scaffold *in vivo* and showed positive for CD31 (A, 1 000×), positive for CD34 (A-1, 1 000×); B: the endothelial cells which were differentiated from BM-MSCs survived for 14 d on ADM scaffold *in vivo* and showed positive for CD34 (1 000×); C: BM-MSCs of the control group on ADM scaffold *in vivo* for 7 d showed negative for CD31 (1 000×); D: BM-MSCs of the control group on ADM scaffold *in vivo* for 14 d showed negative for CD34 (1 000×).

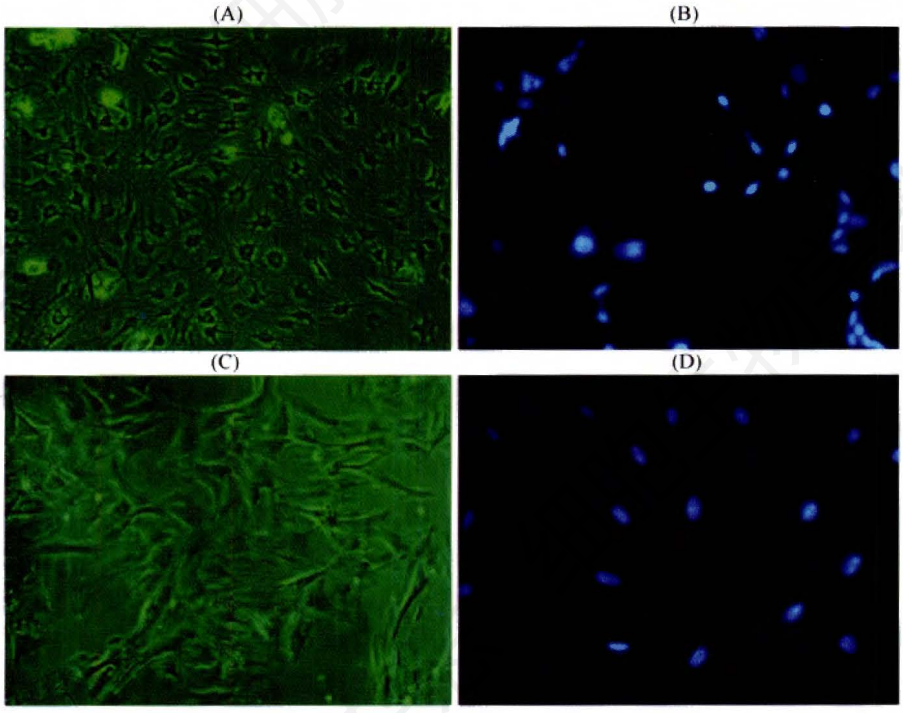

Fig.7　The endothelial cells cultured in the medium without VEGF *in vitro* for 14 d

A, B: the endothelial cells differentiated from BM-MSCs were cultured in EGM-2 medium (EGM-2 group) for 14 d (A, 200×) and did not show FITC-CD34 green fluorescence (B, 400×); C, D: the endothelial cells differentiated from BM-MSCs were cultured in DMEM without VEGF medium (DMEM group) for 14 d (C, 200×) and did not show FITC-CD34 green fluorescence (D, 400×).

黄色颗粒(图 5A、图 5B); 对照组细胞呈多边形, 不表达 CD31、CD34, 胞浆内未见阳性反应(图 5C、图 5D)。

2.3 内皮细胞表型的稳定性检测

2.3.1 体内实验　在SD大鼠肌肉组织内生长7天的ADM支架组织周缘出现少量蚕食状吸收和纤维组织增生, 14 天时 ADM 支架周边吸收明显, 伴有纤维组织包裹。在 7 天与 14 天的 ADM 支架组织中央区域, 内皮细胞清晰可辨, 细胞呈圆形或椭圆形, 胞浆丰富, 对抗 CD31 和 CD34 反应阳性(图 6A、图 6B)。衬里在 ADM 支架裂隙边缘的内皮细胞呈梭形, 平行排列(图 6A-1)。对照组细胞呈椭圆形、三角形或梭形, 细胞对抗 CD31 和 CD34 反应阴性, 胞浆内未见棕黄色颗粒(图 6C、图 6D)。

2.3.2 体外实验　诱导形成的内皮细胞在 EGM-2 培养液中培养 14 天后, 细胞排列疏松, 有多个细长突起, 类似“蜘蛛”样, 细胞对 FITC-CD34 反应阴性(图 7A、图 7B); 在不含 VEGF 的低糖 DMEM 培养液中培养 14 天后, 细胞形态变为多边形多突起, 类似未诱导的对照组“海星”样细胞, FITC-CD34 标记未见绿色荧光(图 7C、图 7D)。继续培养 14 天的诱导组与对照组, 细胞形态及 FITC-CD34 标记没有发生变化。

2.3.3 ELISA 检测　结果显示, 诱导组细胞上清液中 ET-1 含量(0.86±0.02) ng/ml 高于 DMEM 组(0.42±0.12) ng/ml、EGM-2 组(0.25±0.06) ng/ml 和对照组(0.29±0.06) ng/ml (P<0.05); 而 DMEM 组和 EGM-2 组与对照组比较, 差别无显著性(P>0.05)。诱导组细胞上清液中 F-VIIIAg 含量(0.74±0.1) ng/ml 高于 DMEM 组(0.48±0.12) ng/ml、EGM-2 组(0.50±0.08) ng/ml 和对照组(0.36±0.05) ng/ml (P<0.05)。DMEM 组和 EGM-2 组与对照组比较, 差别无显著性(P>0.05) (图 8)。

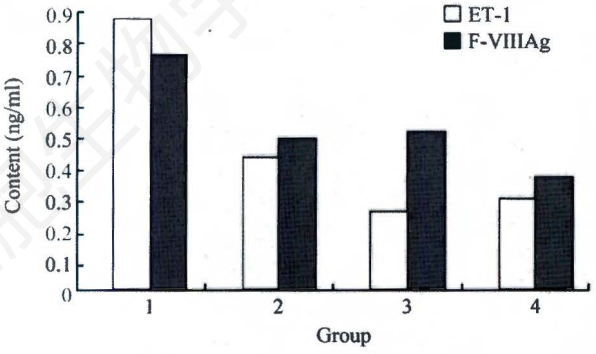

Fig.8 The contents of ET-1 and F-VIIIAg in the different groups detected by ELISA

1: induced group, the endothelial cells differentiated from BM-MSCs were cultured in DMEM with VEGF medium; 2: DMEM group, the endothelial cells were cultured in DMEM without VEGF medium; 3: EGM-2 group, the endothelial cells were cultured in EGM-2 medium; 4: control group, BM-MSCs were cultured in DMEM without VEGF.

3 讨论

VEGF 是血管细胞迁移和增殖的重要刺激因子。在机体内, BM-MSCs 虽不表达 VEGF 受体, 但 VEGF 可刺激血小板来源的生长因子(platelet-derived growth factor receptors, PDGFRs)调节 BM-MSCs 增殖, 通过 VEGF/PDGFRs 信号通路, 在疾病和组织再生过程中调节血管细胞的修复和增殖[7]。在糖尿病多神经病(diabetic polyneuropathy, DPN)大鼠肌肉内移植 BM-MSCs, 放入 VEGF, 能明显改善移植部位的毛细血管数量与肌纤维的比率[8]。本研究采用 VEGF 诱导大鼠 BM-MSCs 分化形成内皮细胞, 使其具有典型的内皮细胞超微结构和免疫细胞化学特征。在诱导完成后, 内皮细胞分别培养于不含 VEGF 的 DMEM 培养基和内皮细胞培养基 EGM-2 中 14 天, 细胞逐渐失去内皮细胞特征, 恢复到未诱导(对照组)的细胞形态和免疫细胞化学特征, 对内皮细胞荧光标记物 FITC-CD34 反应阴性。ET-1 与 F-VIIIAg 的含量测定表明, DMEM 组与 EGM-2 组明显低于诱导组, 而与对照组差别无显著性。表明 VEGF 诱导形成的内皮细胞在体外培养, 其表型的稳定性有赖于 VEGF 的维持。

BM-MSCs 能生长在三维的透明质酸韧带支架上[9]或 PDO/PVA [polydioxanone/poly (vinyl alcohol)] 支架上, 形成关节软骨样组织[10]。也可生长在三维管状支架上, 诱导形成毛细血管样结构或平滑肌细胞[11]。本研究将诱导形成的内皮细胞接种到ADM三维支架上, 在体外含有VEGF的DMEM培养基中培养7天, 细胞生长良好, 并稳定表达 CD31 和 CD34。Chen 等[12]比较骨髓与脐带的 MSCs, 在 Matrigel 共培养的血管生成实验中均能诱导分化为内皮细胞, 形成毛细血管网。然而, 体外诱导分化的细胞能否在体内环境中生长和长期生存是组织工程种子细胞研究所关注的焦点。De Bari 等[13]研究表明, 在体外特殊情况下诱导的滑膜来源 MSCs 具有软骨细胞样表型, 但在体内不能形成异位软骨。Mirza 等[14]将 BM-MSCs 种植到聚氨酯补片(polyurethane patch)上, 在大鼠体内生长两周, 发现 BM-MSCs 在补片上伸展开来, 形成类似主动脉壁中层的结构, 并表达平滑肌蛋白, 因此认为体内环境能诱导平滑肌蛋白成熟。诱导形成的内皮

细胞在体内能否长时间生存？目前文献报道较少。本研究将SD大鼠BM-MSCs诱导形成的内皮细胞，接种到三维的ADM支架上，移植回SD大鼠肌肉组织内，避免了诱导细胞与机体组织之间的免疫排斥反应。本研究发现，诱导形成的内皮细胞在失去VEGF诱导的体内环境中生长14天，细胞存活下来，并保留其形态特征和免疫细胞化学特性，对内皮细胞标记物CD31和CD34均反应阳性。证实体内环境能够适应诱导形成的内皮细胞较长时间生存，这为组织工程化血管内皮种子细胞的选定提供了实验室数据。Lozito等[15,16]研究发现，内皮细胞产生的内皮基质能诱导BM-MSCs分化成为内皮细胞，不需要VEGF或IV型胶原及纤维结合素(fibronectin)的存在。此外，血管紧张素II (AngII)能通过细胞外信号调节激酶1/2 (extracellular signal-regulated kinase 1/2, ERK1/2)等途径刺激BM-MSCs合成VEGF[17]。在机体内，卵巢、睾丸、肾上腺、胰腺和胎盘等器官的细胞均可以产生和表达VEGF[18~20]，机体内环境的体液中自然存在生理性的VEGF，能够为细胞生存提供必需条件。使得在体外诱导形成的内皮细胞，能够在失去VEGF诱导的体内环境中存活下来，并保留其特征。而在体外培养基中生长的诱导形成的内皮细胞，由于没有内源性VEGF的提供，在失去培养基中的VEGF之后，其内皮细胞特征便难以维持下去。

综上所述，本研究探讨BM-MSCs诱导形成的内皮细胞，在体内外去诱导的环境中，其表型的稳定性。结果表明，诱导形成的内皮细胞在体内环境中能够生存下来，保持其表型的稳定性；而在体外培养基中生长，有赖于VEGF的维持。

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Phenotypical Stability of Endothelial Cells Differentiated from Bone Marrow Mesenchymal Stem Cell *in Vitro* and *in Vivo*

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Abstract The endothelial cells which were differentiated from bone marrow mesenchymal stem cells (BM-MSCs) of Sprague-Dawley (SD) rats induced by vascular endothelial growth factor (VEGF) (induced group) were identified by fluorescein isothiocyanate (FITC) labelled CD34 (FITC-CD34), and assayed by immunocytochemistry against CD31 and CD34. The ultrastructures of the endothelial cells were observed by transmission electron microscope (TEM). After then the endothelial cells were seeded onto a three-dimensional acellular dermal matrix (ADM) scaffold, and transplanted into skeletal muscles of SD rats *in vivo* for 7 d and 14 d. DMEM without VEGF medium (DMEM group) and EGM-2 medium (EGM-2 group) was used respectively to culture the endothelial cells for 14 d *in vitro*, and the contents of both endothelin-1 (ET-1) and VIII factor related antigen (F-VIIIAg) in different groups were detected by the method of enzyme-linked immunosorbent assay (ELISA). The results showed that after being induced by VEGF for 14 d, BM-MSCs were arranged like "paver", which showed green fluorescence of TITC-CD34 and were positive immunocytochemical reactions for CD31 and CD34. TEM observation found that there was typical Weibel-Palade body in the cytoplasm of these cells showed the endothelial features. The endothelial cells survived and kept the endothelial features *in vivo* of SD rat for 7 d and 14 d after transplantation, these cells were positive reactions for CD31 and CD34. In contrast, after being cultured respectively in DMEM without VEGF medium and EGM-2 medium for 14 d, the endothelial cells did not show green fluorescence of TITC-CD34. ELISA results displayed the significantly lower contents of both ET-1 and F-VIIIAg of DMEM group and EGM-2 group than those of induced group ($P<0.05$). This study demonstrated that the endothelial cells which derived from BM-MSCs induced by VEGF could keep the phenotypical stability *in vivo*, but could not maintain the stability in the culture medium without VEGF.

Key words bone marrow mesenchymal stem cell; endothelial cell; inducement; vascular endothelial growth factor

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