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小径聚氨酯人工血管内皮化种子细胞的体外诱导分化及种植

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摘 要:【目 的】探讨体外诱导骨髓单个核细胞分化成为内皮祖细胞,为小径聚氨酯人工血管内皮化提供合适的种子细胞的可行性。【方法】收集健康成人骨髓单个核细胞,置于纤维连接蛋白预衬的DMEM培养基中,用血管内皮生长因子(vascular endothelial growth factor, VEGF)和碱性成纤维细胞生长因子(basic fibroblast growth factor, bFGF)加以诱导,通过荧光显微镜和免疫组化分析等方法观察和鉴定诱导后的细胞。将诱导分化的内皮祖细胞种植到聚氨酯小径人工血管表面,用扫描电镜观察。【结果】在VEGF、bFGF等诱导因子存在的条件下,骨髓单个核细胞分化成为内皮祖细胞,倒置荧光显微镜下呈典形的"纺锤样"梭形细胞,单层细胞贴壁生长融合时呈铺路石样排列,免疫组化示VWF和CD34抗体染色阳性。扫描电镜下,未种植细胞的聚氨酯小径人工血管表面呈典型的多孔蜂窝状结构,孔径大小比较适合内皮祖细胞爬行。种植细胞后,聚氨酯小径人工血管表面有大量的内皮祖细胞黏附、爬行及铺展生长,有时可见内皮祖细胞长入蜂窝状孔径内。【结论】体外诱导骨髓单个核细胞分化为内皮祖细胞可作为小径聚氨酯人工血管内皮化的种子细胞。

关键词: 聚氨酯; 人工血管; 内皮祖细胞; 内皮化; 血管组织工程
中图分类号:Q819;R541 **文献标识码**:A **文章编号**:1672-3554(2005)02-0138-04

In Vitro Induction, Differentiation, and Seeding of Source Cells for Endothelialization of Polyurethane Small Diameter Artificial Blood Vessel

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Abstract【Objective】The study was designed to induce bone-marrow mononuclear cells (MNCS) differentiating into endothelial progenitor cells (EPCs) *in vitro* and to seed them on polyurethane small diameter artificial blood vessel, in order to provide source cells for the endothelization of small diameter artificial vessel. 【Methods】The bone marrow mononuclear cells of healthy adult were acquired and put in DMEM culture which was coated with fibronectin, and induced by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The differentiated endothelial cells were observed and identified by fluorescent microscope and immunohistochemical analysis, seeded on the polyurethane small-diameter artificial vessels, and observed by scanning electronic microscope. 【Results】Under the induction factors such as VEGF and bFGF, bone marrow mononuclear cells differentiated into EPCs. They presented typical "spindle-shaped" appearance and formed a monolayer that arrayed in "cobblestone-like" morphology. Identifications of immunohistochemical analysis indicated that they were positively stained for von Willebrand factor (VWF) and CD34 antigen. The unseeded polyurethane small-diameter artificial vessel showed polyporous honeycomb structure under observation of scanning electronic microscope. The size of the hole suited the crawling of EPCs. After being seeded with the EPCs, the cells showed adhesion, crawling, and spreading on the polyurethane vessel surface. Some cells grew into the honeycomb-like

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holes.【Conclusion】The present study demonstrates that the EPCs may be used as source cells for the endothelization of small diameter artificial vessels.

Key words: polyurethane; artificial blood vessel; endothelial progenitor cells; endothelialization; vascular tissue engineering

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动脉粥样硬化所造成的冠状动脉和外周血管疾病是危害人类健康的主要原因，虽然球囊扩张和支架置入术能部分代替冠脉搭桥术，血管移植仍然是治疗晚期血管疾病的主要措施之一。人工血管作为严重狭窄或闭塞性血管的替代物，在临床上有重要的应用价值和前景。近年来许多研究表明[1,2],骨髓、外周血和脐血中存在能分化为内皮细胞并参与血管新生的内皮祖细胞(endothelial progenitor cells, EPCs)，具有高度的分化增殖活性，在血管再生及各种心脑血管疾病治疗中有着广泛的应用。同时,多孔状聚氨酯(polyurethane, PU)人工血管支架则具有良好的生物相容性和血液相容性,可有效抗血栓形成。本实验探讨体外诱导骨髓单个核细胞分化成为内皮祖细胞的可行性,并种植聚氨酯小径人工血管表面,旨在尝试为小径人工血管的研究提供合适的种子细胞及血管支架材料。

1　资料与方法

1.1　骨髓单个核细胞分离培养

取健康成人骨髓 (n=7) 约 10 mL 置于肝素管中,并以 PBS 稀释。加入 Ficoll-Pague 分离液后离心，小心提取血清和 Ficoll 分离液交界处的单个核细胞层，置于含 200 mL/L 胎牛血清(Invitrogen 公司),VEGF 10 ng/mL (Peprotech 公司),bFGF 2 ng/mL (Peprotech 公司),L-glutamine 2 mmol/L,Na-plyviate 1 mmol/L,且以纤维连接蛋白(Hematologic Technologies 公司)预衬的 DMEM 培养基(Invitrogen 公司)中。将上述含有骨髓单个核细胞的培养基置于含有体积分数 5 %CO_2、37℃孵箱中培养，在倒置荧光显微镜下动态观察细胞形态学变化。

1.2　免疫组织化学

将贴壁细胞胰酶消化后放于小的载玻片上爬行，待铺满后置于 40 mg/L 多聚甲醛中固定 2 h，烘箱内烧干过夜后用 ABC 抗体法检测贴壁细胞的表面特异性抗原 VWF 及 CD34。

1.3　小径微孔聚氨酯人工血管的制备

采用浸渍-沥滤法,制得两条小径聚氨酯微孔人工血管。

1.4　扫描电镜

将贴壁细胞胰酶消化后置于 6 孔细胞培养板中,内置已用酒精消毒好的聚氨酯小径人工血管。定期在倒置荧光显微镜下动态观察细胞形态学变化,并于扫描电镜(日本 Hitachi 公司)下拍片,对 PU 人工血管表面以及黏附内皮祖细胞表面进行动态扫描，观察 PU 人工血管表面蜂窝状结构和内皮祖细胞爬行形态。

2　结　果

2.1　细胞贴壁及生长情况

骨髓单个核细胞在 DMEM 培养基中培养约 1 d 左右,大量小圆形的单个核细胞开始粘壁,并有部分集簇形成。约 6 d 后开始出现"纺锤样"结构的梭形细胞，并逐渐爬行生长,20 d 左右单层贴壁细胞融合形成内皮细胞典型的"铺路石样"排列(图 1)。

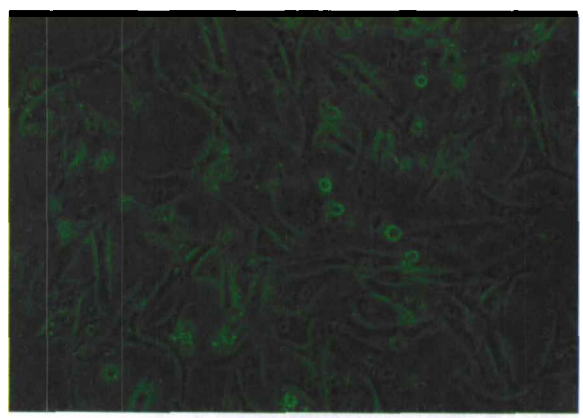

图 1　骨髓单个核细胞诱导分化培养 3 周

Fig.1 The bone marrow mononuclear cells were cultured for 3 weeks(×400)

2.2　免疫组化结果

贴壁细胞 VWF 抗体免疫组化示阳性，其中，浅黄色颗粒以胞浆多见,胞核未见分布(图 2 A),

而 CD34 抗原免疫组化示阳性,胞浆有多量分布,胞核无明显分布(图 3 A),阴性对照则未加抗体(图 2 B,3 B)。

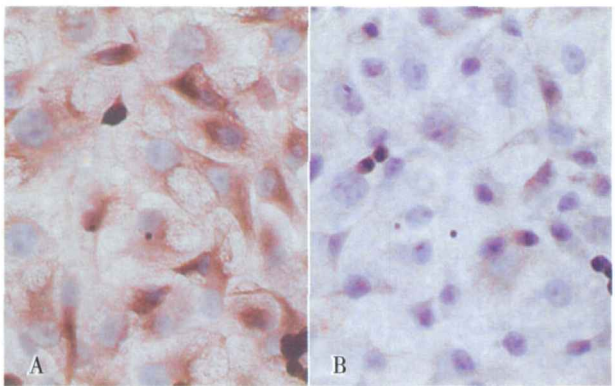

图 2 贴壁细胞 VWF 抗体免疫组化

Fig.2 The immunohistochemical analysis of VWF antigen of adhesive cells(×400)

A: The result was positive; B: Negative control

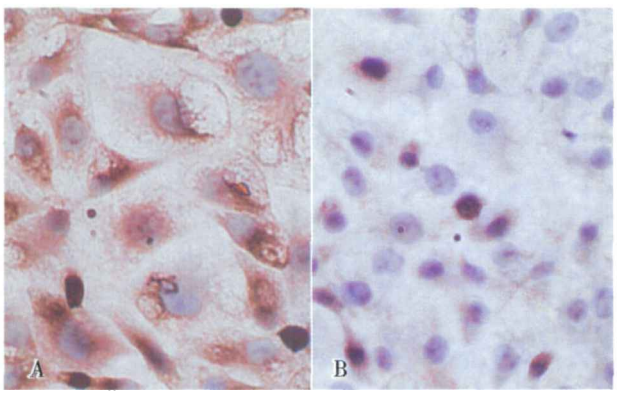

图 3 贴壁细胞 CD34 抗体免疫组化

Fig.3 The immunohistochemical analysis of CD34 antigen of adhesive cells (×400)

A:The result was positive; B:Negative control

2.3 小径微孔聚氨酯人工血管制备后的鉴定

两条小径微孔聚氨酯人工血管的长度为 6~8 cm 不等,内径 4 mm,外径 6 mm,扫描电镜下内腔表面以及断面都呈现多孔蜂窝状结构,孔的分布均匀,孔径在 60~150 μm 范围(图 4)。顺应性分别为 6.38 %/100 mmHg 和 7.39 %/100 mmHg,已接近犬的天然颈动脉的顺应性 7.5 %/100 mmHg。

2.4 扫描电镜观察

种植细胞 4 d 后,聚氨酯小径人工血管表面有大量的内皮祖细胞黏附、生长(图 5),局部可见内皮祖细胞铺展及爬行,并长入蜂窝状孔径内(图 6)。

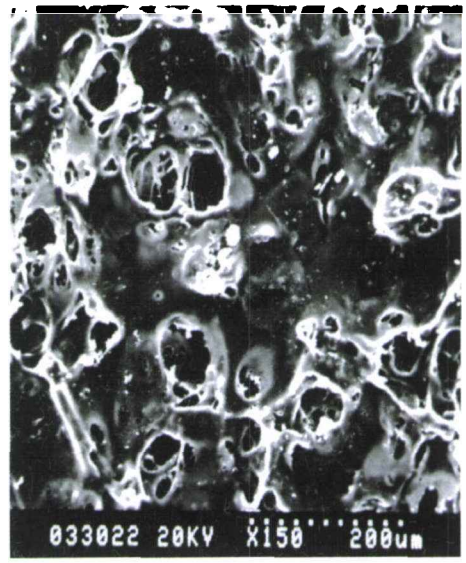

图 4 聚氨酯小径人工血管表面的多孔蜂窝状结构

Fig.4 The polyporous honeycomb structure of polyurethane small-diameter artificial vessel

(scanning electronic microscope, ×150)

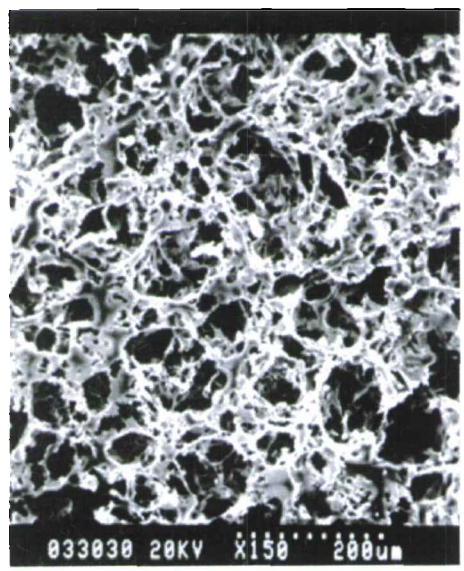

图 5 种植细胞 4 d 后聚氨酯小径人工血管的表面结构

Fig.5 The structure of surface of polyurethane small-diameter artificial vessel after 4 days of seeding cells

(scanning electronic microscope, ×150)

3 讨 论

越来越多研究发现骨髓、外周血和脐血中存在有内皮细胞的前体细胞—内皮祖细胞。内皮祖细胞具有特异性的细胞表面标志,如 $CD34^+$和 VWF 等抗原,可通过免疫组织化学等方法予以鉴定。内皮祖细胞具有分化成为成熟内皮细胞的能

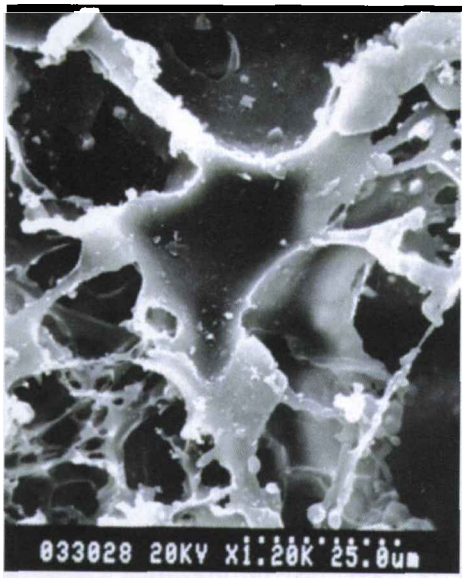

图6　聚氨酯小径人工血管表面内皮祖细胞的铺展

Fig.6　The spreading of endothelial progenitor cells on surface of polyurethane small-diameter artificial vessel

(Observation of scanning electronic microscope, ×1 200)

力，并可参与缺血组织血运重建，有效促进血管新生，增加局部缺血组织血流，是出生后组织血管新生的重要途径[1-4]。骨髓单个核细胞包括骨髓CD34⁺干细胞、间充质干细胞及其他细胞。通过免疫磁珠分离法可将骨髓单个核细胞中的CD34⁺干细胞分离出来，并在特定环境下诱导分化成为内皮祖细胞[5]。用自体内皮祖细胞内皮化的血管移植物进行血管替代手术，可解决传统移植物容易形成血栓、被细菌污染及引起假性内膜增生等问题，同时可避免用异源内皮祖细胞容易引起的免疫排斥反应[6-8]。与成熟的内皮细胞相比，内皮祖细胞具有以下特点：①原位分化增殖能力极强；②黏附、贴壁能力强；③体外扩增培养速度快，且容易获得。因此，内皮祖细胞可替代成熟的内皮细胞作为组织工程小径人工血管内皮化理想的种子细胞来源[9-11]。

本实验通过VEGF、bFGF等刺激因子，可以在体外将骨髓单个核细胞诱导分化成为内皮祖细胞，这与以往报道相似[12,13]。Asahara等[14]报道未经提纯的骨髓单个核细胞的CD34⁺贴壁增殖速度比经免疫磁珠分离后的CD34⁺细胞单独培养快10倍左右，并提出可能CD34⁺与CD34⁻细胞联合培养效果较单纯CD34⁺细胞培养结果为佳。我们认为，与经免疫磁珠分离相比，未经磁珠分离的骨髓单个核细胞诱导分化速度较快，花费成本较低，可作为一种简便易行的内皮祖细胞体外诱导分化途径。

此外，多孔状聚氨酯(polyurethane, PU)人工血管支架可以引起微相分离，其表面微相结构与生物膜相似，具有良好的生物相容性和血液相容性；同时抗张强度好，不易引起血管壁的破裂，机械性能良好。本实验发现，扫描电镜下，未种植细胞的聚氨酯小径人工血管表面呈典型的多孔蜂窝状结构，孔径大小比较适合内皮祖细胞爬行。种植内皮祖细胞后，聚氨酯小径人工血管表面有大量的内皮祖细胞黏附、爬行及铺展生长，有时可见内皮祖细胞长入蜂窝状孔径内，说明诱导分化的内皮祖细胞在聚氨酯小径人工血管上黏附生长状态良好，具有良好的生物相容性，是组织工程小径人工血管内皮化理想的支架。

本研究存在一定的不足之处，第一，采用表面特异性抗原VWF及CD34作为内皮祖细胞的鉴定指标，而未应用内皮祖细胞的最新指标—特异性抗原CD133行进一步的鉴定。这是因为在1997年关于内皮祖细胞的经典实验研究中，Asahara等[14]对单个核细胞来源的内皮祖细胞采用表面特异性抗原VWF及CD34作为鉴定指标，双阳性即为内皮祖细胞，并且用ac-LDL等荧光标记物加以证实。同时，本研究考虑在下一步的实验中进行特异性抗原CD133的鉴定。第二，未做血管移植的动物体内实验，而本研究下一步即将开展体外切应力和动物体内实验。

总之，本实验在体外能将骨髓单个核细胞诱导分化成为内皮祖细胞，而该细胞在小径聚氨酯人工血管上黏附生长状态良好，提示体外诱导骨髓单个核细胞分化为内皮祖细胞可作为小径聚氨酯人工血管内皮化的种子细胞。

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