Caveolin-1对OGD/R人脐带血内皮祖细胞中VEGF表达的影响

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摘要 通过探讨caveolin-1对氧糖剥夺/复氧(oxygen glucose deprivation/reoxygenation, OGD/R)人脐带血内皮祖细胞(endothelial progenitor cells, EPCs)中血管内皮生长因子(vascular endothelial growth factor, VEGF)的影响, 为caveolin-1在脑缺血缺氧性疾病中的应用提供实验依据。采用密度梯度离心法获取脐带血单个核细胞, 接种在鼠尾I型胶原包被的培养板中, 用内皮细胞培养液EGM-2培养, 观察EPCs生长情况, 通过观察细胞形态、双荧光染色法、免疫荧光细胞化学染色法及流式细胞仪等技术对培养的EPCs进行鉴定。选取EPCs高表达的时间点, 构建针对*caveolin-1*基因的RNA干扰真核表达质粒, 建立基因沉默和OGD/R模型, 随机分为正常对照组、OGD/R组、*caveolin-1*基因沉默组、*caveolin-1*基因沉默OGD/R组。免疫荧光法和Western blot检测caveolin-1及VEGF的表达。人脐带血可分离获得内皮祖细胞; 成功构建*caveolin-1*基因沉默模型; caveolin-1及VEGF的表达在*caveolin-1*基因沉默组显著低于正常对照组(P<0.01); OGD/R组caveolin-1及VEGF表达明显高于正常对照组(P<0.01); OGD/R组caveolin-1及VEGF表达明显高于*caveolin-1*基因沉默OGD/R组(P<0.01); *caveolin-1*基因沉默组caveolin-1和VEGF表达量高于*caveolin-1*基因沉默OGD/R组(P<0.05)。以上结果表明, caveolin-1能够影响OGD/R的EPCs中VEGF的表达, 是促进血管细胞损伤修复的可能因素之一。

关键词 caveolin-1; 内皮祖细胞; OGD/R; 基因沉默

Influence of Caveolin-1 on VEGF Expression in OGD/R Human Umbilical Cord Blood Endothelial Progenitor Cells

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Abstract The purpose of this study was to provide experimental basis for the application of caveolin-1 in cerebral hypoxic-ischemic diseases through investigating the influence of caveolin-1 on VEGF expression in OGD/R (oxygen glucose deprivation/reoxygenation) human umbilical cord blood endothelial progenitor cells (EPCs). Density gradient centrifugation was adopted to obtain mononuclear cells from the cord blood, which were then inoculated to the culture plates coated with rat tail collagen-I and cultured using the endothelial cell nutrient solution of EGM-2. The proliferation and growth of EPCs were observed and identified through cellular morphology observation, double fluorescence staining, immunofluorescence cytochemistry staining, flow cytom-

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etry, etc. The time points when EPCs were highly expressed were chosen to establish the *caveolin-1*-targeted RNA interference eukaryotic plasmids and build gene silencing and OGD/R models which were then randomly grouped into the control group, the OGD/R group, the *caveolin-1* gene silencing group and the *caveolin-1* gene silencing OGD/R group. Immunofluorescence and Western blot were adopted to detect the expression of caveolin-1 and VEGF. EPCs could be separated and obtained from human umbilical cord blood; *caveolin-1* gene silencing models were successfully constructed; the expression of caveolin-1 and VEGF in the *caveolin-1* gene silencing group was significantly lower than that in the control group (P<0.01), but higher than that in the *caveolin-1* gene silencing OGD/R group (P<0.05); and the expression of caveolin-1 and VEGF in the OGD/R group was significantly higher than that in the control group and *caveolin-1* gene silencing OGD/R group (P<0.01). The results showed that caveolin-1 could affect the expression of VEGF of OGD/R EPCs, which should be one of favorable factors for vascular cytothesis.

Keywords caveolin-1; endothelial progenitor cells; OGD/R; gene silencing

缺血性脑卒中是由于脑局部供血障碍导致的脑组织缺血缺氧引起的脑组织坏死软化, 从而产生相应的脑功能缺损的临床症状。目前的研究发现, 一些血管生成因子能够通过提高脑血管再生而明显改善缺血性脑卒中的预后, 其中血管内皮生长因子(vascular endothelial growth factor, VEGF)发挥了极为重要的作用[1-2]。Caveolin-1是caveolae的主要结构蛋白, 富含胆固醇和磷脂, 高表达于各类细胞[3-7]。研究显示, caveolin-1参与多种细胞进程, 包括细胞周期调控、信号转导、炎症反应、细胞增殖分化、组织血管再生和组织修复、吞噬以及胆固醇的转运, 同时在肿瘤的进展中也起着重要的作用[7-12]。本课题组的前期动物实验表明, 脑缺血再灌注后脑缺血半暗区caveolin-1和VEGF的表达明显增强, caveolin-1和VEGF的表达呈显著正相关[13-14]。另有研究证实, *caveolin-1*敲除小鼠脑缺血后, 血管功能不能恢复, 对VEGF的刺激无反应, 梗死灶体积增加, 血管再生减弱, 神经功能缺损症状加重, 在这种基因剔除小鼠中导入*caveolin-1*, 上述过程可发生逆转[15-18]。以上研究提示, caveolin-1是VEGF上游的一个关键分子。

本研究在此基础上进一步探讨EPCs体外培养模拟体内缺血再灌注模型即OGD/R模型过程中caveolin-1的变化, 并进一步应用RNA干扰技术下调或沉默体外培养EPCs中caveolin-1的表达后观察VEGF的改变, 借此证实EPCs的增殖生长与caveolin-1的表达强度变化密切相关, 进一步补充和完善caveolin-1对缺血后血管再生的作用机制, 为缺血性脑卒中的临床治疗提供新的实验依据。

1 材料与方法

1.1 材料

1.1.1 细胞来源与培养 无菌条件下采集健康足月新生儿脐带血, 采用密度梯度离心法获取单个核细胞, 接种在鼠尾I型胶原包被的培养板中, 培养液为EGM-2, 培养10~21 d。当出现鹅卵石样细胞集落时, 通过观察细胞形态、双荧光染色法、免疫荧光细胞化学染色法及流式细胞仪等技术成功鉴定内皮祖细胞(endothelial progenitor cells, EPCs)。0.25%胰酶消化细胞进行传代培养, 获取EPCs。

细胞分组: 选取EPCs, 随机分为正常对照组、OGD/R组、*caveolin-1*基因沉默组和*caveolin-1*基因沉默OGD/R组, 共4个小组。

1.1.2 试剂 EGM-2培养基购自美国Lonza公司; 无糖DMEM购自美国Gibco公司; 兔抗caveolin-1抗体购自美国ScienCell公司; 鼠抗VEGF抗体购自英国Abcam公司; FITC荧光标记羊抗兔二抗购自美国Jackson公司(温州长风生物科技公司代理); HRP标记山羊抗小鼠IgG(H+L)、HRP标记山羊抗兔IgG(H+L)购自美国EarthOx公司; RIPA裂解液购自江苏碧云天生物技术研究所; 封闭剂用脱脂奶粉、PVDF膜、Tris、HCl、NaCl、甲醇、甘氨酸、Tuwen 20、底物化学发光ECL、TEMED、SDS、过硫酸铵、丙烯酰胺、预染蛋白Marker均由温州长风生物科技公司代理购买; 慢病毒浓缩液、慢病毒载体、针对*caveolin-1*基因的RNA干扰真核表达质粒等委托英潍捷基(上海)贸易有限公司完成。

1.1.3 仪器 荧光倒置显微镜购自日本Olympus公司; 二氧化碳孵箱购自美国Thermo Forma公司;

FA2104型电子天平购自上海天平仪器厂; 电泳系统: Mini-Proten Tetra System购自美国Bio-Rad公司; 酶标仪(ECX 800)购自BIO-TEK公司; 医学图像分析采用美国Media Cybernetics公司ImagePro Plus 6.0(IPP6.0)系统。

1.2 实验方法

1.2.1 *Caveolin-1*基因沉默模型建立 以*caveolin-1*基因为靶基因, 构建干扰RNA(siRNA)的慢病毒载体, 阳离子脂质体转染EPCs, 沉默*caveolin-1*基因的表达后, 继续培养细胞, 按照上述分组造模。

1.2.2 氧糖剥夺/复氧(OGD/R)模型的建立 选取EPCs高表达时间点, 建立OGD/R模型。先用PBS将培养板中的细胞洗2遍, 加入事先用95% N_2+5% CO_2置换30 min的无糖DMEM, 迅速置于37 ℃、93% N_2+2% O_2+5% CO_2的低氧培养箱中。缺氧缺糖培养8 h后, 按上述分组加入相应培养基, 重新放入37 ℃、5% CO_2的细胞培养箱给予复氧24 h。

1.2.3 免疫荧光染色 PBS浸洗培养板中细胞, 4%多聚甲醛固定10 min, 10%羊血清封闭30 min, 加入工作浓度兔抗caveolin-1一抗, 4 ℃孵育过夜, PBS浸洗, 暗室下加入FITC荧光标记羊抗兔二抗孵育1 h, PBS浸洗, DAPI复染核8 min, PBS浸洗, 加入防荧光淬灭剂, 置于荧光倒置显微镜下观察。同时, 用PBS代替一抗做空白对照以检查免疫反应的特异性, 其余步骤同上。阳性细胞为胞膜胞质出现绿色荧光, 随机选择5个高倍镜下视野(200×), 应用Image-Pro Plus 6.0图像分析软件测定阳性部位的吸光度IOD, 用以代表阳性部位的蛋白表达水平, 计算平均值。IOD值越大, 表示蛋白含量越高。

1.2.4 Western blot检测 提取细胞蛋白、测定含量, 配制上样体系, 蛋白样品变性, –20 ℃保存待用。制备SDS-PAGE胶, 电泳, 电泳转膜, 平衡, 脱脂奶粉封闭2 h, 工作浓度caveolin-1和VEGF一抗孵育16 h, TBST洗涤, HRP标记的羊抗兔、羊抗鼠二抗孵育2 h, TBST洗涤, ECL发光液作用, 曝光、显影。以目的蛋白条带与内参条带IOD之比作为反映蛋白表达水平的相对指标。

1.3 统计学分析

所有计量数据都以均数±标准差(mean±S.D.)表示, 采用SPSS 20.0统计软件对实验数据进行处理。组间比较用*t*检验, 多组间比较用单因素方差(One-Way ANOVA)分析, 两组数据相关性采用Pearson线性相关分析, $P<0.05$为具有显著性差异, $P<0.01$为具有极显著性差异。

2 结果

2.1 基因沉默后EPCs中caveolin-1的表达

Western blot检测caveolin-1的表达, *caveolin-1*基因沉默组对比正常对照组明显下降(图1)。结果表明,

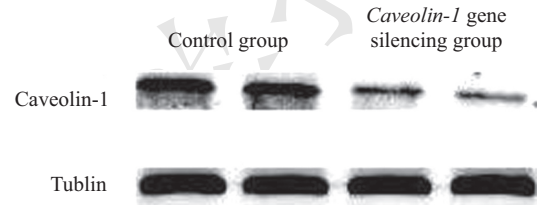
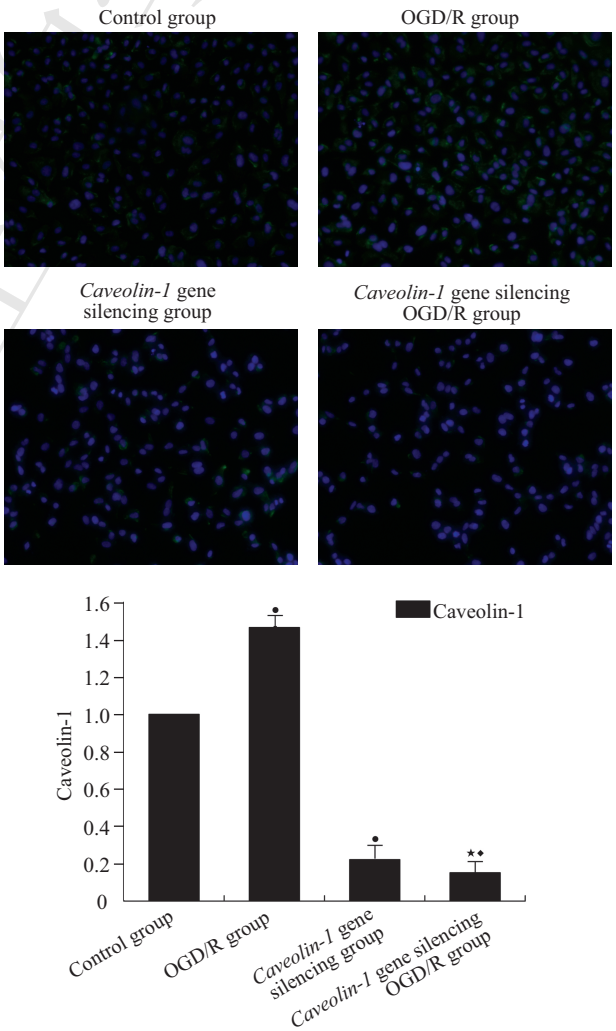


图1 Western blot检测基因沉默后EPCs中caveolin-1的表达

Fig.1 Caveolin-1 in EPCs after gene silencing detected by Western blot

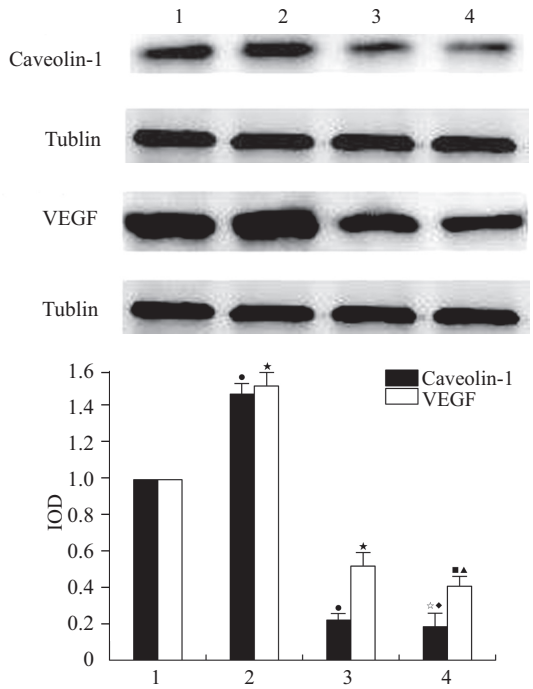


•$P<0.01$, 与正常对照组比较; ★$P<0.01$, 与OGD/R组比较; ◆$P<0.05$, 与*caveolin-1*基因沉默组比较。

•$P<0.01$ *vs* control group; ★$P<0.01$ *vs* OGD/R group; ◆$P<0.05$ *vs caveolin-1* gene silencing group.

图2 各组EPCs中caveolin-1的表达(200×)

Fig.2 The expression of caveolin-1 in each group (200×)



1: 正常对照组; 2: OGD/R组; 3: *caveolin-1*基因沉默组; 4: *caveolin-1*基因沉默OGD/R组。•/★*P*<0.01, 与正常对照组比较; ☆/■*P*<0.01, 与OGD/R组比较; ◆/▲*P*<0.05, 与*caveolin-1*基因沉默组比较。

1: the normal control group; 2: OGD/R group; 3: *caveolin-1* gene silencing group; 4: *caveolin-1* gene silencing and OGD/R group. •/★*P*<0.01 *vs* control group; ☆/■*P*<0.01 *vs* OGD/R group; ◆/▲*P*<0.05 *vs caveolin-1* gene silencing group.

图3 各组EPCs中caveolin-1、VEGF Western blot检测结果

Fig.3 Western blot of caveolin-1 and VEGF in EPCs of each group

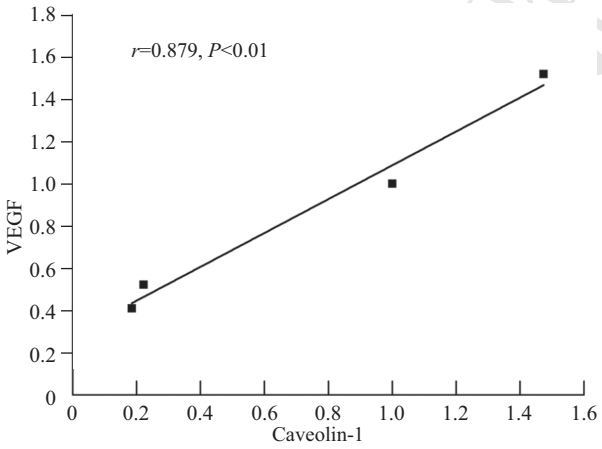


图4 Caveolin-1与VEGF表达的相关性分析

Fig.4 Correlation analysis between expression of caveolin-1 and VEGF

成功构建了*caveolin-1*基因沉默模型。

2.2 OGD/R后EPCs中caveolin-1的免疫荧光染色法检测结果

免疫荧光染色法结果显示, *caveolin-1*基因沉默组、*caveolin-1*基因沉默OGD/R组对比正常对照组细胞数量少, OGD/R组对比正常对照组细胞大量增殖; caveolin-1的表达在*caveolin-1*基因沉默组对比正常对照组明显下降, 有显著性差异(P<0.01); OGD/R组中caveolin-1表达对比正常对照组明显升高, 有显著性差异(P<0.01); OGD/R组中caveolin-1表达对比*caveolin-1*基因沉默OGD/R组明显升高, 有显著性差异(P<0.01); *caveolin-1*基因沉默组caveolin-1表达对比*caveolin-1*基因沉默OGD/R组升高, 差异有统计学意义(P<0.05)(图2)。

2.3 Western blot检测OGD/R后EPCs中caveolin-1、VEGF表达及相关性分析

Western blot检测结果显示, caveolin-1的表达在*caveolin-1*基因沉默组对比正常对照组有显著下降, 差异有统计学意义(P<0.01); OGD/R组中caveolin-1表达对比正常对照组明显升高, 有显著性差异(P<0.01); OGD/R组中caveolin-1表达对比*caveolin-1*基因沉默OGD/R组明显升高, 有显著性差异(P<0.01); *caveolin-1*基因沉默组caveolin-1表达对比*caveolin-1*基因沉默OGD/R组升高, 差异有统计学意义(P<0.05), 与免疫荧光染色法检测结果一致(图3); 各组中caveolin-1与VEGF的表达呈显著正相关(r=0.879, P<0.01)(图4)。

3 讨论

Asahara等[19]于1997年首次从人外周血分离培养出内皮细胞的前体细胞, 并将其命名为内皮祖细胞(EPCs)。此后的研究发现, 脐血和骨髓也存在EPCs, 而且含量远多于外周血, 三者比例约为15:10:1[20]。内皮祖细胞是起源于骨髓的原始细胞, 在一定条件下动员到外周血, 具有迁移、增殖和黏附功能, 不仅参与胚胎血管生成, 也参与出生后血管发生与内皮修复; 同时, EPCs也是多种疾病风险、严重程度及预后的指标。本研究采用密度梯度离心法获取单个核细胞, 接种在鼠尾I型胶原包被的培养板中, 用EGM-2专用培养液培养, 10~21 d出现典型的铺路石样形态, 通过观察细胞形态、双荧光染色法、免疫荧光细胞化学染色法及流式细胞仪等成功鉴定获取EPCs。

小窝蛋白(caveolae)是直径为25~100 nm的烧瓶状膜结构蛋白, 1950年首次通过电子显微镜发现[21]。Caveolae存在于不同细胞类型, 尤其在内皮类细胞中表达丰富, 其存在三个成员(caveolin-1、

caveolin-2和caveolin-3), caveolin-1是其主要结构, 分子量为21~24 kDa[22], 它特有的脚手架结构可区域化多种非活性信号分子, 能与各种信号分子如Src家族酪氨酸激酶、生长因子受体、内皮源性一氧化氮合酶(endothelial nitric oxide synthase, eNOS)、G蛋白及G蛋白偶联受体(G protein-coupled receptors, GPCRs)等结合, 在细胞周期调控、信号转导、炎症反应、耐药产生、吞噬以及胆固醇的转运过程中都起着重要作用, 同时在肿瘤的进展与转移中也起着重要作用[23-27]。在中枢神经系统重, 神经元、星形胶质细胞、内皮细胞等均可产生caveolin-1, 病理状态下(如缺氧、缺血)可诱导其表达上调, 但其具体作用机制仍未明。近年的研究结果表明, caveolin-1可通过多种途径参与受损脑组织的修复, 它的表达和生成对保护和修复脑缺血性损伤具有重要的意义。

VEGF是一种重要的血管生成因子, 能通过促进细胞增殖、提高血管再生而明显改善血管损伤及缺血性脑卒中的预后。本课题组的前期研究显示, 在脑缺血再灌注急性损伤及后期康复过程中caveolin-1大量增加, VEGF的表达与其成正相关, 而注射了caveolin-1特异性抑制剂的模型组大鼠脑组织中VEGF的表达量明显受到抑制。Caveolin-1具有多种细胞功能, 参与胆固醇运输和脂类稳定、信号转导、血管生成等, VEGF及其下游分子NO是血管生成的促进剂。有实验表明, 缺血再灌注后, *caveolin-1*基因剔除小鼠的血管功能不能恢复, 对VEGF的刺激无反应, 且在这种基因剔除小鼠中导入*caveolin-1*, 上述过程可发生逆转[28], 因此, caveolin-1是血管生成关键的作用靶点, 是VEGF上游的关键分子。

本实验采用体外培养EPCs方法模拟体内缺血缺氧模型, 严格控制培养箱氧气浓度(1% O_2、5% CO_2和94% N_2)及细胞培养过程中的无菌操作, 通过RNA干扰基因沉默技术成功构建*caveolin-1*基因沉默模型, 沉默率85%±3.4%。选取EPCs高表达时间点, 随机按实验要求分为正常对照组、OGD/R组、*caveolin-1*基因沉默组和*caveolin-1*基因沉默OGD/R组, 置于相应培养箱中8 h后更换培养基, 放入正常培养箱中继续培养24 h。免疫荧光染色和Western blot检测各组中caveolin-1和VEGF的表达。OGD/R后caveolin-1及VEGF上升, 而基因沉默OGD/R中, VEGF无上升, 细胞损伤严重, 两种方法检测结果一致。实验结果证明, caveolin-1能够影响VEGF的表达, 是促进血管细胞损伤修复的可能因素之一。

本研究仅仅从caveolin-1/VEGF方面比较了OGD/R后*caveolin-1*基因沉默组与正常组间的差异, 其作用机制复杂, 涉及多种信号分子及细胞因子的参与。Caveolin-1的相互作用蛋白即内皮型一氧化氮合酶(endothelial nitric oxide synthase, eNOS)被证明在血管内皮生长因子诱导的血管生成起主导作用。近年来的研究证实, caveolin-1可以通过G蛋白介导、PKC及酪氨酸激酶的级联反应等信号转导系统对EPCs的功能进行调控。另有研究显示, 通过药物干预或剔除*caveolin-1*可使PI3K表达下降, 活化的PI3K可以激活其下游的信号转导通路PI3K/丝氨酸、苏氨酸蛋白激酶(Akt)/eNOS, 而该信号通路下游eNOS又直接影响到EPC的动员、增殖和分化, 对EPC的动员、迁徙及VEGF介导的血管生成功能都有非常重要的调节作用[29]。

目前研究尚无法确定caveolin-1/VEGF通过哪种信号通路对EPCs进行调控、调控有没有时间或是剂量依赖性以及是否可以通过提高caveolin-1的表达改善EPCs的功能及提高VEGF表达促进血管细胞损伤修复, 这些都有待于进一步的研究。

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