

研究报告

对去血清后 HITASY 细胞分子表达及表型分析*

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摘要 以人血管平滑肌细胞克隆株 HITASY 为实验材料, 探讨 HITASY 细胞分子表达与表型转换间的关系, 为阐明血管新生内膜形成及再狭窄病理机制提供实验依据. 实验表明, 在含血清或去血清培养条件下, 平滑肌细胞于体外发生表型转换. 去血清后细胞外基质蛋白合成中止, 增殖及移行能力趋于降低, 细胞特异性标志物平滑肌 α 肌动蛋白、肌球蛋白重链及钙调结合蛋白等的表达随去血清时间延长而增加. 进一步实验证实, 在血管活性介质作用下, 胞液钙离子浓度骤增产生膜信号级联反应, 引发细胞面积减小而显示收缩功能. 上述处于分化表型的细胞经补加血清后其表型特征又恢复到原有去分化型, 提示体外培养人平滑肌细胞可发生表型转换. 为验证去血清诱导表型转换过程中相关基因的表达变化, 用差异显示 PCR 筛选出 E1A 激活基因阻遏子, 在细胞处于分化表型时表达上调并对细胞增殖伴有较强抑制作用.

关键词 平滑肌, 血管, 表型, 细胞分化, 收缩

学科分类号 Q555.7, R34

血管损伤后平滑肌细胞 (SMCs) 由分化表型转变为去分化表型的过程, 是导致细胞增殖和迁移的起始步骤, 也是增生性血管疾病的共同病理基础^[1]. 近年研究发现, 分化的血管 SMCs 仍维持高度可塑性, 通过表型转换的细胞, 其形态与功能亦发生相应改变^[2]. 已有报道, 体外培养的血管 SMCs 表型转换过程中分别表达特异性收缩蛋白及其相关基因^[3], 但体外显示细胞表型与功能转换的直接证据却知之甚少. 我们以人胸廓内动脉平滑肌 HITASY 克隆细胞系作为研究表型转换机制的细胞模型, 分别以平滑肌特异性收缩蛋白、收缩调节蛋白以及 SM- α 22 基因表达为 SMCs 表型转换标志, 探讨了体外去血清诱导 HITASY 表型转换过程中细胞标志特征与细胞收缩功能的改变, 并对细胞去分化过程中与增殖相关的基因表达进行了初步筛选, 进而为阐明病理性血管新生内膜的形成和再狭窄的发生机制及其防治提供实验依据.

1 材料与方法

1.1 材料

HITASY 克隆细胞系由本室保存. 差异显示 PCR 分析采用 Clontech 公司 Delta Differential Display Kit (K1810-1). 反转录 PCR 采用 TaKaRa 公司的 RNA PCR Kit (DR019A). 蛋白质印迹化学发光试剂 (ECL, RPN2106) 和辣根过氧化物酶标记第二抗体购自 Amersham 公司. 平滑肌 α -肌动蛋白

(SM α -actin) 单抗、肌钙结合蛋白 (calponin) 单抗、钙调结合蛋白 (H-caldesmon) 单抗、Fura-2 购自 Sigma 公司. 平滑肌肌球蛋白重链 (SMMH) 单抗购自 Santa Cruz Biotechnologies 公司. FITC 荧光标记第二抗体购自 Jackson Immunoresearch Laboratories 公司. Hoechst 33258 购自 Molecular Probes 公司. 血小板源性生长因子 (PDGF-BB)、M199 和胎牛血清 (FBS) 购自 GIBCO BRL 公司. ³H-亮氨酸 (NET004001MC) 及 ³³P-dATP (NEG-312H) 购自 NEN 公司.

1.2 方法

1.2.1 免疫荧光染色: 将细胞培养在清洁灭菌的盖玻片上, 用 4% 多聚甲醛固定 15 min, PBS 洗 3 次, 再用冷甲醇: 丙酮 (体积比 1:1) 固定通透 15 s, 经 PBS 缓冲液洗涤 3 次后, 用 5% 山羊血清封闭 30 min. 加 α -actin 抗体室温孵育 2 h, 随后用 PBS 缓冲液洗涤 3 次, 每次 5 min, 加入荧光标记第二抗体孵育 1 h, 用 Hoechst 33258 (2 mg/L) 复染细胞核 10 s, 经 PBS 洗涤后甘油封片. 在荧光显微镜 (Olympus IX-70) 下观察、摄像.

1.2.2 基因表达分析: 用 Trizol 一步法提取细胞总 RNA, 采用半定量 RT-PCR 分析 SM- α 22 基因转

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录活性, 引物序列如下: SM- α 22 上游引物 5' CTATGCCTGGTAGATTCTGA, 下游引物 5' CTCT-ACCTTCAAAGAGGTCA; GAPDH 上游引物 5'-TATTGGGCGCCTGGTCACCA, 下游引物 5'-CCACC-TTCTTGATGTCATCA. 以 GAPDH 为内参照, 参考 TaKaRa RNA PCR Kit 说明书实施 RT-PCR, PCR 产物经 1.5% 琼脂糖凝胶电泳, 溴乙锭染色后于 Bio-Rad 2000 图像分析仪扫描分析.

1.2.3 细胞外基质合成分析: 实验分组依培养液组成的不同分为含血清、去血清和恢复血清三个实验组. 指定培养时间 7 天内, 用 ^3H -亮氨酸掺入法测定细胞外基质合成^[4]. HITASY 细胞密度 5 000/孔, 于 24 孔板培养 48 h, 换液, 每孔加 M199 (含 $1.48 \times 10^5 \text{ Bq L}^{-1} [^3\text{H}]$ -亮氨酸) 250 μl , 培养 16 h, 每 3 天换液一次, 补加 $1.48 \times 10^5 \text{ Bq L}^{-1} [^3\text{H}]$ -亮氨酸继续培养 16 h. 将分组细胞经预冷 PBS 洗涤后置含 1 mmol/L PMSF, 10 mg/L 亮抑肽酶和 1 mmol EDTA 的 0.25 mol/L NH_4OH 液中溶解 30 min, 收集上清, 组合所有收集液、洗涤液和溶解液, 经 10% TCA 沉淀后, 离心 12 000 r/min $\times$ 20 min, 50 mmol/L Tris-HCl (pH 7.4, 含 1 mol/L NaCl) 高盐溶液提取沉淀 15 min, 经高盐提取后不溶解的细胞外基质组分溶于 1 mol/L NaOH, 在 4 $^\circ\text{C}$ 加入终浓度 10% TCA 使其沉淀, 乙酸中和后用液体闪烁计数器 (BCS, Amersham) 测量其 cpm 值, 每组实验平行测定三次. 蛋白质浓度采用 Bradford 法测定^[5].

1.2.4 蛋白质印迹分析: 按文献 [6] 实施 SDS-聚丙烯酰胺凝胶电泳 (SDS-PAGE)、转膜、抗体结合及显色. 分离胶浓度分别为: SM α -actin、Calponin 为 10%; H-caldesmon、SMMH 为 6%. 所用第一抗体分别为抗 SM α -actin 单抗, Calponin 和 H-caldesmon 单抗, SMMH 单抗, 经辣根过氧化物

酶标记第二抗体孵育后, 按 ECL 试剂盒说明书进行曝光、显色.

1.2.5 细胞内 Ca^{2+} 浓度测定: 将细胞接种于预先放置盖玻片的细胞培养皿上, 加入荧光负载试剂 Fura-2/AM, 终浓度 0.2 $\mu\text{mol/L}$. 加药方式采用微滴管将 1 $\mu\text{mol/L}$ 组胺加压注射到单细胞表面, 微滴管尖端距细胞 50 μm . 单细胞胞液游离 Ca^{2+} 浓度 ($[\text{Ca}^{2+}]_i$) 的测量以落射荧光为光源, 通过 Delta scan 波长切换器, 在激发波长 345 nm 和 380 nm 交替照射下, 于发射波长 510 nm 处测定 Fura-2 与细胞液 Ca^{2+} 作用后生成的发射光强度. 在减去背景荧光强度后, 细胞 $[\text{Ca}^{2+}]_i$ 由 345 nm 波长下的荧光强度除以 380 nm 波长下的荧光强度计算得出^[7,8].

1.2.6 PCR 差异显示分析: 用 Trizol 提取细胞 RNA, 在变性琼脂糖电泳, 可见清晰 28 S 和 18 S rRNA 区带, 其比率为 2:1, 未见 RNA 降解. 采用 Delta 差异显示试剂盒, 将 RNA 逆转录为 cDNA, 在含 8 mol 尿素的 5% 聚丙烯酰胺变性凝胶上分离 PCR 样品, 转移凝胶 cDNA 到 Whatman 滤纸上, 75 $^\circ\text{C}$ 干胶 40 min, 曝光. 将差异显示的区带切下来, 行 PCR 扩增和序列测定, 将测得的 DNA 序列于 GenBank 进行 Blast 分析比较. 然后实施 RNA 印迹. 按常规方法, 先提取样品 RNA, 行甲醛变性电泳, 随后将 RNA 转移到尼龙膜. 扩增差异表达的 PCR 产物, 经 ^{32}P 标记 dATP 的寡核苷酸标记后与样品 mRNA 杂交, 通过 X-胶片显示 mRNA 分子的大小和灰度.

2 结 果

2.1 去血清培养对细胞增殖与形态的影响

将细胞低密度铺片, 采用免疫荧光染色观察不同血清条件下细胞形态 (图 1). HITASY 在 10%

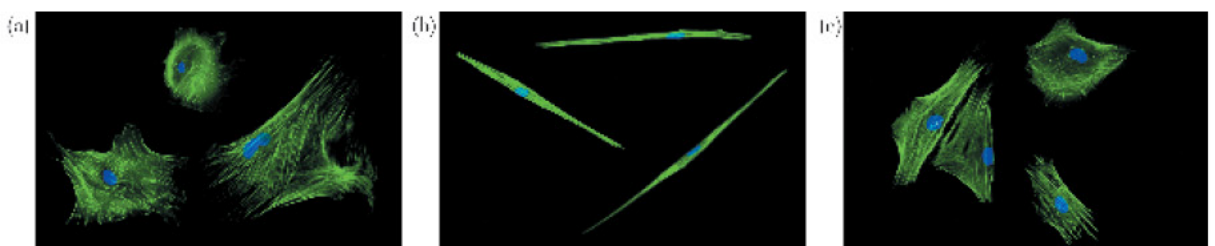


Fig. 1 Morphological features of HITASY cells before and after serum withdrawal

Immunofluorescence images of individual cell in M199 with 10% FBS (a), 7 days after FBS withdrawal (b), and 3 days after readdition of 10% FBS (c). Cells were fixed and stained with secondary antibody-FITC labeled against SM α -actin antibody (green) and Hoechst 33258 (blue) and observed by fluorescence microscopy. Magnification is $\times 400$.

FBS M199 条件下生长良好, 细胞呈梭形、可重叠生长形成峰谷排列, 细胞密度达 80% 融合时去血清培养 7 天, 细胞形态由比例正常的宽梭形转变为长梭形, 呈良好分化状态, 有形成组织之趋势. 此形态至少可维持 1 周. 补加 10% 血清后, 细胞形态逐渐恢复到原始宽梭形.

随培养条件改变细胞表型可在分化和去分化间相互转变. 细胞接种后培养 48 h 达对数生长期, 用细胞计数法观察细胞倍增时间约为 71 h, 去血清后细胞增殖速率降低 58%, 补加 10% FBS 后细胞增殖恢复到原有基础水平. 去血清条件下加入 PDGF-BB 15 $\mu\text{g/L}$ 后, 未见对细胞增殖产生明显影响 (图 2).

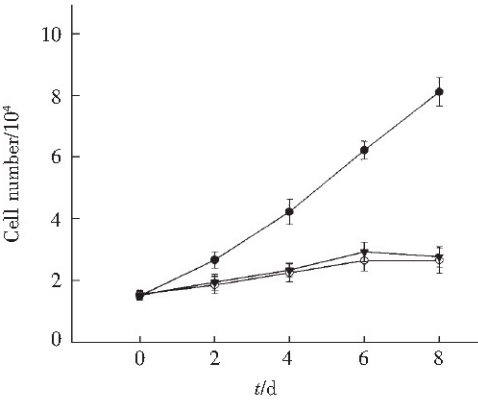


Fig. 2 Effect of serum withdrawal on HITASY cells proliferation

HITASY cells were incubated with M199 with 10% FBS for 2 days, during which time they initiated logarithmic growth. The medium was then replaced with either FBS-free M199 or 15 $\mu\text{g/L}$ PDGF M199. Incubations were followed by analysis of cell number with a hemocytometer. Data are ($\bar{x} \pm s$), $n = 4$. ●—●: +10% FBS; ○—○: -10% FBS; ▼—▼: 15 $\mu\text{g/L}$ PDGF.

2.2 细胞分化特异性标志物在表型转换中的变化及其相关基因的筛选

为进一步验证在去血清培养条件下, HITASY 细胞表型转换过程中平滑肌特异性标志物的变化, 用蛋白质印迹分析平滑肌特异性收缩蛋白和收缩调节蛋白的表达 (图 3).

处于分化表型的 HITASY 细胞表达较多 SMMH, H-Caldesmon, 其水平随去血清时间的延长而增多, 平滑肌 α 肌动蛋白和 Calponin 的表达也呈增加趋势. RT-PCR 分析亦显示平滑肌特异性 SM- α 22 基因表达随去血清培养时间延长而上调 (图 4). 提示 HITASY 在去血清后转换成为分化表型, 其不同标志物的表达时序有所不同.

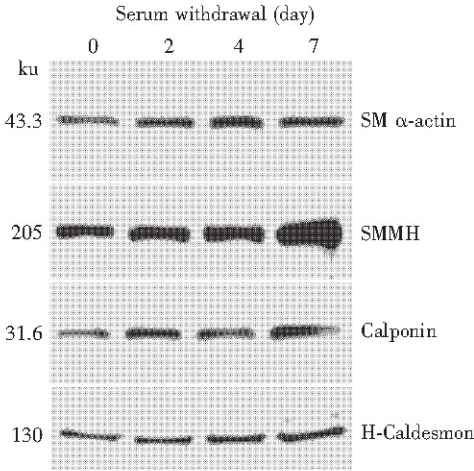


Fig. 3 Expression of smooth muscle- specific markers in HITASY cells after serum withdrawal

Confluent HITASY cells were incubated in M199 FBS withdrawal for 7 days. Cell lysates of equal protein content were separated on polyacrylamide gel, trans to nitrocellulose, and immublotted with anti-SM - α -actin, anti-SM myosin heavy chain, anti-calponin and anti-high- molecular- mass Caldesmon antibodies.

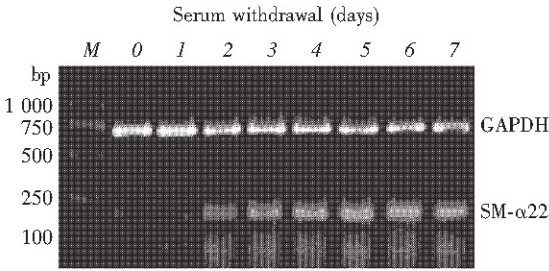


Fig. 4 Analysis of SM- α 22 gene expressed by RT-PCR

The expression level of SM- α 22 mRNA was measured by RT-PCR. Equal amount of total RNA isolated from HITASY cells at indicated times were used for PCR amplification, and the RNA concentration of each samples was normalized by GAPDH.

采用差异显示 PCR 筛选出差异表达条带 33 条, 回收差异显示明显的条带测序, 测得的 DNA 序列于 GenBank 进行同源序列检索分析, 证实为 CREG 的编码基因. 说明去血清 3 天后 CREG mRNA 表达开始上调, 经 RNA 印迹分析证实 CREG mRNA 表达时序与差异显示分析相一致 (图 5).

2.3 表型转换对细胞外基质合成的影响

在 10% FBS 培养条件下, 处于去分化型细胞外基质蛋白合成率很高; 去血清后, 细胞外基质蛋白合成明显减少, 与分化型成熟平滑肌细胞相似, 蛋白质合成率处于相对静止状态. 恢复血清后细胞外基质蛋白合成明显增加, 但尚未完全恢复到去血清前的合成水平 (图 6).

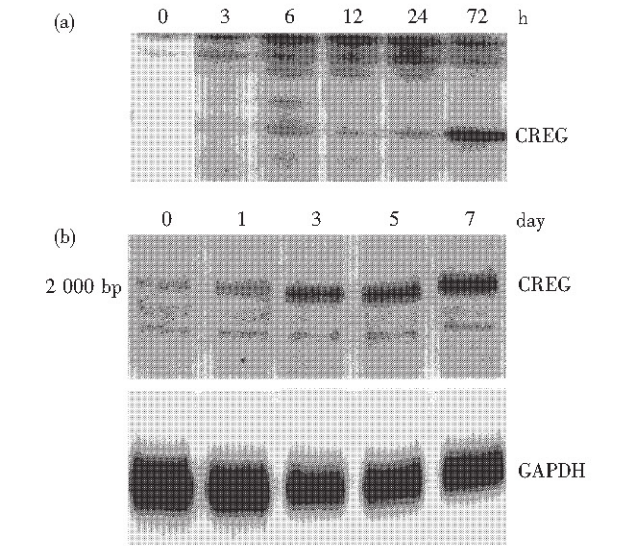


Fig. 5 Upregulation of CREG mRNA in HITASY cells after serum withdrawal
(a) Differential display-PCR product from HITASY cells in M199 serum withdrawal for 0 to 72 h. Total RNA prepared from cultured HITASY cells was reversed transcribed with an anchored oligo (dT) T primer and an arbitrary P primer. PCR products were resolved on 5% DNA-sequencing gels. Differences in PCR product patterns indicate a change in expression of CREG gene. (b) Northern blot as a confirmation of differential display-PCR result. Total RNA extracted from HITASY cells at indicated times were electrophoresed, blotted and hybridized to a CREG probe.

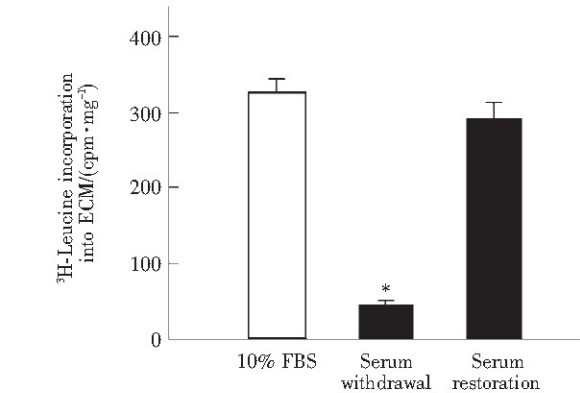


Fig. 6 Effect of serum withdrawal on the extracellular matrix synthesis
³H-leucine incorporation into ECM fractions of HITASY cells cultures. Cells were cultured in M199 supplemented, sequentially, with 10% FBS for 48 h, with 0% FBS for 3 days, and restored with 10% FBS for 2 more days. After each change of growth medium, leucine incorporation was determined by pulsing cells with 1.48×10^5 Bq ³H-leucine for 16 h. The experiment was done in quadruplicate. * $P < 0.01$ vs 10% FBS-supplemented conditions.

2.4 血管活性介质对细胞收缩功能的影响

鉴于体外传代血管平滑肌细胞的收缩反应是判断其分化表型的重要依据，图 7 可见，本实验以降

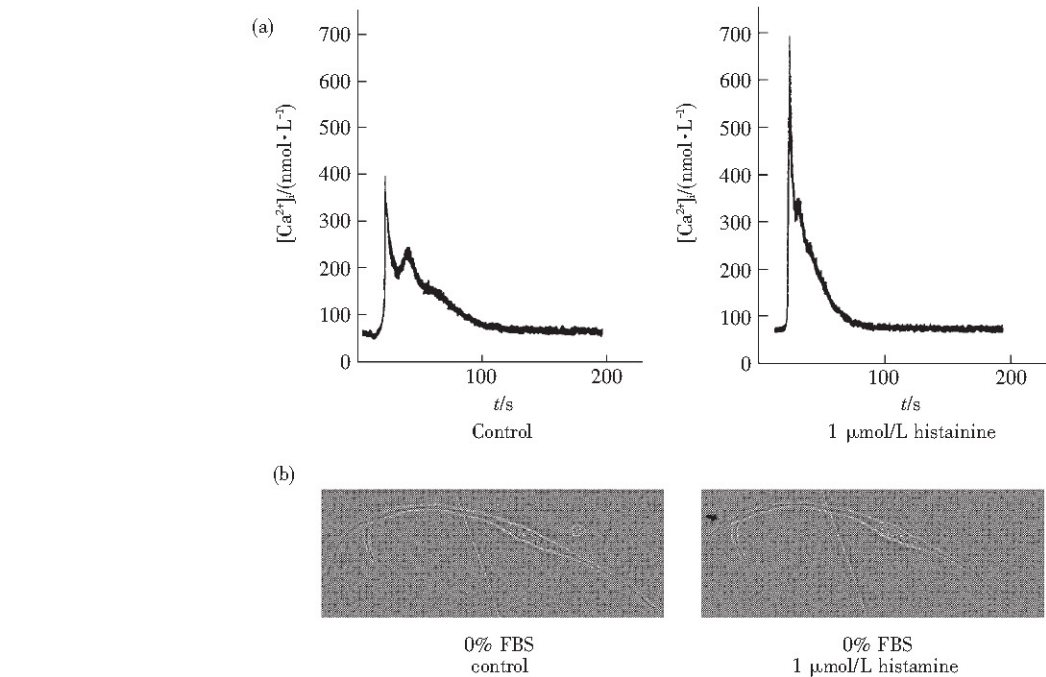


Fig. 7 Vasoactive agonist stimulated robust cytoplasmic calcium transients coupled cell contract in HITASY SMCs
Cells were grown on glass coverslips and $[Ca^{2+}]_i$ was measured using fura-2. Histamine was applied to individual cells using pressure ejection from a micropipette, at the concentrations noted (a). Lines under each $[Ca^{2+}]_i$ trace indicate the duration of agonist application. Results are representative of 5 experiments. Cells adherent to the culture dish were stimulated with 1 mmol/L histamine. Phase-contrast photomicrographs of HITASY cells before (top panel) and after (bottom panel) application of histamine showing contraction (b). The cell was driven to a mature state by serum-deprived for 3 days. Arrow indicate bulge blebs treated with histamine. Magnification is $\times 400$.

低 HITASY 细胞培养密度, 经去血清诱导 7 天, 然后用组胺 $1 \mu\text{mol/L}$ 刺激, 发现胞液 $[\text{Ca}^{2+}]_i$ 骤增, 分别由未刺激对照时 $(397 \pm 79) \text{ nmol/L}$ 增加至 $(693 \pm 92) \text{ nmol/L}$ (图 7a). 图 7b 显示经血管活性物质刺激后, 胞膜周围形成泡状隆凸, 细胞面积减小, 长度缩短, 组胺 $1 \mu\text{mol/L}$ 引起的细胞收缩可被组胺 H1 受体拮抗剂苯海拉明 $1 \mu\text{mol/L}$ 所抑制. 说明处于分化表型的 HITASY 细胞经血管活性物质作用仍可引起受体介导的收缩反应.

3 讨 论

有关平滑肌细胞功能与表型转换存在两种学说, Wissler^[9]首次提出血管 SMCs 表型可逆转换假说, 但迄今对体外培养的 SMCs 尚缺乏可逆转换的实验证据. 虽然通过适当的培养条件可以鉴定 SMCs 表型的标志特征, 但还不能验证体外培养的 SMCs 具有收缩功能. 根据动脉壁 SMCs 生长特征表明 SMCs 是非均一的细胞群体, 细胞亚群选择性增殖假说则认为 SMCs 不同亚群分布及其所实施功能亦不相同, 细胞增殖系来自去分化表型的细胞亚群, 不需经过由分化表型向去分化表型转换的过渡^[10,11], 该假说从另一角度解释了动脉壁 SMCs 亚群分布与其功能间的特点, 这对表型转换学说提出不同见解.

本研究通过去血清培养发现, 人血管 HITASY 具有表型转换的生理特性, HITASY 是克隆化的细胞株, 其分化与去分化转换特性归于单克隆细胞表型转换而不是处于不同分化阶段的细胞亚群选择性增殖. 用细胞自身的生理性收缩功能作为表型转换的鉴定依据可以印证体内表型转换规律, 替代以往细胞超微结构和特异性标志物等指标. 尽管本实验结果尚不足以说明 SMCs 在病理情况下呈去分化表型是源于表型转换抑或细胞亚群选择性增殖, 但体外实验证实 HITASY 是可以发生表型转换的.

在体外含血清培养的 HITASY 细胞呈去分化表型, HITASY 细胞倍增时间较长、移行能力亦弱, 对多种趋化因子应答降低, 可能系克隆细胞自身固有的生理特性. HITASY 细胞经去血清诱导后转换为分化表型, 能对化学物质刺激产生收缩应答, 启动钙离子级联反应引起平滑肌收缩^[12], 显示出功能上的明显变化与细胞分化趋于成熟相一致. 尽管这些指标证实 HITASY 细胞处于分化状态, 但多数指标表达上调是在细胞完成分化表型转换数天前开始增加的, 推测这种表达上调可能不是其表型转换

的主导机制, 其机制还有待进一步研究.

HITASY 细胞在血清培养中不具备收缩功能, 去血清培养后外基质蛋白合成中止, 细胞重组排列转换为分化型, 恢复血清再培养, 细胞逆转为去分化型, 但细胞外基质蛋白尚未恢复到初始基础水平. 推测 HITASY 细胞可能是介于去分化型与分化型之间呈中间表型. 已有研究表明, 鼠损伤的颈动脉新生内膜细胞系合成型的中间表型, 在表达各种细胞外基质基因的同时亦表达平滑肌特异性基因 Calponin 和 SM- $\alpha 22$ ^[13], 经球囊损伤的颈动脉 SMCs 含有丰富的内质网和肌丝纤维, 提示血管间质细胞的合成功能是起源于中间表型而不是原始或胎儿分化表型^[14].

外源性腺病毒蛋白 E1A 和哺乳动物体内转录因子 E2F 均能和几个调节其活性的蛋白质结合, 正向调节其活性, 协同活化细胞周期调节基因, 刺激哺乳动物体内某些细胞增殖^[15]. CREG 是近年新发现的一种分泌型糖蛋白, 可直接与 E1A 及 E2F 家族蛋白竞争性结合在靶基因的启动子上, 从而阻遏 E1A 和 E2F 对靶基因的激活^[16], 抑制细胞增殖. 本实验发现 HITASY 细胞分化过程中 CREG 表达上调, 与细胞增殖受抑呈负相关^[17]. 推测 CREG 可能参与平滑肌细胞表型调控, 作为关键阻遏子在表型转换过程中起开关作用, 但其详细作用机制有待进一步研究证实.

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Expression of Smooth Muscle-specific Molecules and Phenotypic Changes in HITASY Cells by Serum Withdrawal*

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Abstract Human internal thoracic artery cells (HITASY) were used to explore the vascular smooth muscle cells (SMCs) converted from a synthetic into a contractile phenotype characterized by serum withdrawal, which may contribute to neointimal formation and restenosis after vascular injury. Confluent monolayer of HITASY cells cultured in M199 serum-free medium exhibited morphological and functional characteristics consistent with a differentiated phenotype. Smooth muscle cell contractile phenotype was determined by observing the expression of smooth muscle myosin heavy chain (SMMHC, a marker of fully differentiated smooth muscle) and smooth muscle alpha-actin (a marker for all smooth muscle, including immature smooth muscle). Serum withdrawal induced a prominent decrease in extracellular matrix protein synthesis resulting in a 58% lower cell number. Nonsignificant proliferation HITASY cells treated with platelet derived growth factor (PDGF) was detected. Western blot analysis revealed a reversible upregulation of smooth muscle α -actin, calponin, caldesmon and SMMHC. Further, RT-PCR also indicated the expression of smooth muscle alpha 22 gene in HITASY cells after serum withdrawal. Vasoactive agonist stimulated robust calcium oscillations that coupled cell contraction in HITASY cell area or length on average. Differential display PCR was used to screen differential expression genes in HITASY cells, and the data showed that HITASY cells have the potential to express cellular repressor of E1A-stimulated genes, only after cultured in medium serum-free. Following readdition 10% serum, SMCs underwent a reversible dedifferentiation, followed by proliferation. These findings support the pivotal role of SMCs phenotype modulation *in vitro*.

Key words smooth, muscle, vascular, phenotype, cell differentiation, contracts

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