·论 著·

凝血酶和脂多糖诱导小牛主动脉平滑肌细胞表达单核细胞趋化蛋白- 1

孟 峰 邓仲端

(同济医科大学病理学教研室, 武汉 430030)

主题词 单核细胞趋化蛋白- 1; 趋化因子; 凝血酶; 脂多糖; 肌, 平滑, 血管; 牛; 动脉粥样硬化

摘 要 为探讨凝血酶和脂多糖是否可诱导血管壁平滑肌细胞表达单核细胞趋化蛋白- 1, 用贴块法培养小牛主动脉平滑肌细胞至 3~ 5 代, 用 *dot blot* 分析测定对照组、凝血酶(2 *ku*/*L*)组和脂多糖(100 μg/L)组单核细胞趋化蛋白- 1 *mRNA* 表达情况; 并用夹心酶联免疫吸附法检测各组条件培养基中单核细胞趋化蛋白- 1 蛋白表达情况。结果发现, 凝血酶和脂多糖均能增加单核细胞趋化蛋白- 1 *mRNA* 表达(分别为对照组的 2.1 倍和 1.6 倍), 但夹心酶联免疫吸附法显示仅凝血酶能增加单核细胞趋化蛋白- 1 蛋白表达(是对照组的 1.4 倍, $P< 0.05$)。结果提示, 凝血酶能诱导平滑肌细胞表达单核细胞趋化蛋白- 1 而参与动脉粥样硬化发生中单核细胞在动脉内膜中的聚集, 而脂多糖对平滑肌细胞表达单核细胞趋化蛋白- 1 的作用不能肯定。

Thrombin and Lipopolysaccharide Induce Expression of Monocyte Chemoattractant Protein- 1 in Calf Aortic Smooth Muscle Cells

MENG Feng and DENG Zhong- Duan

(*Department of Pathology, Tongji Medical University, Wuhan* 430030, *China*)

MeSH Monocyte Chemoattractant Protein- 1; Chemotactic Factors; Thrombin; Lipopolysaccharide; Muscle, Smooth, Vascular; Bufferloes; Atherosclerosis

ABSTRACT **Aim** Monocyte chemoattractant protein- 1 (MCP- 1) lays an important role in migration of monocytes into subendothelial space. The purpose of this study is to examine whether thrombin and lipopolysaccharide (LPS) induce expression of MCP- 1 mRNA and protein in calf aortic smooth muscle cells (SMCs). **Methods** Calf aortic SMCs were cultured by a substrate- attached explant method. The SMCs at the third to fifth passage which became confluent were used for the experiment. After a four- hour exposure to 2 ku/L thrombin and 100 μg/L LPS respectively, total RNA of SMCs of different groups were extracted by guanidinium isothiocyanate method. On the other hand, the SMCs of different groups after exposed to the above- mentioned inducers for 24 h, respectively, the conditioned media were collected. The expression of MCP- 1 mRNA in SMCs was examined by dot blot analysis using a probe of γ- ^{32}P- labelled 35 mer oligonucleotide, and the MCP- 1 protein in the conditioned media was determined by sandwich ELISA. **Results** Dot blot analysis showed that cultured SMCs were able to express MCP- 1 mRNA at a low level. A 4 h exposure of SMCs to LPS and thrombin, respectively, induced a 1.6- fold and 2.1- fold increase in MCP- 1 mRNA expression in the cells. After a 24 h exposure to thrombin, ELISA showed that the MCP- 1 protein content in the conditioned media were also markedly increased (1.4- fold, $P< 0.05$), while the MCP- 1 protein content was not significantly increased (1.1- fold, $P> 0.05$) after exposed to LPS for 24 h. **Conclusions** Thrombin is able to induce expression of MCP- 1 mRNA and protein in cultured SMCs. It suggests that thrombin may play an important role in atherogenesis through increasing recruitment of monocytes in the arterial intima. The effect of LPS on the expression of MCP- 1 in SMCs, however, is equivocal.

已知单核细胞粘附于内皮并随之迁入内皮下间隙是动脉粥样硬化(atherosclerosis, As)发病中的一个关键性早期事件。单核细胞趋化蛋白- 1 (monocyte chemoattractant protein- 1, MCP- 1)在该过程中发挥重要作用, MCP- 1 不但对于单核细胞具有强大的趋化作用, 而且还能使其激活并表达整合素[1]。血栓形成参与粥瘤形成的假说早已得到公认。斑块中纤维素的存在提示斑块处有原位血栓的形成[2]。凝血酶作为凝血过程中的最终效应物, 它在血栓形成过程中以活性形式存在。在动物实验性气囊损伤中具酶活性的凝血酶在血管壁中持续存在达 10 日[3]。凝血酶除了在凝血过程中起关键作用外, 还具有其它多种生物活性, 包括促进血管壁平滑肌细胞(smooth muscle cell, SMC)增殖及血小板源性生长因子 A 链(platelet derived growth factor- A, PDGF- A)基因表达[4], 促进单核细胞在内皮细胞表

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面粘附及内皮释放 PDGF[5]。文献[6]报道,凝血酶可促进血管内皮细胞及血液单核细胞释放 MCP-1。已知内毒素脂多糖对血管壁细胞有毒性作用,但它是否参与 As 发生,不甚明了。本实验旨在探讨凝血酶和脂多糖是否也诱导小牛主动脉 SMC 表达 MCP-1 mRNA 及蛋白,以阐明其在 As 发生中的作用。

1 材料和方法

1.1 试剂

M199 培养基、胎牛血清和 MCP-1 标准品购自 Gibco 公司。凝血酶、脂多糖(lipopolysaccharide, LPS)和异硫氰酸胍购自美国 Sigma 公司。γ-^{32}P ATP 购自北京福瑞公司。兔多克隆 MCP-1 抗体由中国军事医学科学院提供。

1.2 平滑肌细胞的培养及鉴定

用贴块法培养小牛主动脉 SMC,实验所用细胞均为第 3~5 代。所用培养基为含 10% 胎牛血清的 M199。倒置显微镜下观察细胞生长状态,用 α 抗平滑肌肌动蛋白(α-sm-actin)单克隆抗体通过免疫细胞化学方法鉴定 SMC。

1.3 实验分组

当 SMC 生长汇合后随机分为三组:对照(control)组,在其培养基中不加任何诱导剂;④脂多糖(LPS)组,在其培养基中加入终浓度为 100 μg/L 脂多糖;㈣凝血酶(thrombin)组,在其培养基中加入终浓度为 2 ku/L 凝血酶。

1.4 总 RNA 的提取及 dot blot 分析

各组细胞不加或加入诱导剂后培养 4 h 收集细胞,用异硫氰酸胍法[7]分别提取各组细胞的总 RNA。每组取总 RNA 20 μg 用多孔过滤加样器点样到硝酸纤维素膜上。MCP-1 探针是由 35 对碱基组成的寡核苷酸(由西安医科大学免疫病理研究室合成)。用 γ-^{32}P ATP 对 MCP-1 寡核苷酸探针进行末端标记。将点好样品的硝酸纤维素滤膜 80℃干烤 2 h,57℃下预杂交 4 h。预杂交液含 0.75 mol/L NaCl、0.15 mol/L Tris.HCl(pH 8)、10 mmol/L EDTA、5×Dehardt 溶液(1×Dehardt 溶液:0.02% 牛血清白蛋白、0.02% Ficoll、0.02% 聚乙烯吡咯烷酮)、0.1% 十二烷基磺酸钠(SDS)、0.1% $Na_2P_2O_7$ 和 100 mg/L 变性断裂的鲑精 DNA。预杂交后加入 γ-^{32}P 标记的 MCP-1 寡核苷酸探针,57℃下杂交过夜。杂交完后用清洗液 0.2×SSC(1×SSC:0.15 mol/L NaCl 和 15 mmol/L 柠檬酸钠,pH 7.0)和 0.1% SDS 57℃下洗膜 4 次。凉干后在-70℃下对感光胶片曝光 72 h,常规显影定影后用 TJTY-300 型医用图像处理系统检测各样本的积分吸光度(A)值。

1.5 夹心酶联免疫吸附实验(ELISA)

各组细胞在加入诱导剂(对照组不加其他物质)培养 24 h 后收集培养基。参照 Evanoff 等[8]报道的夹心酶联免疫吸附法检测各组细胞条件培养基中 MCP-1 蛋白含量。从 MCP-1 标准曲线确定 MCP-1 蛋白的浓度(波长 490 nm)。确定的数据用单因素方差分析进行统计处理。

2 结果

2.1 血管平滑肌细胞的鉴定

实验所用的细胞生长呈典型的"峰"与"谷"特征。免疫细胞化学方法显示出胞浆内有棕黄色细肌丝,即 α-sm-actin 抗原阳性。

2.2 Dot blot 分析

培养的 SMC 能够低水平地表达 MCP-1 mRNA,其积分吸光度值为 43.52,SMC 暴露于凝血酶和脂多糖后,其 MCP-1 mRNA 表达均能增强,其积分吸光度值为 89.38 和 67.70(分别是对照组的 2.1 倍与 1.6 倍)。杂交结果见图 1(Figure 1),各组积分吸光度值见图 2(Figure 2)。

图 1. 小牛主动脉平滑肌细胞暴露于脂多糖和凝血酶后其单核细胞趋化蛋白-1 mRNA 的表达

Figure 1. Expresion of MCP-1 mRNA by calf aortic SMCs exposed to LPS and thrombin

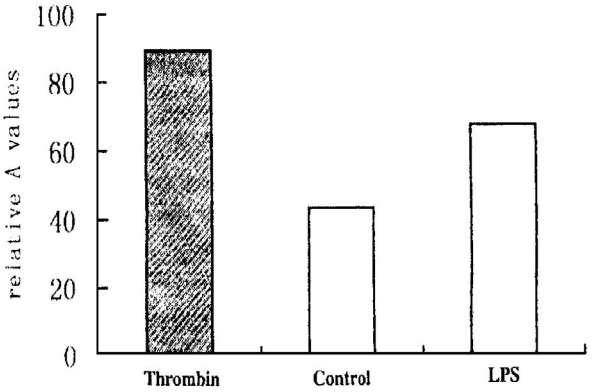

图 2. 单核细胞趋化蛋白-1 mRNA 斑点的光密度扫描的吸光度值

Figure 2. Absorbance (A) values by densitometry scanning of the dot blots of MCP-1 mRNA

2.3 条件培养基中的单核细胞趋化蛋白- 1 蛋白含量测定

用夹心酶联免疫吸附法测定 SMC 条件培养基中 MCP- 1 蛋白含量。结果发现,凝血酶能明显诱导 MCP- 1 蛋白的产生(是正常对照组的 1.4 倍, $P < 0.05$),而脂多糖诱导 SMC 产生 MCP- 1 的作用不明显(仅为对照组的 1.1 倍, $P > 0.05$)。结果见表 1 (Table 1)。

表 1. 平滑肌细胞条件培养基中的单核细胞趋化蛋白- 1 蛋白含量

Table 1. The protein content of MCP- 1 in the SMC conditioned media ($\bar{x} \pm s$, μg/L)

Groups	n	MCP- 1 protein
Control	12	1.45 ±0.15
LPS	12	1.54 ±0.07[a]
Thrombin	12	2.01 ±0.18[b]

a: $P > 0.05$, b: $P < 0.05$, compared with control group

3 讨 论

在 As 发病中,外周血液中单核细胞迁移入内皮下间隙是一个极为重要的环节。尽管这个过程是受多种趋化因子影响,但最主要的是 MCP- 1[9]。NelKen 等[10]用原位杂交法检出 As 斑块中 SMC 有 MCP- 1 mRNA 表达。Strieter 等[11]用免疫组织化学方法检出 As 斑块中只有少数的内皮细胞表达 MCP- 1,而 SMC(33% ~ 50%)表达较高水平的 MCP- 1。Cushing 等[9]的研究证明,培养的 SMC 能产生 MCP- 1,且轻度修饰 LDL 能促进其 MCP- 1 的生成及其 mRNA 的表达。然而其他研究者的实验表明,VLDL 及细胞因子 IL- 1β 和 TNF- α 均能促进 SMC 表达 MCP- 1 mRNA[12, 13]。

许多实验表明,对血管内膜和血管壁的损伤,致使局部的促凝物如胶原及组织因子等暴露,造成局部凝血酶产生[8]。同时,通过对人动脉粥样硬化组织行原位杂交发现,在斑块内富含巨噬细胞的区域,血管 SMC 及间质样内膜细胞增殖区域的凝血酶受体表达水平升高。目前,冠状动脉成形术后,中膜 SMC 迁入内膜并增殖,形成所谓新内膜而造成再狭窄,仍然是困扰临床医师的棘手问题。凝血酶是否参与再狭窄的过程,不甚明了。尽管很多学者的研究显示,凝血酶能够促进 SMC 增殖[4, 14],但最近的资料表明中晚期粥瘤中 SMC 增殖率很低[15]。提示凝血酶在 As 发生发展中除引起 SMC 增殖外还应具有其它功能。因此探讨凝血酶是否同样可以促进 SMC 表达 MCP- 1 具有十分重要的意义。我们的实验表明,凝血酶能明显增加 SMC 表达 MCP- 1 mRNA 和蛋白。我们的结果与 Wenzel 等[16]实验一致。Wenzel 等报道,凝血酶可以诱导兔和人 SMC 表达 MCP- 1 基因和表现 MCP- 1 生物学活性,同时其 mRNA 表达呈浓度依赖性。我们的实验和其他实验[6, 15]结果表明,凝血酶能够促进血管壁内皮细胞、巨噬细胞和 SMC 表达 MCP- 1,从而促进单核细胞在新内膜聚集,使再狭窄加剧;同时在不稳定型心绞痛时使用抗凝疗法提供了实验依据。虽然,文献[17]报道和我们过去的实验表明,脂多糖能诱导内皮细胞表达 MCP- 1 mRNA 和蛋白,但本实验表明,脂多糖对 SMC 的诱导作用不明显。

综上所述,我们的研究表明凝血酶可诱导 SMC 表达 MCP- 1 mRNA 和蛋白。凝血酶能够促进 SMC 表达 MCP- 1 而吸引外周血液单核细胞迁入内皮下间隙参与 As 的发生与发展。

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