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Original Research

Inhibitory effect of recombinant TGF_{α}-PE40 on vascular smooth muscle cell proliferation[1]

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KEY WORDS vascular smooth muscle; epidermal growth factor-urogastrone receptors; transforming growth factor alfa; *Pseudomonas aeruginosa*; exotoxins; cultured cells

ABSTRACT

AIM: To study inhibitory effect of recombinant transforming growth factor α-*Pseudomonas* exotoxin fusion protein (TP40) on proliferation of the cultured vascular smooth muscle cells (SMC). **METHODS**: Expression of epidermal growth factor receptor (EGFR) mRNA and EGFR in cultured proliferating and quiescent SMC was analyzed with Northern blot and immunohistochemistry. Inhibitory effects of TP40 on SMC proliferation and protein synthesis were analyzed with crystal violet staining and [^{3}H]leucine incorporation. Competition assays were performed by the addition of 100-fold excess of EGF. **RESULTS**: Expression of EGFR mRNA and EGFR in rapidly proliferating SMC increased than that in quiescent SMC. When the concentration of TP40 was 10 or 100 $\mu g \cdot L^{-1}$, inhibitory effects of TP40 on rapidly proliferating SMC proliferation and protein synthesis were much higher than that on quiescent SMC ($P < 0.01$), and the IC_{50} of [^{3}H]leucine incorporation against rapidly proliferating and quiescent SMC were 8.01 (5.05 - 12.69) and 121.95 (90.98 - 163.47) $\mu g \cdot L^{-1}$. Excess EGF completely blocked inhibitory effects of TP40. **CONCLUSION**: The rapidly proliferating SMC express EGFR at a high level. TP40 selectively inhibited the proliferation of rapidly proliferating SMC. The cytotoxic effects of TP40 were specifically mediated by EGFR.

INTRODUCTION

Percutaneous transluminal coronary angioplasty (PTCA) has become a successful and widely used treatment for patients with coronary artery disease. Nevertheless, its major limitation lies in restenosis, which occurs in 30 % - 50 % of the patients within 6 months[1,2]. One of the primary mechanisms responsible for restenosis was identified as activation of medial smooth muscle cells (SMC), a process that leads to their proliferation and migration to the neointima, where they continue to proliferate and secret extracellular matrix, with ultimate encroachment on the vessel lumen[3,4]. Despite intensive clinical and experimental investigations over the past decade, the attempts to inhibit the events leading to this proliferation have met little success. There is no drug that consistently inhibits this response clinically. Once vascular SMC are activated postinjury, they overexpress several cell surface growth factor receptors than nonactivated SMC. This differential expression of

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growth factor receptors provides a rationale for the use of growth factor receptor-specific cytotoxic agents to target the activated, proliferating SMC that typically constitute a restenotic lesion. It was the purpose of the present investigation to determine whether epidermal growth factor (EGF) receptor-directed cytotoxicity, using the recombinant transforming growth factor α-*Pseudomonas* exotoxin 40 (TP40) fusion protein, could inhibit the proliferation of SMC.

MATERIALS AND METHODS

SMC culture Cultured SMC were isolated from New Zealand white rabbits (2.5 - 3.5 kg) by explant method[5], identificated by typical morphological characteristics and immunohistochemical staining for antimuscle actin.

EGFR expression of SMC Rapidly proliferating SMC compared with quiescent SMC were obtained using SMC (passage 3) grown in MEM (Gibco) supplemented with either 20 % or 0.5 % calf serum. The total RNA was extracted from SMC using guanidinium isothiocyanate method. EGFR mRNA expression in SMC was examined by Northern blot analysis using a random-primed [^{32}P] DNA probe[6]. The plasmid (Ph $EGFR_{4a}$) was kindly supplied by Dr Richard POULSON, Laboratory of ICEF Histopathology, UK. α-[^{32}P]-dATP was obtained from Amersham Corp. After SMC were fixed with 4 % polyformaldehyde, they were stained with a polyclonal antibody to EGFR (kindly presented by Dr XU Yong-Hua) with immunohistochemical ABC method.

Effect of TP40 on SMC proliferation SMC (passage 4 and 5) at confluence were seeded in 96-well plates at a desity of 5×10^3/well (6 wells each group) in MEM supplemented with 20 % calf serum after trypsinization and incubated at 37 ℃ in 5 % CO_2 for 24 h to permit attachment. Quiescent SMC were incubated in MEM supplemented with 0.5 % calf serum for another 72 h. The medium was replaced with medium containing TP40 (0.1, 1, 10, and 100 $\mu g \cdot L^{-1}$; contructed and supplied by Dr XU Yong-Hua). After incubation for 48 h, cell number was measured by using a modification of Flick's procedure[7]. Inhibitory rate was calculated: Inhibitory rate (%) = [(*A* of the control group-*A* of the TP40 group)/*A* of the control group] × 100 %

Effect of TP40 on SMC protein synthesis SMC (passage 4 and 5) seeding and concentration of TP40 were the same as those described above. After SMC were incubated with TP40 for 48 h, [^{3}H]leucine (Amersham Corp) 18.5 kBq/well was added to each well. SMC were incubated at 37 ℃ for 4 h, washed 3 times with PBS, and extracted with a cell harvester. Radioactivity was measured by a liquid scintillation counter (Shanghai Institute of Nuclear Research, Chinese Academy of Sciences). The results were expressed as a % of control without toxin.

Competition assay of EGF to TP40 SMC (passage 4 and 5) were seeded in 96-well plates at a density of 5×10^3/well in MEM supplemented with 20 % calf serum (6 wells each group). After incubation for 24 h, excess EGF and acidic fibroblast growth factor (aFGF) (the gift of Dr ZHANG Jian-Jun, Department of Cardiology, Shanghai First People Hospital) 1000 $\mu g \cdot L^{-1}$ (100-fold) were added. TP40 10 $\mu g \cdot L^{-1}$ was then added after incubation for another 2 h. Only TP40 was added in positive control, medium without TP40 and other growth factors was added in negative control. After a 48-h incubation period, protein synthesis was estimated by the [^{3}H]leucine incorporation assay.

Statistical analysis Between-group comparisons were made with the *t* test at the same concentrations.

RESULTS

EGFR expression of SMC There was very little expression of EGFR mRNA in quiescent SMC. The expression of EGFR mRNA increased in rapidly proliferating SMC. The rapidly proliferating SMC exhibited the brown immunoperoxidase reaction indicative of EGFR. The reaction product was located mainly on the membrane of the SMC. The quiescent SMC showed no immunoreactivity (Fig 1, 2).

Inhibition of SMC proliferation by TP40 When the concentration of TP40 was 0.1 or 1 $\mu g \cdot L^{-1}$, growth suppression to rapidly proliferating SMC was slightly higher than that to quiescent SMC ($P > 0.05$). When the concentration of TP40 was 10 or 100 $\mu g \cdot L^{-1}$, growth suppression to rapidly proliferating SMC was much higher than that to quiescent SMC ($P < 0.01$) (Fig 3).

Inhibition of SMC protein synthesis by

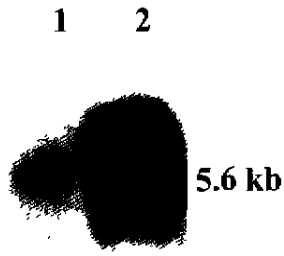

Fig 1. Detection of EGFR mRNA expression in cultured SMC by Northern blot. 1) Quiescent SMC. 2) Rapidly proliferative SMC.

Fig 2. EGFR expression of cultured proliferating SMC by immunohistochemical staining. ×400.

TP40 When the concentration of TP40 was 0.1 or 1 $\mu g \cdot L^{-1}$, the inhibitory effect of TP40 on rapidly proliferating SMC protein synthesis was slightly higher than that on quiescent SMC protein synthesis ($P > 0.05$). When the concentration of TP40 was 10 or 100 $\mu g \cdot L^{-1}$, the inhibitory effect of TP40 on rapidly proliferating SMC protein synthesis was much higher than that on quiecent SMC protein systhesis ($P < 0.01$). The IC_{50} values (95 % confidence limits) against rapidly proliferating and quiescent SMC were 8.01 (5.05 – 12.69) and 121.95 (90.98 – 163.47) $\mu g \cdot L^{-1}$, respectively (Fig 3).

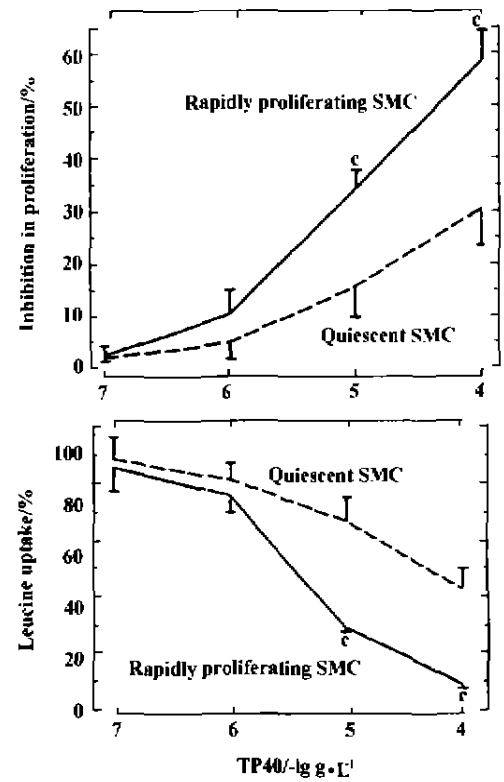

Fig 3. Inhibitory effects of TP40 on rapidly proliferating and quiescent SMC proliferation, and protein synthesis. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* quiescent SMC.

Competition assay of EGF to TP40 [^{3}H]leucine incorporation of the excess EGF group which approached that of blank control ($P > 0.05$) was much higher than that of excess aFGF and TP40 groups ($P < 0.01$). It illustrated that excess EGF completely blocked inhibitory effects of TP40 and pointed out that the cytotoxic effects of TP40 were specifically mediated by its binding to the EGFR (Tab 1).

Tab 1. Competition of cytotoxicity of TP40 by coincubation with excess of EGF and aFGF. $n = 6$ wells. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* EGF/TP40.

	[^{3}H]leucine incorporation/Bq
Blank control	65.9 ± 4.8[a]
TP40	26.3 ± 1.6[c]
aFGF/TP40	28.8 ± 2.4[c]
EGF/TP40	61.9 ± 3.3

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DISCUSSION

A key feature of restenosis in many patients is the accumulation of intimal vascular SMC. Although it is not known how SMC proliferation is initiated, substantial *in vitro* data as well as indirect evidence from the study of human atherosclerotic arteries support the concept that growth factor play an important role in this process. Growth factors such as EGF, FGF, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF_β), insulin-like growth factor Ⅰ (IGF-Ⅰ) and IGF-Ⅱ may be involved in this process[5]. Many studies have provided evidence that proliferating SMC express a high number of receptors for EGF, FGF, PDGF, and IGF-Ⅰ than quiescent SMC. In the present study, we found that proliferating SMC expressed high levels of EGFR mRNA and its protein. This increase in EGFR gene expression and function may play an important role in the proliferation of SMC that follows angioplasty. We have attempted to take advantage of this increase in the EGFR by targeting a cytotoxic agent to the proliferating SMC.

Transforming growth factor α (TGF_α) has a 30 % amino acid homology to EGF including conservation of all six cysteines. The two ligands are recognized by the binding site of the EGFR with practically identical affinities[5]. They both activate EGFR protein kinase activity and stimulate cell growth. *Pseudomonas exotoxin* (PE) is an extremely potent toxin, cytotoxicity mediated by its three domains. Although the intact exotoxin molecule is cytotoxic to virtually all cells, its cytotoxic effects are remarkably reduced when its cell binding-domain (domain Ⅰ) is deleted to produce a 40-kDa protein (PE40)[8,9]. When the gene encoding TGF_α is fused to the gene encoding PE40, a fusion protein containing TGF_α and PE40 is produced. The recombinant protein termed TP40 can be made in *E coli*[5,10]. TP40 has the ability to target cells bearing EGFR. The steps involved in TP40 mediated cytoxicity include: (1) binding of the TGF_α-toxin complex to EGFR; (2) translocaton of the entire complex to the cytosol in clathrin-coated vesicles; (3) cleavage of the toxin into the translocation domain (domain Ⅱ) and the protein inhibition domain (domain Ⅲ); and (4) ADP-ribosylation of elongation factor 2 by domain Ⅲ, an effect that inhibits protein synthesis and thereby causes cell death[11]. The issue whether TP40 can induce proliferating SMC apoptosis remains controversial.

The activities of inhibition to cell proliferation by TP40 depend on the amount of EGFR on target cell surface[10]. In this study, we observed that TP40 inhibited cell protein synthesis and proliferation after it was added to cultured rapidly proliferating SMC which expressed EGFR at a high level, an effect greater than that seen against quiescent SMC that expressed EGFR at a low level. Competition studies using excess EGF indicated that cytotoxic effects of TP40 were specifically mediated by the EGF receptor. According to the different EGFR expression in proliferating SMC and quiescent SMC, there is a possibility to select a suitable dose which can kill proliferating SMC and less influence quiescent SMC. TP40 that selectively targets actively proliferating SMC might be applied to preventing restenosis after PTCA.

REFERENCES

1 Rozenman Y, Gilon D, Welber S, Sapoznikov D, Wexler D, Lotan C, *et al*. Total coronary artery occlusion late after successful coronary angioplasty of moderately severe lesions: incidence and clinical manifestations. Cardiology 1994; 85: 222-8.
2 Sathe S, Alt C, Black A, Manolas E, Warren R, Valentine P. Initial and long-term results of percutaneous transluminal balloon angioplasty for chronic total occlusions: an analysis of 184 procedures. Aust N Z J Med 1994; 24: 277-81.
3 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362: 801-9.
4 Casscells W. Migration of smooth muscle and endothelial cells. Critical events in restenosis. Circulation 1992; 86: 723-9.
5 Epstein SE, Siegall CB, Biro S, Fu YM, FitzGerald D, Pastan I. Cytotoxic effects of recombinant chimeric toxin on rapidly proliferating vascular smooth cells. Circulation 1991; 84: 778-87.
6 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a labroatory manual, 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989. p 363-71.
7 Zeng GQ, Rui YC. Effects of anisodamine and dauricine on proliferation, DNA synthesis, and calcium influx in bovine anterior cerebral arterial smooth muscle cells in culture. Acta Pharmacol Sin 1991; 12: 308-11.
8 Fu YM, Mesri EA, Yu ZX, Kreitman RJ, Pastan I, Epstein SE. Cytotoxic effects of vascular smooth muscle cells of the chimeric toxin, heparin binding TGF_2-*Pseudomonas* exotoxin. Cardiovasc Res 1998; 27: 1691-7.

9 Pastan I, Chaudhary V, FitzGerald DJ. Recombinant toxins as novel therapeutic agents. Annu Rev Biochem 1992; 61: 331 – 54.
10 Xu YH, Peng SF, Jiang WL, Zhu L, Chandhary V, FitzGerald D, *et al*. Gene cloning, expression of fusion protein TGF_{α}-PE40 and its inhibition activity on cancer cell growth. Acta Biol Exp Sin 1993; 26: 289 – 95.
11 Phillips PC, Levow C, Catterall M, Colvin OM, Pastan I, Brem H. Transforming growth factor α-*Pseudomonas* exotoxin fusion protein (TGF_{α}-PE38) treatment of subcutaneous and intracranial human glioma and medulloblastoma xenografts in athymic mice. Cancer Res 1994; 54: 1008 – 15.

重组 TGF_{α}-PE40 对血管平滑肌细胞增殖的抑制作用[1]

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关键词：血管平滑肌；表皮生长因子-尿抑胃激素受体；转化生长因子α；铜绿假单胞菌；外毒素类；培养的细胞

目的：探讨基因重组转换生长因子α-绿脓杆菌外毒素融合蛋白（TP40）对血管平滑肌细胞（SMC）增殖的抑制作用. **方法**：用 RNA 印迹法和免疫组化检测原代培养增殖期 SMC 及静止期 SMC 表皮生长因子受体（EGFR）的表达，用结晶紫染色法和 [^{3}H]亮氨酸掺入法检测 TP40 对增殖期 SMC 和静止期 SMC 增殖及蛋白质合成的抑制作用，用过量 EGF（TP40 浓度的 100 倍）竞争拮抗 TP40 的细胞毒作用. **结果**：增殖期 SMC EGFR 的 mRNA 及受体蛋白质的表达显著高于静止期 SMC. TP40 浓度为 10 及 100 $\mu g \cdot L^{-1}$时，对增殖期 SMC 增殖及蛋白质合成的抑制作用较静止期 SMC 强（$P < 0.01$），对[^{3}H]亮氨酸掺入抑制的 IC_{50}及其 95 %可信限分别为 8.01 （5.05 – 12.69）和 121.95 （90.98 – 163.47）$\mu g \cdot L^{-1}$. 过量 EGF 能完全拮抗 TP40 的细胞毒作用. **结论**：增殖期 SMC 能高水平地表达 EGFR，TP40 对增殖期 SMC 的增殖具有导向抑制作用，作用部位为细胞的 EGFR.

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