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Inhibitory effect of recombinant TGF_{α}-PE40 on neointimal proliferation after arterial balloon injury[1]

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KEY WORDS balloon angioplasty; vascular smooth muscle; transforming growth factor α; *Pseudomonas aeruginosa*; exotoxins

ABSTRACT

AIM: To investigate inhibitory effects of recombinant transforming growth factor α-*Pseudomonas* exotoxin 40 fusion protein (TGF_{α}-PE40; TP40) on neointimal proliferation after arterial balloon injury. **METHODS**: Forty male rabbits fed a cholesterol rich diet were randomly divided into TP40 15 μg, 30 μg, 60 μg, physiologic saline control, and normal artery groups ($n = 8$). Rabbits in the treatment groups were treated by local administration of TP40 (15 μg, 30 μg, and 60 μg per rabbit) 24 h postinjury, and those in the control group were treated by physiologic saline 24 h postinjury. Remained 8 rabbits in normal artery group were treated by TP40 (60 μg). Optical microscope, electron microscope, and computer image analysis were used to study arterial segments 2 weeks after treatment. **RESULTS**: Irregular thickening of the arterial intima, large amounts of smooth muscle cells (SMC) within the neointima, and stenosis of the arterial cavity were observed in the physiologic saline control group. Great inhibition of intimal proliferation and prevention of stenosis of the arterial cavity were observed in the TP40-treated groups that were examined 2 weeks postinjury by optical microscope. The uninjured carotids were histologically normal. Lots of destructural and necrotic SMC were observed in media in TP40 60 μg group by electron microscope. Computer image analysis showed that the neointimal area of the TP40-treated groups was markedly smaller than that of the saline control group ($P < 0.01$). **CONCLUSION**: Recombinant TP40 greatly inhibited neointimal proliferation after arterial balloon injury.

INTRODUCTION

Percutaneous transluminal coronary angioplasty (PTCA) has become a successful and widely used treatment for patients with coronary artery disease. However, its major limitation is restenosis, which occurs in 30 % - 50 % of 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]. Once vascular SMC are activated postinjury, they overexpress several cell surface growth factor receptors than nonactivated SMC. This differential expression of 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.

Transforming growth factor α (TGF_{α}) has 30 % amino acid homology to epidermal growth factor (EGF). The two ligands are recognized by the binding site of the EGF receptors (EGFR) with practically identical affinities[5]. *Pseudomonas* exotoxin (PE) is an extremely potent toxin with activity mediated by its three domains. Domain Ⅰ is the cell binding-domain, domain Ⅱ translocates the molecule into the cell cytosol, and domain Ⅲ catalyses the ADP ribosylation of elongation fac-

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tor-2 that inhibits protein synthesis leading to cellular death. Although the intact exotoxin molecule is cytotoxic to all cells virtually, when its cell binding-domain is either deleted or mutated (PE40), its cytotoxic effects are markedly reduced[6,7]. Hence, when the gene encoding TGF_α is fused to the gene encoding PE40, a fusion protein containing TGF_α and PE40 has been expressed in *E coli*. The recombinant TGF_α-PE40 (TP40) has the ability to target cells bearing EGFR and translocate PE40 into cells. PE40 can arrest protein synthesis effectively and cause cell death[5,8]. It has been demonstrated that proliferating rabbit vascular SMC expressed 10-fold more EGFR per cell than quiescent SMC *in vitro*[5]. The cytotoxic effect of TP40 on proliferating SMC was much higher than that on quiescent SMC in culture, and this effect is mediated by the EGFR[9]. The purpose of the present investigation is to determine whether TP40 could inhibit neointimal proliferation after arterial balloon injury *in vivo*.

MATERIALS AND METHODS

Animal groups Forty male New Zealand white rabbits (weighing 3.0 kg ± 0.5 kg, grade Ⅱ, certificate № 02-64, provided by Experimental Animal Center of Second Military Medical University) were randomly divided into TP40 15 μg, 30 μg, 60 μg, physiologic saline control, and normal artery groups ($n = 8$). The rabbits were fed on a cholesterol-rich diet 10 d before artery injury till the end of the study. Each animal was fed 50 g cholesterol-rich forage daily, which consisted of cholesterol 1 g, yolk powder 10 g, lard 10 g, and ordinary mixed forage 29 g. Others received ordinary mixed forage. The total cholesterol in serum of eight rabbits selected randomly was examined before and after 1 or 2 weeks on the cholesterol-rich diet.

The rabbit balloon-injury model[10] Anesthesia was achieved with intravenous pentobarbital (30 mg/kg). The distal right common carotid artery and the region of the bifurcation were surgically exposed under aseptic conditions. By a small arteriotomy, a Fogarty 4F balloon dilatation catheter (3.0 mm in diameter and 20 mm in length, NAMIC Corp) was introduced through the external carotid artery. The balloon was inflated to a pressure of 304 kPa (3 atmosphere) and passed three times up and down the common carotid artery to produce a deendothelializing injury. The catheter was then removed, and the external carotid artery was temporarily ligated.

Administration Twenty-four hours after balloon injury, the injured artery was exposed again in anesthesia and aseptic condition. A tinny catheter (Cuff tube) was advanced into injured segment of the common carotid artery through the external carotid artery and TP40 (constructed and supplied by Prof XU Yong-Hua, stored at −20 ℃, dissolved in physiologic saline and kept at 4 ℃ before used) 15 μg, 30 μg, and 60 μg in 30 μL physiologic saline or physiologic saline 30 μL were injected. The common carotid artery was occluded by proximal and distal ties. TP40 or physiologic saline were incubated for 20 min, followed by release of the ligtures. In another type of control experiment, normal arteries were treated with TP40 60 μg according to the above method. The external carotid artery was ligated and the surgical incision was closed. Experimental arterial segments were harvested 2 weeks after treatment and histologic sections were stained with hematoxylin-eosin. Small arterial pieces 2.0 mm × 1.5 mm taken from TP40 60 μg treatment group and physiologic saline control group were fixed with 2 % glutaraldehyde for electronic microscope ultrastructural analysis.

Computer image analysis In each group, three sections were chosen at different parts from each injured arterial specimen. Cross-sectional area of the neointima, cross-sectional area of the media, and the ratio of the cross-sectional area of neointima to media of the injured carotid artery were measured by using VIDAS Image Analysis System (OPTON Co).

Statistical analysis Values were given as $x \pm s$. Comparisons of groups were made with an unpaired t-test.

RESULTS

Dynamic observation of total serum cholesterol The total serum cholesterol was (1.1 ± 0.4) mmol/L before the rabbits were fed the cholesterol-rich diet and rose to (10.4 ± 1.8) mmol/L after 1 week on the cholesterol-rich diet ($P < 0.01$), and after 2 weeks to (21.5 ± 3.4) mmol/L ($P < 0.01$).

Morphological analysis Light microscopic examination demonstrated that marked intimal thickening and lumen narrowing occured in the physiologic saline control group 2 weeks postinjury, characterized by SMC proliferation, accumulation of extracellular matrix, and infiltration of many foam cells into the neointima and the media. SMC were identified by antimuscle actin. Inti-

mal thickness was greatly less in the TP40-treated groups 2 weeks postinjury than in the physiologic saline control group. When TP40 15 μg was locally applied, there was a decrease in neointimal accumulation compared with the physiologic saline control group. In segments receiving TP40 30 μg and 60 μg, no neointima was detected. But TP40 60 μg caused apparent SMC death in some areas in the medial layer. The inhibitory effects of TP40 on neointimal proliferation postinjury were dose-dependent. The uninjured carotids were histologically normal even if treated by TP40 60 μg (Fig 1). Electron micrographs also showed ultrastructural changes. In physiologic saline control group, SMC in neointima had oval nucleus. There were many well developed rough endoplasmic reticulum, polyribosomes, myoneme, and large numbers of lipid droplets in cytoplasm. In TP40 60 μg-treated group, there were lots of destructural and necrotic SMC in media, and most of them close to intima. Cellular structure was not clear and neucleus were disintegrated. The shape of endoplasmic reticulum and mitochondria became vacuole, and myoneme were lost. Some of necrotic SMC had splitted into pieces (Fig 2).

Computer image analysis Treatment with TP40 strikingly inhibited neointimal proliferation of the rabbit common carotid artery postinjury (Tab 1).

DISCUSSION

The balloon-injured rabbit carotid artery model[10], namely a primary injury model, has been used in this study. Endothelial denudation of vessel and in the setting of hypercholesterolemia were able to creat great intimal thickening in a relatively short period of time. We observed that proliferating SMC in injured carotid arteries began to express high levels of EGFR 24 h postinjury[11]. When TP40 was delivered locally to injured arteries, a dose response effect on neointimal proliferation was observed. At the 15 μg dose, there was a partial inhibition of the proliferative response indicating the dose was not high enough. When 60 μg was given, there was complete inhibition of neointimal proliferation, but some areas of apparent SMC death were noted in the medial layer, indicating that the dose was too high leading to risk of aneurysm or rupture. At the 30 μg dose, there was complete inhibition of proliferation of the neointimas without detectable SMC damage. So we thought that TP40 30 μg was the suitable dose for this study. In this study, TP40 was given locally one time, 24 h after balloon injury. Its effect was very pronounced 2 weeks

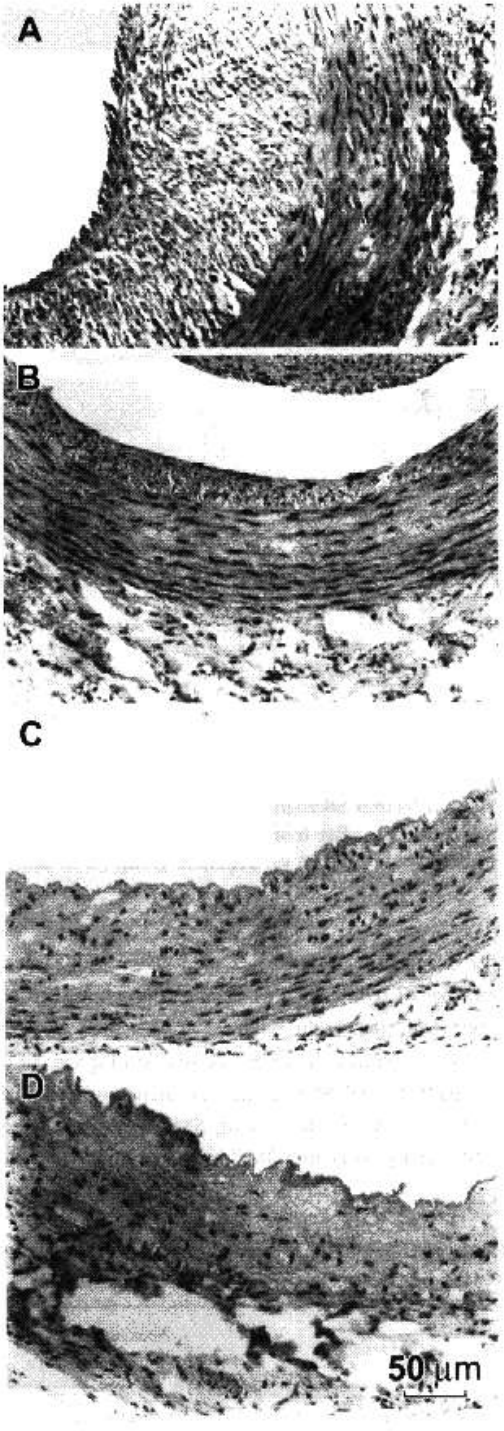

Fig 1. The photomicrograph of histologic cross-section of rabbit common carotid artery 2 weeks after TP40 treatment. (HE stain, × 200). Saline control group (A), TP40 15 μg (B), 30 μg (C), and 60 μg (D) group.

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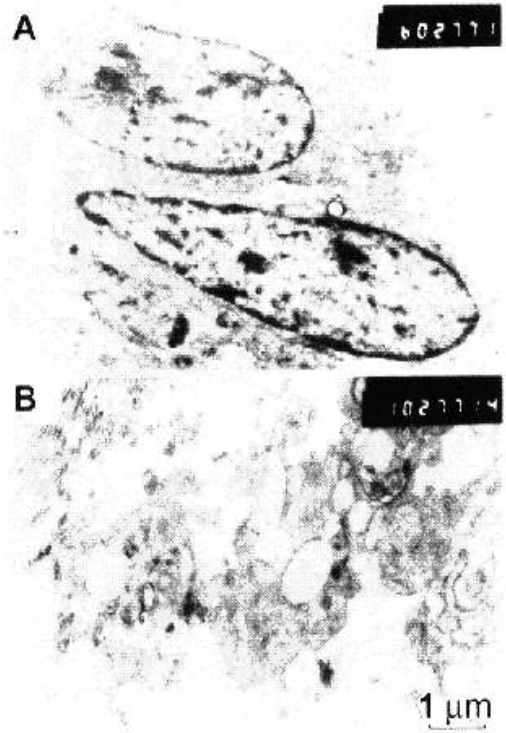

Fig 2. Electron micrographs of rabbit common carotid artery 2 weeks after treatment. A: Saline control group (×6000); B: TP40 60 μg treatment group (×10 000).

after treatment. If TP40 had been given locally several times before proliferating SMC in media returned to quiescent state, the results might have been even more impressive. Because the EGFR expression of quiescent endothelial cells and SMC in normal arteries was markedly lower than that of proliferating SMC in injured artery, TP40 killed proliferating SMC in balloon-injured arteries but had no apparent effect on normal arteries in animals. We observed that the uninjured carotid arteries were histologically normal even if treated with high dose of TP40 60 μg. The amount of TP40 needed to inhibit protein synthesis is dependent on the amount of EGFR on the target cell surface[8]. Because there is a difference in EGFR expression in proliferating SMC compared to quiescent SMC, it is possible to select a suitable dose which can kill proliferating SMC and have little effect on normal quiescent SMC.

Other approaches to growth factor-targeted killing of SMC have been investigated *in vivo*. Pastore *et al* observed that systemic or local administration of EGF-diphtheria toxin fusion protein ($DAB_{389}EGF$) 0.15 mg appeared to inhibit injury-induced neointimal proliferation. $DAB_{389}EGF$ is highly cytotoxic to vascular SMC proliferating in media and this effect is mediated by EGFR. The mechanism of cytotoxicity for $DAB_{389}EGF$ is identical to that of native diphtheria toxin. It catalyzes the covalent linkage of ADP-ribose to elongation factor-2. This step arrests protein synthesis, causing cell death[12]. They did not further illustrate wheather higher dose of $DAB_{389}EGF$ could cause medical necrosis. Recent studies have established that proliferating SMC in culture or *in vivo* also express a higher number of basic fibroblast growth factor receptors (bFGFR) than quiescent SMC. Casscells *et al* targeted bFGFR with a fusion protein bFGF-saporin and achieved a significant reduction in neointimal proliferation in a dose-dependent form in balloon-injured rat arteries. Saporin is a kind of vegetal toxin, which can cause cellular ribosome inactivity. When bFGF-saporin were given in high dose (100 μg/kg, iv, at 24, 48, and 72 h postinjury or 1-10 μg/kg locally applied), however, extensive medial necrosis was noted[13]. Both of above fusion proteins had no effect on the uninjured artery.

Tab 1. Neointimal area, medial area, and neointimal area/medial area after TP40 treatment. $n = 24$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Group	Neointimal area mm²	Media area mm²	Neointimal area/Media area (%)
Saline control	0.43 ± 0.13	0.65 ± 0.16	66 ± 15
TP40 15 μg	0.044 ± 0.028^c	0.52 ± 0.10	7.9 ± 1.7^c
TP40 30 μg	0.0020 ± 0.0007^c	0.55 ± 0.09	0.43 ± 0.09^c
TP40 60 μg	0^c	0.61 ± 0.15	0^c

Biro put forward an idea that the cytotoxic effects of the fusion protein might cause dying SMC to release growth factors which could stimulate contiguous SMC to proliferate and also saturate local growth factor receptor and thereby inhibit the influence of the fusion protein on other cells[14]. But his idea has not been confirmed by experiment up to date. Pastore thought that this was an unlikely consequence of DAB_{389}EGF treatment. Halting protein synthesis is an attractive means of killing proliferating SMC to inhibit neointimal proliferation, and it will lead to a depletion of intracellular growth factors that could otherwise have a mitogenic effect on nearby SMC following lysis of the targeted SMC. The abrupt release of intracellular contents of SMC may be potentially mitogenic. In fact, the mechanism of SMC death did not appear to involve acute disruption of cell membrane integrity. This notion was supported by his experimental observation. The inhibitory effect of treatment with DAB_{389}EGF on neointimal proliferation 2 weeks was the same as 3 weeks postinjury[12]. Location of SMC binding TP40 and cytotoxic mechanism of TP40 are identical to those of DAB_{389}EGF. In this study, neointimal proliferation was inhibited greatly in the TP40 group compared with physiologic saline control group 2 weeks postinjury, and the significant "rebound" proliferation of neointima was not detected.

Casscells and Pastore have demonstrated that local delivery of fusion protein not only was superior for inhibiting neointimal proliferation but also avoided the deleterious effects associated with systemic toxicity[12,13]. Systemic toxicity of local delivery of TP40 60 μg was evaluated by measurement of rabbit weight, examination of blood cells, hepatic and renal function, and histological observation of main organs. We did not find any abnormal phenomena compared with those of physiologic saline control group (unpublished data). So local delivery of TP40 is safe to inhibit neointimal proliferation postinjury in rabbits. The inhibitory effect of TP40 on neointimal proliferation after artery balloon injury will provide a possible new approach to the prevention of restenosis clinically after PTCA.

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重组 TGF_{α}-PE40 对动脉球囊损伤后内膜增生的抑制作用[1]

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关键词 气囊血管成形术；血管平滑肌；转化生长因子α；铜绿假单胞菌；外毒素类

目的：探讨基因重组转化生长因子 α-绿脓杆菌外毒素融合蛋白（TGF_{α}-PE40；TP40）对动脉球囊损伤后内膜增生的抑制作用. **方法**：40 只雄性兔随机分为 TP40 15 μg、30 μg、60 μg、正常动脉及生理盐水对照组（$n=8$），均高脂饲养. 在兔右颈总动脉行球囊内膜剥脱术后 24 h，治疗组于动脉损伤段局部给予 TP40 15 μg、30 μg 及 60 μg 治疗；对照组给予生理盐水. 正常动脉组局部给予 TP40 60 μg. 于治疗后第 2 周取实验动脉段行光镜、电镜观察及计算机图像分析. **结果**：光镜观察见对照组动脉内膜不规则增厚，新生内膜可见大量平滑肌细胞（SMC），管腔狭窄；TP40 治疗组动脉内膜增生被明显抑制，管腔无狭窄；TP40 对正常动脉影响不大；电镜观察见 TP40 60 μg 治疗组的动脉中膜有较多变性、坏死的 SMC；计算机图像分析显示动脉损伤后第 2 周 TP40 治疗组内膜截面积及内膜与中膜截面积的比值显著小于生理盐水对照组（$P<0.01$）. **结论**：TP40 对动脉球囊损伤后的内膜增生具有抑制作用.

（责任编辑 朱倩蓉）