Cytotoxic Effects of a Recombinant Chimeric Toxin on Rapidly Proliferating Vascular Smooth Muscle Cells

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Background. Restenosis after percutaneous transluminal coronary angioplasty is associated with activation of medial smooth muscle cells (SMCs); they proliferate, migrate to the subintima, and narrow the vessel lumen. Cancer cells often express more cell surface receptors than do normal cells. This has allowed tumor cells to be specifically targeted using cytotoxic agents. We have examined whether a similar concept can be applied to rapidly proliferating but nontransformed SMCs. Pseudomonas exotoxin (PE; MW, 66 kDa) is a potent toxin that kills cells by inhibiting protein synthesis; its toxicity is diminished when its cell recognition domain is deleted to produce a 40-kDa protein (PE40).

Methods and Results. A complementary DNA encoding transforming growth factor α (TGFα) was ligated to that encoding PE40 and the chimeric toxin TGFα-PE40, which is cytotoxic to cancer cells displaying epidermal growth factor (EGF) receptors, was expressed in Escherichia coli. The ability of this toxin to kill proliferating SMCs was tested. When cells were seeded at low density (2,500 cells/cm²) and grown in medium supplemented with 10% fetal bovine serum, they were found to be rapidly proliferating; these cells were very sensitive to the cytotoxic effects of TGFα-PE40 (ID₅₀, 4.0±0.17 ng/ml). In contrast, cytotoxicity was 30-fold less (ID₅₀, 125±23 ng/ml; p<0.0004) when cells were in a quiescent state (grown in medium supplemented with 0.5% fetal bovine serum).

Conclusions. Competition studies using excess EGF indicated that the cytotoxic effects of TGFα-PE40 are specifically mediated by the EGF receptor. EGF receptor binding analysis demonstrated that rapidly proliferating SMCs display 10-fold more EGF receptors than do quiescent SMCs in vitro. Thus, a chimeric toxin targeted toward the EGF receptor can selectively kill rapidly proliferating SMCs. Whether this toxin or other chimeric toxins directed against other cell surface receptors will effectively inhibit SMCs proliferating in vivo or be useful in preventing restenosis remains to be determined. (Circulation 1991;84:778–787)

Percutaneous transluminal coronary angioplasty (PTCA) has become a critically important modality in the treatment of coronary artery disease. However, although the initial success rate of PTCA for opening narrowed coronary vessels is very high, late restenosis occurs in 25–50% of patients.¹⁻⁵ This complication has proven refractory to a diverse array of therapeutic attempts, and it remains the major limitation of this procedure.

A great advance in understanding the basic pathophysiological processes of restenosis was made when one of the primary mechanisms responsible for restenosis was identified as activation of medial smooth muscle cells. This event is characterized by the proliferation and migration of smooth muscle cells from the media to the subintima, with ultimate encroachment on the vessel lumen.⁶⁻¹⁰ Identification of this mechanism has led to the evaluation of pharmacological approaches designed to inhibit the processes causing activation of smooth muscle cells.

Our strategy of inhibiting smooth muscle cell proliferation and migration focused on recombinant DNA approaches that have the potential to facilitate...
the development of highly specific targeted therapy. This strategy is based on a hypothesis derived from newer innovative therapeutic approaches to treating cancer, which take advantage of the fact that cancer cells often express high levels of receptors that are usually either absent or present at a low density.11-14 This characteristic of certain transformed cells stimulated the development of cytotoxic agents specifically targeted to the cancer cells that overexpress these receptors.15-20 We speculated that because activated smooth muscle cells also express higher numbers of certain surface receptors than do nonactivated cells, their excessive growth might be controlled by an analogous therapeutic approach.

One group of cytotoxic molecules that has been designed by recombinant DNA techniques to target certain cancer cells is derived from genetically modified Pseudomonas exotoxin A (PE). PE is one of a class of ADP-ribosyl transferases that includes diphtheria toxin, Escherichia coli enterotoxin, and cholera toxin. PE binds to its cell surface receptor, is internalized by coated pits into endocytic vesicles, and is then translocated into the cell cytoplasm.21,22 Once in the cytoplasm, PE inhibits protein synthesis by catalyzing the transfer of the ADP-ribosyl moiety of oxidized nicotinamide-adenine dinucleotide onto elongation factor 2.23,24 Because this factor is required for adding amino acids onto developing protein chains during messenger RNA translation, its inactivation results in cell death. The intact PE molecule, which has a molecular weight of 66 kDa, is cytotoxic to virtually all cells25,26; its toxicity is markedly reduced, however, when its cell recognition domain (domain I) is deleted to produce a 40-kDa protein (PE40; see “Methods”).27 Although the cytotoxicity of this altered molecule is minimal because its cell-binding capacity is lost, it retains its translocating (domain II) and ADP-ribosylating (domain III) activities.28

By expressing the gene fusion created by ligating complementary DNAs encoding various growth factors to the gene fragment encoding PE40, chimeric molecules are produced. These molecules are cytotoxic to cells displaying the ligand-specific growth factor receptor because the cell-killing domain of PE enters the cell when the ligand portion of the fusion protein binds to its receptor. It was the purpose of the present investigation to determine whether epidermal growth factor (EGF) receptor-directed cytotoxicity, using the chimeric toxin TGFα-PE40, could be applied to rapidly proliferating smooth muscle cells, which appear to be an essential component of the restenosis process.

Methods

Bacteria and Plasmids

Competent E. coli strain HB101 cells from Bethesda Research Laboratories were used for transformation and for amplification of plasmids. E. coli BL21 (ADE3) cells, which carry a T7 RNA polymerase gene in a lysogenic and inducible form,29 were used as host for expression of the plasmids carrying the fusion genes.

The cloning strategy for TGFα-PE40 has been described in detail.20 Briefly, the gene encoding PE, which has a molecular weight of 60 kDa, was mutated by deleting domain I, which is the binding domain of PE. The resulting truncated PE gene has a molecular weight of 40 kDa and is referred to as PE40. PE40, which retains domains II and III that encode the translocating and ADP-ribosylating activities of PE, respectively, was ligated to an expression plasmid downstream of the bacteriophage T7 late promoter. The complementary DNA encoding TGFα was ligated immediately 5' to the PE40 gene. The resulting plasmid is depicted in Figure 1. A mutated form of TGFα-PE40, TGFα-PE40<sup>669</sup> (which contains no ADP-ribosylation activity and serves as a control for toxin-mediated cytotoxicity), was constructed by ligating the 0.45-kb BamHI-EcoRI fragment (encapping the Asp553 mutation) derived from a previously described ADP-ribosylation mutant interleukin 6 (IL-6)-PE40<sup>669</sup>, with the 3.5-kb BamHI-EcoRI DNA fragment derived from TGFα-PE40.<sup>30</sup>

Expression of Recombinant Protein

To express the chimeric protein TGFα-PE40, E. coli BL21 (ADE3) cells that were transformed with the plasmid carrying the gene fusion (TGFα-PE40) were cultured to OD<sub>650</sub> of 1.0 and induced for 90 minutes with 1 mM isopropyl-thiogalactoside. The total cell pellet was fractionated, and inclusion bodies, which contained the fusion protein, were isolated from the spheroplast fraction. The details of these procedures have been previously described.<sup>20</sup>

Purification of Recombinant Protein

TGFα-PE40 was found mainly in insoluble form located in the inclusion bodies. The fusion protein was denatured in guanidine-HCl and renatured by

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**Figure 1. Structure of plasmid expressing transforming growth factor α-Pseudomonas exotoxin A with cell recognition domain deleted to produce a 40-kDa protein (TGFα-PE40).** Sequence at junctions between inducible T7 promoter and TGFα-PE40 is shown. Horizontal arrow indicates direction of transcription.
rapid dilution in phosphate-buffered saline. Purification was performed by anion-exchange chromatography (Mono Q, Pharmacia) followed by gel filtration using a TSK-250 column. Fractions containing ADP-riboseylation and cell-killing activity were used for subsequent experiments.20

Other Toxins and Toxin Mutants

Native PE was expressed in a fashion similar to that outlined for TGFα-PE40 and purified from the periplasm fraction of E. coli;31 TGFα-PE40asp553, which has a single amino acid mutation at residue 553 (Glu to Asp), was also expressed and purified in the same way as TGFα-PE40. This mutation eliminates the ADP-riboseylation capacity of domain III, thereby rendering the molecule nontoxic.30

Vascular Smooth Muscle Cells

Cultured vascular smooth muscle cells were isolated from New Zealand White rabbits (weight, 2.5–3 kg) or Sprague-Dawley rats (weight, 200–250 g) by explant method. Briefly, the thoracic aortas were removed in a sterile manner. The adventitia was completely stripped off with fine forceps, and the endothelium was denuded by scraping it with a gauze. The remaining media was minced into approximately 1-mm³ pieces. The media explants were placed into tissue culture dishes containing a small volume of medium-199 (Biofluids) supplemented with 10% fetal bovine serum (FBS; Biofluids), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Five to 7 days later, smooth muscle cells began to grow out from the explants. At confluence they were transferred. The cultures exhibited typical morphological characteristics of vascular smooth muscle cells (spindle shape and hill-and-valley pattern). Furthermore, identification of vascular smooth muscle cells was confirmed by antimuscle actin (HHF-35; kindly provided by Dr. Allen M. Grown) immunocytochemical staining.

Assessment of Capacity to Inhibit Protein Synthesis

Incorporation of [³⁵S]leucine by cultured cells reflects their rate of protein synthesis. The cytotoxic activity of TGFα-PE40 was determined by its capacity to inhibit tritiated leucine incorporation, which correlates with the toxin's cell-killing activity. Thus, aortic smooth muscle cells were seeded in 24-well plates at a density of 2,500 cells/cm² in medium-199 supplemented with 10% FBS. Cells were exposed to various concentrations of toxin 24 hours after seeding; they were incubated at 37°C and remained in contact with the toxins for 48 or 96 hours. The original media was then removed, and leucine-poor media (5 μM leucine) with 10% FBS plus 3.75–5 μCi [³⁵S]leucine were added to each well. The cells were then incubated at 37°C for 4 hours, after which the rate of [³⁵S]leucine incorporation was measured. [³⁵S]Leucine was obtained from Amersham Corp., Arlington Heights, Ill. Six dose–response curves, each determined in duplicate, were performed using rat aortic smooth muscle cells (passages 16 and 17), and five dose–response curves were performed in duplicate using rabbit aortic smooth muscle cells (passages 5 and 6).

Competition Assay

Competition assays were performed by the addition of an excess of recombinant EGF. TGFα-PE40 was added to the smooth muscle cells 24 hours after seeding in either the presence or absence of excess EGF (500 ng/ml). Cells were seeded at a density of 2,500 cells/cm² and grown in medium supplemented with 10% FBS. After a 48-hour incubation period at 37°C, protein synthesis was estimated by the [³⁵S]leucine incorporation assay. For these experiments, cell passage numbers of the smooth muscle cells derived from rat and rabbit aortas were 17 and 5, respectively.

Cell Counting

TGFα-PE40 was found to inhibit leucine uptake of cells grown in 10% FBS. To determine whether the decrease in leucine uptake caused by TGFα-PE40 in 10% FBS reflected a direct effect of TGFα-PE40 on cell viability, the absolute numbers of cells were counted in an additional experiment. Four days after seeding rat aortic smooth muscle cells (2,500 cells/cm²; passage 2), TGFα-PE40 was added. At the end of a 96-hour incubation with the chimeric toxin, cells were counted manually using a hemocytometer.

PE was found to inhibit leucine uptake in both 10% and 0.5% FBS. The question arose as to whether the decreased leucine uptake of quiescent cells (those grown in 0.5% FBS) reflects decreased cell viability or whether decreased protein synthesis in such cells could occur and not lead to cell death. Therefore, in another experiment, which was performed in triplicate, absolute numbers of cells were counted to assess the direct effect of PE on cell viability in 0.5% FBS. Rat aortic smooth muscle cells (50,000 cells/cm²; passage 9) were used; the protocol was like that of the TGFα-PE40 cell-counting experiment, except that cells were counted by Coulter counter.

Thymidine Incorporation

Four days after seeding rat aortic smooth muscle cells (passage 2), [³²P]thymidine was added (4 μCi/ml, 6.7 Ci/mmol; Du Pont Co., Boston). After 24-hour incubation, the cells were washed three times with PBS and extracted with a PHD cell harvester; radioactivity was then measured by a liquid scintillation counter (model 1500 TRI-CARB, Hewlett-Packard Co.).

Characterization of Rapidly Proliferating and Quiescent Smooth Muscle Cells

In the second part of the present study, smooth muscle cells were cultured under conditions facilitating either high or low proliferation rates by growing the cells in medium supplemented with various concentrations of FBS. Thus, rabbit aortic smooth muscle cells (passage 8) were seeded at a density of 2,500 cells/cm² in medium-199 supplemented with 10% FBS to facilitate attachment. After 24 hours, the
medium was changed so that it contained either 0.5%, 1%, 2%, 5%, or 10% FBS. Four days after seeding, one of the following procedures was performed: 1) tritiated thymidine incorporation assay, 2) cell counting using individual wells counted in triplicate through day 10, or 3) EGF binding assay. A growth curve for rat aortic smooth muscle cells (passage 2) was performed using identical conditions. With the rat cells, however, only cell-counting studies were performed, and these were done in the absence of toxins. Cells grown under low serum conditions had a lower doubling time and a lower rate of tritiated thymidine incorporation compared with the values derived from cells grown in medium supplemented with higher concentrations of FBS (Figure 2 and Table 1). Based on these results, the relative effects of the fusion toxin and of PE on quiescent compared with rapidly proliferating smooth muscle cells were obtained using cells (rat cells; passage 2) grown in medium supplemented with either 0.5% or 10% FBS. Toxins were added at day 4; tritiated leucine incorporation assay was performed 48 hours later, and cell-counting studies were performed 96 hours later.

Receptor Binding Assay

Analysis was performed 4 days after seeding rat aortic smooth muscle cells (passage 4), as previously detailed. Cells were grown in either 0.5% or 10% FBS. Binding of \(^{125}\)I-EGF (specific activity, 150–200 \(\mu\)Ci/\(\mu\)g; New England Nuclear, Boston) to intact cultured smooth muscle cells was carried out in 35-mm culture dishes or multiwell plates (six to 12 wells per plate). Cultured smooth muscle cells were washed twice with binding buffer (Dulbecco's modified Eagle's medium, 50 mM BES, and 0.1% bovine serum albumin) and incubated for 4 hours at 4°C with various amounts of \(^{125}\)I-EGF in the presence or absence of 100-fold excess of unlabeled EGF (Upstate Biotechnology, Inc., Lake Placid, N.Y.). The cells were then washed four times with binding buffer and solubilized with 0.5–1.0 ml lysing buffer (0.01 M Tris, 0.5% sodium dodecyl sulfate, and 1 \(\mu\)M EDTA). Total cell-bound radioactivity was measured in a gamma counter. Data were fitted to Scatchard plots by least-squares analysis.

**Results**

Purification of TGF\(\alpha\)-PE40

The purification procedure described in “Methods” resulted in a homogenous monomeric protein with a molecular weight of 46 kDa, visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and staining with Coomassie blue. Its identification as TGF\(\alpha\)-PE40 was confirmed by immunoblot analysis using antibodies to PE (Figure 3).

Cytotoxic Activity

Effects of TGF\(\alpha\)-PE40 on rapidly proliferating smooth muscle cells. Sparsely plated cells (2,500 cells/cm\(^2\)) cultured in medium containing 10% FBS were found to be proliferating at relatively rapid rates (Figure 2 and Table 1). Dose–response curves depicting the inhibitory effects on \(^{3}H\)leucine incorporation of TGF\(\alpha\)-PE40 against rapidly proliferating rabbit and rat smooth muscle cells are shown in Figure 4. Because no significant differences were found between experiments in which the cells were exposed to toxin for 48 compared with 96 hours, these data were pooled. The ID\(50\) values of TGF\(\alpha\)-PE40 against rabbit and rat aortic smooth muscle cells were 15 and 20 ng/ml, respectively.

Direct evidence of cell-killing activity was documented by cell counting: fewer cells survived 96 hours of incubation when they were exposed to the chimeric toxin. In these experiments, the amount of TGF\(\alpha\)-PE40 that reduced the number of living cells by 50% was 7 ng/ml (Figure 5).

Comparative effects of TGF\(\alpha\)-PE40 on rapidly proliferating versus quiescent smooth muscle cells. Culturing cells in medium with low serum concentration encouraged a quiescent state as indicated by the flat growth curve (Figure 2) and low tritiated thymidine incorporation (Table 1). The cytotoxic effect of TGF\(\alpha\)-PE40 on these quiescent cells was significantly lower than that observed on rapidly proliferating cells (Figure 6). The ID\(50\) values against rapidly proliferating and quiescent smooth muscle cells were

![Figure 2. Plot of effects of varying concentrations of fetal bovine serum (FBS) on growth curve of rabbit aortic smooth muscle cells (passage 2) plated at a density of 2,500 cells/cm\(^2\) in 24-well plates. Vertical bars, ±SD.](image)
4.0 ± 0.17 (± SEM) and 125 ± 23 ng/ml, respectively (p < 0.0004).

Effects of native Pseudomonas exotoxin on rapidly proliferating versus quiescent smooth muscle cells. The influence of different rates of smooth muscle cell proliferation on the cytotoxic effects of PE was small (Figure 6). The ID_{50} values against rapidly proliferating and quiescent smooth muscle cells were 1.04 ± 0.25 and 1.68 ± 0.58 ng/ml, respectively (p = NS).

Direct evidence that the decreased leucine uptake reflected cell-killing activity, even in quiescent cells (those growing in 0.5% FBS), was documented by cell counting; fewer cells growing in medium supplemented with 0.5% FBS survived 96 hours of incubation when exposed to PE. In these experiments, the amount of PE that reduced the number of living cells by 50% was 0.7 ng/ml (Figure 5).

Receptor Mediation of Cytotoxic Effects of TGFα-PE40

To determine whether the cytotoxic effects of TGFα-PE40 are specifically mediated by its binding to the EGF receptor, a competition assay using EGF was performed on smooth muscle cells growing in 10% FBS. Excess EGF completely blocked the inhibitory effects of TGFα-PE40 (Figure 7), whereas EGF alone exerted no effect on leucine incorporation (data not shown).

EGF Receptor Analysis of Rapidly Proliferating Versus Quiescent Smooth Muscle Cells

Quantitation of EGF receptor sites was performed using rat smooth muscle cells grown under conditions of either rapid or quiescent growth. Specific binding of [¹²⁵I]EGF was determined (Figure 8A) and found to constitute approximately 67% of total bound [¹²⁵I]EGF for quiescent smooth muscle cells and 94% of total bound [¹²⁵I]EGF for rapidly proliferating smooth muscle cells. Scatchard analysis is depicted in Figure 8B. The quiescent cells displayed a single set of high-affinity binding sites estimated to be 4,200 sites/cell with a dissociation constant of 2 × 10^{-10} M. The rapidly proliferating cells displayed two receptor classes as determined by Scatchard analysis. The number of high-affinity binding sites per cell was estimated to be 12,000, whereas the number of sites...
and together, on smooth muscle protein synthesis were determined by using the same molar concentrations used for the chimeric protein; no cytotoxic activity was demonstrated (Figure 9). To demonstrate that the cytotoxic effects of TGFα-PE40 were a result of the chimeric protein and not of a bacterial contaminant derived during protein expression (e.g., E. coli endotoxin), dose-response curves using an inactive TGFα-PE40 mutant were determined (TGFα-PE40asp553). No cytotoxic effect of this molecule on the smooth muscle cells was found despite the fact that it was cloned, expressed, and purified using methods identical to those used for TGFα-PE40 (Figure 9).

Discussion

A major breakthrough in understanding the pathophysiological processes involved in restenosis after PTCA was the recognition that a critical step in the chain of events leading to restenosis is injury-induced activation of medial smooth muscle cells, which causes their proliferation and migration to the sub-intima6-10; if this "healing" response is excessive, it leads to luminal narrowing and restenosis.

A further key to our understanding of the restenosis process, and thereby of its treatment, was the finding that smooth muscle cells have the capacity to express, either constitutively, in response to injury, or in response to being placed in culture, growth factors and cytokines such as platelet-derived growth factor (PDGF); fibroblast growth factor, both acidic and basic forms (FGF); interleukin 1 (IL-1); interleukin 6 (IL-6); insulin-like growth factors I and II (IGF-I and IGF-II); and transforming growth factor β (TGFβ).32-41 Furthermore, smooth muscle cells also exhibit a mitogenic response to several of these factors, indicating that receptors for some of these ligands must be present. In fact, recent studies have provided evidence that smooth muscle cells do express receptors for PDGF, EGF, FGF, and IGF-I.40,42-47

**Figure 5.** Plot of cytotoxic effects of transforming growth factor α-Pseudomonas exotoxin A with cell recognition domain deleted to produce a 40-kDa protein (TGFα-PE40) on rapidly proliferating rat aortic smooth muscle cells (passage 2, growing in medium supplemented with 10% fetal bovine serum [FBS]) and of PE in quiescent rat aortic smooth muscle cells (passage 9, growing in medium supplemented with 0.5% FBS). Smooth muscle cells were exposed to toxins for 96 hours, at which time cell number was determined.

**Figure 6.** Plot of relative cytotoxic effects of transforming growth factor α-Pseudomonas exotoxin A with cell recognition domain deleted to produce a 40-kDa protein (TGFα-PE40) and of PE on rapidly proliferating cells (grown in medium supplemented with 10% fetal bovine serum [FBS]) compared with quiescent smooth muscle cells (grown in medium supplemented with 0.5% FBS). Bars, ±SD. For these experiments, rat smooth muscle cells (passage 2) were used.

**Figure 7.** Plot of competition of cytotoxic activity of transforming growth factor α-Pseudomonas exotoxin A with cell recognition domain deleted to produce a 40-kDa protein (TGFα-PE40) by coincubation with an excess of epidermal growth factor (EGF). Rabbit (passage 5) and rat (passage 17) aortic smooth muscle cells (SMC) were used. Each curve represents data from one experiment performed in duplicate.
The strategy for inhibiting the restenosis process we were particularly interested in exploring was based on the observation that when cancer cells express much larger numbers of certain cell surface receptors than their normal counterparts, they can be selectively targeted and killed. Thus, chimeric toxins, produced by expressing the recombinant genes encoding a growth factor (TGFα, IL-2, IL-4, or IL-6) fused to PE40, have been used successfully for specific targeting and killing of cancer cells overexpressing the growth factor’s receptor.\(^{11,17-20,48,49}\)

Given this background, we tested our hypothesis that a similar targeting strategy might be successful when directed to rapidly proliferating but nontransformed smooth muscle cells. To this end, we determined whether TGFα-PE40, a chimeric toxin previously found to be extremely potent in killing certain cancer cells,\(^{20}\) would be cytotoxic to rapidly proliferating smooth muscle cells.

Several potentially important observations derive from our investigation. First, we found that TGFα-PE40 is cytotoxic in vitro to aortic smooth muscle cells derived from rats and rabbits, exhibiting an ID\(_{50}\) of 4–20 ng/ml when directed against smooth muscle cells plated at low density (2,500 cells/cm\(^2\)) and cultured in serum-rich media (Figures 4 and 5). The cells growing under these conditions have a high tritiated thymidine uptake (indicative of a high rate of DNA synthesis) and a rapid doubling time (Table 1 and Figure 2). The cytotoxic effects were observed with cells of early or late passage numbers. This is most easily seen by comparing Figure 4 (rat smooth muscle cells; passages 16 and 17) with Figure 6 (rat smooth muscle cells; passage 2).

TGFα has 30% amino acid homology to EGF, including the conservation of all six of EGF’s cysteines. The two ligands are recognized by the binding site of the EGF receptor with practically identical affinities.\(^{50-54}\) This latter fact undoubtedly explains the finding that cytotoxic activity of TGFα-PE40 is lost when it is coincubated with an excess of EGF (Figure 8) (e.g., its cell-killing effect results from its binding to the EGF receptor).

If TGFα-PE40 killed all smooth muscle cells with which it came into contact, it would have little therapeutic potential. However, we found that the molecule had considerably less cytotoxicity against quiescent compared with rapidly proliferating smooth muscle cells (Figure 6). The greater cytotoxic capacity against rapidly proliferating cells could result from any of several possible mechanisms. For example, the rapidly proliferating cells could more efficiently internalize the toxin or more efficiently translocate it from the endocytic compartment to the cytoplasm. This possibility is unlikely because there was no statistically significant difference in cytotoxicity when native Pseudomonas exotoxin was added to either rapidly proliferating or quiescent smooth muscle cells (Figure 6). Alternatively, the EGF receptor
could be upregulated or have a higher binding affinity during the culture conditions that caused rapid cell proliferation. In this regard, cultured smooth muscle cells in the exponential phase of growth have been shown to bind more EGF than do quiescent cells.\(^4\)\(^7\)

Other studies have shown that cell surface receptors for several growth factors, including EGF, are fewer in number in cells grown at high density than in those grown at low density.\(^5\)\(^5\)\(^–\)\(^5\)\(^9\) Although these latter differences were ascribed to effects of cell density on receptor number, an effect of proliferation rate was not ruled out. Our data clearly show that rapidly proliferating smooth muscle cells express 10-fold more EGF receptors per cell (44,000 versus 4,200) than quiescent smooth muscle cells (Figure 8), which may result in more EGF binding and thereby account for some of the increased cytotoxicity of rapidly growing smooth muscle cells to TGF\(\alpha\)-PE40. Our data therefore suggest that in vitro rapidly proliferating smooth muscle cells overexpress EGF receptors and a chimeric toxin can be specifically targeted to such cells. These findings may provide the basis by which smooth muscle cells that have responded to vascular injury after PTCA by rapidly proliferating—that is, those cells destined to compromise vascular patency and cause restenosis after PTCA—can be selectively killed.

Because the gene fusion encoding TGF\(\alpha\)-PE40 is expressed in E. coli, contamination with E. coli endotoxin is inevitable. It was therefore imperative to determine that the cytotoxic effects of TGF\(\alpha\)-PE40 result from its intrinsic activity rather than from contaminating bacterial products. That this is the case is demonstrated by several lines of evidence. First, the cytotoxic effects of TGF\(\alpha\)-PE40 are eliminated when the chimeric toxin is co-incubated with an excess of EGF (Figure 8), which competes with TGF\(\alpha\)-PE40 for the EGF receptor. This effect should be specific for TGF\(\alpha\)-PE40 and should not be seen if the cytotoxic effect were the result of a bacterial product. Second, TGF\(\alpha\)-PE40\(^{6}^\)\(^5\)\(^5\), a mutant of TGF\(\alpha\)-PE40 genetically engineered to be devoid of cytotoxic activity\(^6\)\(^0\) that was cloned, expressed, and purified in a manner identical to that used to produce TGF\(\alpha\)-PE40, was found to exert no cytotoxic action on smooth muscle cells (Figure 9).

TGF\(\alpha\)-PE40 is just one of many possible ligand-toxin fusions that might be used to target rapidly proliferating smooth muscle cells. Preliminary results in our laboratory have shown that other ligands that have been genetically fused to PE40 (e.g., acidic FGF and IGF-I) exert cytotoxic effects on smooth muscle cells (personal observation); so also does the agent produced by chemical conjugation of basic FGF to saporin (a potent plant toxin).\(^6\)\(^0\)

The in vitro studies we have reported raise the intriguing possibility that use of recombinant techniques to produce “killer” molecules that selectively target actively proliferating cells might be applied to the problem of restenosis after PTCA. It remains to be seen, however, not only whether the selective cytotoxic effects achieved in vitro can be achieved in vivo but also whether they can be achieved without the complication of important deleterious actions on either the healing response to vascular injury, the vascular wall, or nonvascular cells and tissues.

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