In Vitro Effects of a Recombinant Toxin Targeted to the Fibroblast Growth Factor Receptor on Rat Vascular Smooth Muscle and Endothelial Cells

Sadatoshi Biro, Clay B. Siegall, Ya-Min Fu, Edith Speir, Ira Pastan, and Stephen E. Epstein

The dominant mechanism responsible for restenosis after angioplasty is believed to be the activation of medial smooth muscle cells (SMCs), leading to their proliferation, migration to the subintima, and further proliferation. To develop novel strategies that might inhibit or prevent restenosis, we previously used a chimeric toxin composed of transforming growth factor-α (which targets the epidermal growth factor receptor) and mutated Pseudomonas exotoxin to preferentially recognize and kill rapidly proliferating, versus quiescent, vascular SMCs. We have recently cloned and expressed a recombinant gene encoding Pseudomonas exotoxin with a mutated (nonfunctional) cell recognition domain fused with the ligand acidic fibroblast growth factor, termed aFGF-PE664 GIUKDEL; thus, this recombinant toxin targets the fibroblast growth factor receptor. In the present study, we evaluated the relative effects of this chimeric toxin on quiescent versus rapidly proliferating vascular SMCs and also determined whether aFGF-PE664 GIUKDEL exerted different effects on SMCs versus endothelial cells. Rapidly proliferating SMCs (grown in 10% fetal bovine serum) were very sensitive to the cytotoxic effects of aFGF-PE664 GIUKDEL, whereas cytotoxicity was significantly less when the SMCs were in a quiescent state (grown in medium supplemented with 0.5% fetal bovine serum). The chimeric toxin was also significantly less cytotoxic against endothelial cells. Competition studies using excess acidic fibroblast growth factor indicated that the cytotoxic effects are specifically mediated by the fibroblast growth factor receptor. Thus, the present studies suggest a potentially expanded role of recombinant toxin therapy in restenosis: multiple receptors can be targeted, and cytotoxic effects, at least in vitro, can be preferentially directed to rapidly proliferating vascular SMCs, with relative sparing of vascular endothelial cells. It will next be necessary to test this strategy for inhibiting restenosis in an in vivo model of vascular injury and SMC proliferation.

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Key Words • percutaneous transluminal coronary angioplasty • restenosis • Pseudomonas exotoxin • acidic fibroblast growth factor • angioplasty • vascular smooth muscle cells

Percutaneous transluminal coronary angioplasty (PTCA) is an increasingly important approach to the treatment of ischemic heart disease. However, its major limitation is restenosis, which occurs in 25–50% of patients within 6 months of PTCA.1–5 A high incidence of restenosis also follows laser angioplasty,6 atherectomy,7 and implantation of stents.8 Pathological observations in patients after coronary angioplasty have revealed that smooth muscle cells (SMCs) are present in restenotic lesions,9–11 and experimental studies have shown that the major response to experimental vascular injury is activation of SMCs, which is characterized by SMC proliferation, migration to the subintima, and further proliferation.12–14 It is this event that is now believed to be the dominant mechanism responsible for restenosis occurring after angioplasty.

We have attempted to inhibit SMC proliferation by targeting rapidly proliferating SMCs with chimeric toxins that recognize receptors that are expressed in high numbers during rapid cell growth. The “killer” molecule we have used is a mutated form of Pseudomonas exotoxin (PE). After binding to its cell surface receptor, PE is internalized into endocytic vesicles and then is translocated into the cell cytoplasm, where it inhibits protein synthesis by ADP ribosylation of elongation factor 2.15–19 Native PE is cytotoxic to most cells. However, by deleting or mutating its cell binding domain (domain I), the molecule’s cytotoxicity is markedly reduced, even though it retains its translocation (domain II) and ADP-ribosylation (domain III) activities.20

A targeted killer molecule can be created by expressing the gene fusion produced by ligating the mutated PE gene to cDNAs encoding ligands recognizing any of several cell surface receptors. The resulting chimeric proteins are toxic to cells displaying the specific cell surface receptor recognized by the ligand. Using this strategy, we previously demonstrated that a chimeric

From the Cardiology Branch (S.B., Y.-M.F., E.S., S.E.), and the Laboratory of Molecular Biology (I.P.), Division of Cancer Biology, Diagnosis and Centers National Cancer Institute, National Institutes of Health Bethesda, Md., and Bristol-Myers Squibb (C.B.S.), Pharmaceutical Research Institute, Wallingford, Conn.

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toxin, which contains a ligand that recognizes the epidermal growth factor (EGF) receptor, preferentially targets and kills in vitro rapidly proliferating versus quiescent SMCs.21

We have recently cloned and expressed a recombinant gene encoding PE with a mutated (nonfunctional) cell recognition domain fused with the ligand acidic fibroblast growth factor (aFGF), termed aFGF-PE66GluKDEL, which is targeted to the fibroblast growth factor (FGF) receptor.22 In an attempt to expand our options for targeting SMCs with recombinant toxins, we have evaluated in the present study the relative effects of this chimeric toxin on quiescent versus rapidly proliferating SMCs. In addition, endothelial cells usually exert a dominant inhibitory effect on SMC proliferation.23 It would thus be desirable, in the context of restenosis, that the fusion toxin exert less of a cytotoxic effect on endothelial cells than on SMCs. Therefore, a second important goal of this investigation was to determine whether such a differential effect exists.

Materials and Methods

Vascular Smooth Muscle Cells

Cultured vascular SMCs were isolated from Sprague-Dawley rats (200–250 g) by the explant method, as previously described.21 SMCs were maintained in medium 199 (M199, Biofluids, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum (FBS, Biofluids), 100 units/ml penicillin, and 100 μg/ml streptomycin. Identification of SMCs was confirmed by typical morphological character (spindle shape, hill and valley pattern) and immunocytochemical staining for antimuscle actin (HHF35, kindly provided by Dr. Allen M. Gown, Department of Pathology, University of Washington, Seattle). Vascular SMCs were used between passages 4 and 9. Rapid cell proliferation was achieved by culturing cells in M199 containing 10% FBS after plating at 2,500 cells/cm². Quiescent cells were studied by plating at 10,000 cells/cm² and maintaining them in M199 containing 0.5% FBS.

Vascular Endothelial Cells

Rat vascular endothelial cells were isolated from the supernatant of collagenase-digested aortas. Briefly, minced aortas without adventitia were incubated with 0.1% collagenase (198 units/mg type II, Worthington Biochemical Corp., Freehold, N.J.) for 1 hour with agitation. The cells in suspension were seeded in culture dishes. Several days later, actively growing colonies were transferred to new dishes by aspirating the cells with a pipette (Eppendorf Inc., Fremont, Calif.) fitted with a sterile 200-μl tip. Endothelial cells were grown in Dulbecco’s minimum essential medium (Biofluids) with high glucose (4.5 g/l), 10% FBS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.24 Identification of vascular endothelial cells was confirmed by typical morphology (polygonal and monolayer cells) and acetylated low density lipoprotein staining. Vascular endothelial cells were used between passages 4 and 9. They were seeded at a density of 2,500 cells/cm², grown in 10% FBS, and exposed to various concentrations of aFGF-PE66GluKDEL under the exact protocol used for SMCs.

Chimeric Toxin

The plasmid encoding aFGF-PE66GluKDEL (Figure 1) was constructed as described.22 Briefly, this plasmid contains a mutated PE gene (which encodes a protein with a molecular mass of 66 kd that has glutamates substituted for basic amino acids at residues 57, 246, 247, and 249 of PE). These mutations in domain I inactivate the cell binding domain of PE and thereby reduce its toxicity in animals.25 In addition, the mutated PE has REDLK at positions 609–613 replaced by KDEL (single-letter representation of amino acids). KDEL is an endoplasmic reticulum retention sequence that renders the molecule more potent.26 The plasmid containing the mutated gene PE66GluKDEL was digested with the restriction enzymes Xba I and HindIII. The 5,000-bp DNA fragment was isolated and ligated to a 400-bp DNA fragment isolated from the plasmid encoding aFGF-PE4022 and digested with the same restriction enzymes. The resulting plasmid contains, in 5' to 3' order, the T7 late promoter, a cDNA encoding aFGF, a cDNA fragment encoding PE66GluKDEL, and a transcription terminator.

Mutant Toxin

aFGF-PE66GluAsp553KDEL, which has a single amino acid mutation at residue 553 (Glu to Asp), was expressed and purified in the same way as aFGF-PE66GluKDEL. This mutation eliminates the ADP-ribosylation capacity of domain III, thereby rendering the molecule nontoxic.27

Expression and Purification of the Chimeric Toxins

Escherichia coli BL21 (ADE3) cells were transformed with the relevant plasmids. The cells were grown in super broth; when the OD value of absorbance at the 650-mm wavelength reached 1.5 in the medium, the cells were induced with 1 mM isopropyl thiogalactoside. After 90 minutes, the total cell pellet was harvested, and inclusion bodies, which contain the fusion protein, were isolated from the spheroplast fraction. The details of these procedures have been previously described.22

To purify the chimeric toxins, the inclusion bodies were denatured in guanidine HCl and renatured by rapid dilution in phosphate-buffered saline. The refolded protein was dialyzed and applied onto a Q Sepharose (fast flow) column attached to a Pharmacia fast protein liquid chromatography column at a flow rate of 4 ml/min. Pooled fractions from Q Sepharose
columns were subjected to heparin affinity chromatography. Purified proteins were demonstrated by Western blot analysis.22

Assessment of Capacity to Inhibit Protein Synthesis

The cytotoxic activity of aFGF-PE6664GluKDEL was determined by its ability to inhibit the cells' capacity to incorporate radiolabeled leucine into protein. Cells were seeded in 48-well plates in medium supplemented with 10% FBS (for rapidly proliferating cells) or 0.5% FBS (for quiescent cells), and 4 days later, various concentrations of aFGF fusion toxin were applied for 48 hours. The original medium was then removed, and leucine-poor medium (5 μM leucine) with 0.5% or 10% FBS plus 5 μCi [³H]leucine was added to each well. The cells were then incubated at 37°C for 4 hours, after which the rate of [³H]leucine incorporation was measured. [³H]Leucine was purchased from Amersham Corp., Arlington Heights, Ill.

Competition Assay

Competition assay was performed by adding excess aFGF (1,000-fold) with aFGF fusion toxin to the smooth muscle cells 4 days after seeding. Cells were seeded at a density of 2,500 cells/cm² and grown in medium with 10% FBS. After 48 hours of incubation, protein synthesis was estimated by the [³H]leucine incorporation assay.

Cell Counting and Cell Staining

Four days after seeding, cells were exposed to various concentrations of aFGF fusion toxin for 96 hours. For cell counting, the cells were then washed twice with phosphate-buffered saline (Biofluids), trypsinized, and counted with an electronic cell counter (Particle Data, Inc., Elmhurst, Ill.). For cell staining, the cells were washed with phosphate-buffered saline, stained with methylene blue (0.5% in 50% phosphate-buffered saline and 50% methanol) for 15 minutes, and washed with H₂O. Methylene blue only stains living cells.

Cell-Killing Assay

Rat SMCs were seeded in 96-well microtiter plates (2×10⁴ cells/100 μl per well) and incubated overnight. aFGF-PE6664GluKDEL (100 ng/ml) was added to triplicate wells and incubated for 24 or 48 hours before analysis. To count the number of dead cells versus the number of viable cells, all medium was removed, and 100 μl trypsin-EDTA was added, followed by 50 μl trypan blue dye. Cells were dispersed and counted on a hemocytometer based on whether they took up the trypan blue stain (dead) or excluded the stain (viable). Three fields were averaged for each data point; there were approximately 40 cells per high-power field after 24 hours of exposure to aFGF-PE6664GluKDEL and 70 cells at 48 hours. The data are presented as the percentage of total cells that were dead per high-power field.

Statistical Analysis

Concentration–response curves were compared using the t test within analysis of variance.

Results

Effects of aFGF-PE6664GluKDEL on Smooth Muscle Cells

Concentration–response curves depicting the inhibitory effects on [³H]leucine incorporation of aFGF-PE6664GluKDEL against rapidly proliferating versus quiescent SMCs are shown in Figure 2; the toxin was markedly cytotoxic to rapidly proliferating SMCs and significantly less so against quiescent cells (at 10 ng/ml, p<0.05; at 100 ng/ml, p<0.001).

To determine whether protein synthesis inhibition reflected cell killing, we performed cell-staining and cell-counting assays. Cells were treated with aFGF-PE6664GluKDEL and counted or stained with methylene blue or with trypan blue as described in “Materials and Methods.” Cell counting and cell staining with methylene blue demonstrated fewer SMCs when cells were exposed to aFGF-PE6664GluKDEL, an effect that was dose related (Figures 3 and 4). Since these results could be due to inhibition of proliferation rather than to cell killing, a cell-killing assay was performed using the trypan blue dye exclusion assay. Of the total cells counted per high-power field, 11% were dead at 24 hours, and 32% were dead at 48 hours. However, no dead cells were detected in the control groups.

Specificity of the Effects of aFGF Fusion Toxin on Protein Synthesis Inhibition

To determine whether the cytotoxic effects of aFGF-PE6664GluKDEL were due to the chimeric toxin itself and not to a bacterial contaminant derived during protein expression (such as E. coli endotoxin), dose–response curves using an inactive aFGF fusion toxin mutant were determined (aFGF-PE6664GluKDEL). No cytotoxic effect of this molecule on the SMCs was found at doses up to 1,000 ng/ml (Figures 3 and 5).

Receptor Mediation of Cytotoxic Effects of aFGF Fusion Toxin

To determine whether the cytotoxic effects of aFGF fusion toxin are specifically mediated by its binding to the FGF receptor, a competition assay using aFGF was performed on SMCs growing in 10% FBS. Excess aFGF...
Effects of aFGF-PE66\textsuperscript{Glu}KDEL on Endothelial Cells

Dose–response curves depicting the inhibitory effects on [\textsuperscript{3}H]leucine incorporation of aFGF-PE66\textsuperscript{Glu}KDEL against endothelial cells are shown in Figure 6. The sensitivity of endothelial cells to the fusion toxin was significantly less (\(p<0.0025\)) than that of rapidly proliferating SMCs at 10 ng/ml and at 100 ng/ml (Figure 6). Most interestingly, the concentration of toxin that exerted a nearly 50% inhibitory effect on rapidly proliferating SMCs (10 ng/ml) exerted no inhibitory effect on endothelial cells.

Discussion

We previously showed that rapidly proliferating SMCs overexpress the EGF receptor and that the chimeric toxin TGF\(\alpha\)-PE40 (transforming growth factor-\(\alpha\) [TGF\(\alpha\)] is recognized by the EGF receptor) has a 10-fold greater capacity to kill, in vitro, rapidly proliferating versus quiescent SMCs.\textsuperscript{21} These findings raised the possibility that such an approach might be used to inhibit the development of restenosis after coronary angioplasty.

However, we were also eager to determine whether the repertoire of ligand-directed cytotoxic molecules produced by recombinant DNA technology and directed against rapidly proliferating versus quiescent SMCs could be expanded. Our reasons were to ensure that the particular receptor chosen to be targeted by a fusion toxin would lead to the greatest cytotoxic effects against rapidly proliferating SMCs and to identify other receptors that, when targeted by a fusion toxin, might have additive or synergistic effects with TGF\(\alpha\)-PE40. Moreover, TGF\(\alpha\)-PE40 is toxic to the liver, undoubtedly because liver cells express large numbers of EGF recep-

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\caption{Photograph showing cytotoxic activity of recombinant toxin aFGF-PE66\textsuperscript{Glu}KDEL and an aFGF-PE66\textsuperscript{Glu}KDEL mutant against smooth muscle cells. The cells were plated in medium with 10% fetal bovine serum, incubated with aFGF-PE66\textsuperscript{Glu}KDEL (row A) or with an aFGF-PE66\textsuperscript{Glu}KDEL mutant (aFGF-PE66\textsuperscript{Glu} Ap553KDEL) (row B) for 96 hours, and stained with methylene blue. Acidic fibroblast growth factor (aFGF) toxin concentrations were as follows: column 1, no protein added; column 2, 1 ng/ml; column 3, 10 ng/ml; column 4, 50 ng/ml; column 5, 250 ng/ml; column 6, 1,000 ng/ml.}
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\caption{Graph showing relative cytotoxic effects of recombinant toxin aFGF-PE66\textsuperscript{Glu}KDEL (aFGF-PE), aFGF-PE66\textsuperscript{Glu}KDEL mutant (aFGF-PE66\textsuperscript{Glu} Ap553KDEL [aFGF-PE553]), and aFGF-PE66\textsuperscript{Glu}KDEL plus excess (\(\times1,000\)) acidic fibroblast growth factor (aFGF-PE+aFGF) on rapidly proliferating smooth muscle cells (grown in medium with 10% fetal bovine serum). The data represent the mean±SD of triplicate samples.}
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\begin{figure}
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\includegraphics[width=\textwidth]{fig5}
\caption{Graph showing effect of acidic fibroblast growth factor (aFGF) fusion toxin on rapidly proliferating smooth muscle cells, assessed by cell counting. The data represent the mean±SD of triplicate samples.}
\end{figure}
DOSE OF FUSION TOXIN

FIGURE 6. Graph showing relative cytotoxic effects of recombinant toxin aFGF-PE66KDEL on rapidly proliferating smooth muscle cells (SMCs) and rapidly proliferating endothelial cells (ECs). aFGF-PE66KDEL is less cytotoxic to ECs (passage 7) than to SMCs (passage 7). The data represent the mean ± SEM of the percentage of control.

Therefore, it was our aim to develop fusion toxins that would allow us, to the greatest extent possible, to selectively target and kill rapidly proliferating SMC cells versus cells of all other normal tissues.

In the present investigation, we assessed the effects of a chimeric toxin produced from a gene fusion containing the cDNA encoding aFGF and a mutated form of the gene encoding PE. (Mutations of PE at positions 57, 246, 247, and 249 inactivate the cell binding domain of PE, and replacement of REDLK with KDEL at the carboxy end increases its cytotoxic activity.) We found that aFGF-PE66KDEL was markedly cytotoxic to rapidly proliferating SMCs, an effect significantly greater than that seen against quiescent SMCs. Its approximate ID₅₀ against rapidly proliferating SMCs (15 ng/ml) is similar to that found using TGFα-PE40 (4.0 ng/ml). Its effects are receptor mediated, as the cytotoxic effects of the fusion toxin are greatly reduced when it is coinoculated with an excess of recombinant aFGF. Additionally, aFGF-PE66KDEL, a mutant of aFGF fusion toxin genetically engineered so that it is devoid of ADP-ribosylation activity but cloned, expressed, and purified in an identical manner to that used to produce the parent active molecule, had no cytotoxic effects on SMCs (Figures 3 and 5).

Our finding that aFGF-PE66KDEL was considerably more cytotoxic against rapidly proliferating SMCs than against quiescent SMCs may be of practical importance, because aFGF-PE66KDEL would have little therapeutic potential if it killed all SMCs. The greater cytotoxic activity against rapidly proliferating cells raises the possibility that there may be a therapeutic window for the fusion toxin, in that it might be possible to target and selectively kill those SMCs that are activated and hence proliferate after angioplasty.

Although endothelial cells secrete substances that can either inhibit or stimulate SMC proliferation, they normally inhibit SMC proliferation. When angioplasty causes intimal denudation of endothelial cells, the regrowth of such cells (if they are functionally normal) would be expected to inhibit the subsequent development of excessive SMC proliferation and, thereby, the development of restenosis. Therefore, we determined whether the cytotoxic activity of the aFGF fusion toxin against SMCs is different from that against endothelial cells. We found that endothelial cells are significantly less sensitive to the cytotoxic effects of the fusion toxin than are SMCs (Figure 6).

These in vitro studies provide further evidence suggesting a role for recombinant DNA–derived targeted therapy in restenosis. The main cause of restenosis after PTCA is injury-induced activation of SMCs, leading to their proliferation, migration to the subintima, and further proliferation. Although the mechanisms responsible for these events are poorly understood, the present investigation demonstrates the in vitro effectiveness of targeting the FGF receptor with a fusion toxin, and our prior study demonstrated the effectiveness of targeting the EGF receptor. Moreover, preliminary results reported by Cassells et al. indicate that the agent produced by the chemical conjugation of basic FGF to saporin (a potent plant toxin) also exerts cytotoxic effects on SMCs. Thus, multiple receptors can be targeted, and cytotoxic effects, at least in vitro, can be preferentially directed against rapidly proliferating SMCs, with relative sparing of quiescent SMCs and endothelial cells.

It must be emphasized, however, that the in vivo situation is considerably more complex. For example, the cytotoxic effects of the fusion toxins might cause dying SMCs to release FGF and other growth factors, which could stimulate contiguous SMCs to proliferate, and also saturate local FGF receptors and thereby inhibit the influence of the fusion toxin on other cells. Clearly, the next step in testing this strategy for inhibiting restenosis will be to determine whether the success achieved in vitro can also be achieved in vivo.

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