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Research Article

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Prevention of Smooth Muscle Cell Outgrowth from Human Atherosclerotic Plaque by a Recombinant Cytotoxin Specific for the Epidermal Growth Factor Receptor

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Abstract

Smooth muscle cell proliferation in the intima of arteries is a principal event associated with vascular narrowing after balloon angioplasty and bypass surgery. Techniques for limiting smooth muscle cell proliferation, however, have not as yet yielded any therapeutic benefit for these conditions. This may reflect the present lack of sufficiently potent and specific inhibitors of smooth muscle cell proliferation. DAB_{389} EGF is a genetically engineered fusion protein in which the receptor-binding domain of diphtheria toxin has been replaced by human epidermal growth factor. We evaluated the effect of this fusion toxin on human vascular smooth muscle cells in culture. Incubation of proliferating cells with $DAB_{389}EGF$ yielded a dose-dependent inhibition of protein synthesis, as assessed by uptake of [^{3}H]leucine, with an IC_{50} of 40 pM. The cytotoxic effect was inhibited in the presence of excess EGF or with monoclonal antibody to the EGF receptor. We further studied the effect of the fusion toxin on smooth muscle cell outgrowth from human atherosclerotic plaque. Outgrowth was markedly inhibited after as little as 1 h of exposure to the fusion protein. Furthermore, complete inhibition of proliferation of cells within the plaque could be attained. These results demonstrate that $DAB_{389}EGF$ is highly cytotoxic to human smooth muscle cells proliferating in culture and can prevent smooth muscle cell outgrowth from "growth-stimulated" human atherosclerotic plaque. $DAB_{389}EGF$ may therefore be of therapeutic value in vascular diseases characterized by smooth muscle cell accumulation. (*J. Clin. Invest.* 1993. 91:724–729.) Key words: vascular smooth muscle cell • atherosclerosis • fusion protein • epidermal growth factor receptor

Introduction

Approximately 40% of atherosclerotic arteries treated by balloon angioplasty develop a recurrent lesion at the site of the angioplasty procedure in the six months following this intervention (1, 2). In animals, a principal response to vascular injury is the rapid accumulation of smooth muscle cells in the intima of the vessel wall (3–5). In human lesions that develop after angioplasty (restenosis lesions), an abundance of smooth muscle cells is a consistent histologic feature (6–9). Rapid accumulation of intimal smooth muscle cells is therefore considered to be an important process contributing to the occurrence of restenosis after angioplasty, and to the formation of other rapidly developing lesions, such as those that develop after coronary and peripheral artery bypass surgery, cardiac transplantation, and carotid and coronary endarterectomy (10). These forms of atherosclerosis have, to date, been refractory to a variety of mechanical and pharmacological therapies. This may reflect, in part, the present lack of sufficiently potent and specific inhibitors of smooth muscle cell accumulation. Furthermore, although certain agents demonstrate efficacy in animal models, there are no reports demonstrating efficacy specifically with human vascular smooth muscle cells, including those within or derived from atherosclerotic lesions.

EGF is a mitogen for vascular smooth muscle cells. Recently, smooth muscle cells proliferating in culture were found to express a higher number of binding sites for EGF than nonproliferating smooth muscle cells in culture (11). A differential expression of EGF receptors may provide the opportunity for using cytotoxic agents that specifically target proliferating smooth muscle cells, and thereby inhibit their repopulation of the arterial intima. Targeted delivery of cytotoxins to specific cell surface receptors may be accomplished with recombinant fusion proteins. One such molecule, $DAB_{389}EGF$, has recently been engineered and has been shown to be cytotoxic to EGF receptor–bearing tumor cells (12). The hybrid gene for this protein consists of the diphtheria toxin gene with the sequences encoding the receptor binding domain replaced by those coding for human EGF. The mechanism of toxicity is similar to that of native diphtheria toxin (12–15). After receptor binding and internalization of the protein, the enzymatically active fragment A of diphtheria toxin catalyzes the nicotinamide adenine dinucleotide-dependent ADP-ribosylation of elongation factor 2. This reaction arrests protein synthesis, causing cell death.

We have investigated the effect of this recombinant toxin on proliferating human vascular smooth muscle cells. We found that $DAB_{389}EGF$ is highly cytotoxic to human smooth muscle cells and we demonstrated that this effect is mediated by the EGF receptor. Furthermore, the fusion protein can inhibit smooth muscle cell outgrowth from explanted human atheroma, and can prevent smooth muscle cell proliferation within these growth-stimulated lesions.

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Methods

Bacteria strains. Escherichia coli strain DH5αF′ (Bethesda Research Laboratories, Gaithersburg, MD) was used for M13 cloning. Strain SCS1 (Stratagene Inc., La Jolla, CA) was used as the host for the production of $DAB_{389}EGF$.

Construction of vectors. Construction of the plasmid used to express $DAB_{389}EGF$ has been described previously (12). Briefly, this entailed modifying a parental plasmid, pSI123, which encodes the fusion protein $DAB_{486}IL\text{-}2$, consisting of the first 485 amino acids of diphtheria toxin fused to a synthetic IL-2 gene (16). A synthetic gene for EGF was annealed and ligated to M13mp18. A clone designated M13EGF#2 was confirmed to have the correct sequence and was used to replace the IL-2 coding region from pSI123 with the EGF coding region. This yielded the plasmid pSE1 which has been used to express $DAB_{486}EGF$ (12). The gene encoding $DAB_{389}EGF$ was constructed by digestion of the plasmid pSE1 with ClaI and SphI and ligation with the ClaI/SphI fragment of pDW27 (17). The resulting plasmid, pSE5, contains a 97 amino acid deletion of the diphtheria toxin. The expressed fusion protein, $DAB_{389}EGF$, has been shown to be 10–100-fold more cytotoxic to EGF receptor-expressing tumor cell lines than $DAB_{486}EGF$ (12).

Expression and purification of $DAB_{389}EGF$. To express $DAB_{389}EGF$, *E. coli* strains were transformed with plasmid pSE5, then grown for 13–17 h at 30°C, and induced with isopropyl-β-D-thiogalactopyranoside. Cells were collected and lysed and the fusion protein was purified as previously reported (12). This entailed a three-stage protocol that included immunoaffinity, anion exchange, and gel filtration chromatographies. Endotoxin levels of the final preparation were < 30 endotoxin units per mg of protein.

Cell culture. Vascular smooth muscle cells were cultivated by explant outgrowth from unused portions of internal mammary arteries retrieved at the time of open heart surgery. Cell cultures were grown in medium 199 (M199; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum, unless otherwise indicated. Experiments were performed using cells in the second or third subculture. Smooth muscle cell identity was confirmed by positive immunostaining with monoclonal antibody to smooth muscle α actin (clone 1A4; Sigma Chemical Co., St. Louis, MO) and lack of staining with monoclonal antibody to Factor VIII–related antigen (Signet Laboratories, Dedham, MA).

Smooth muscle cell protein synthesis. Cells were seeded on to fibronectin-coated wells (10 $\mu g/cm^2$) at a density of 5,000 cells/cm^2 and

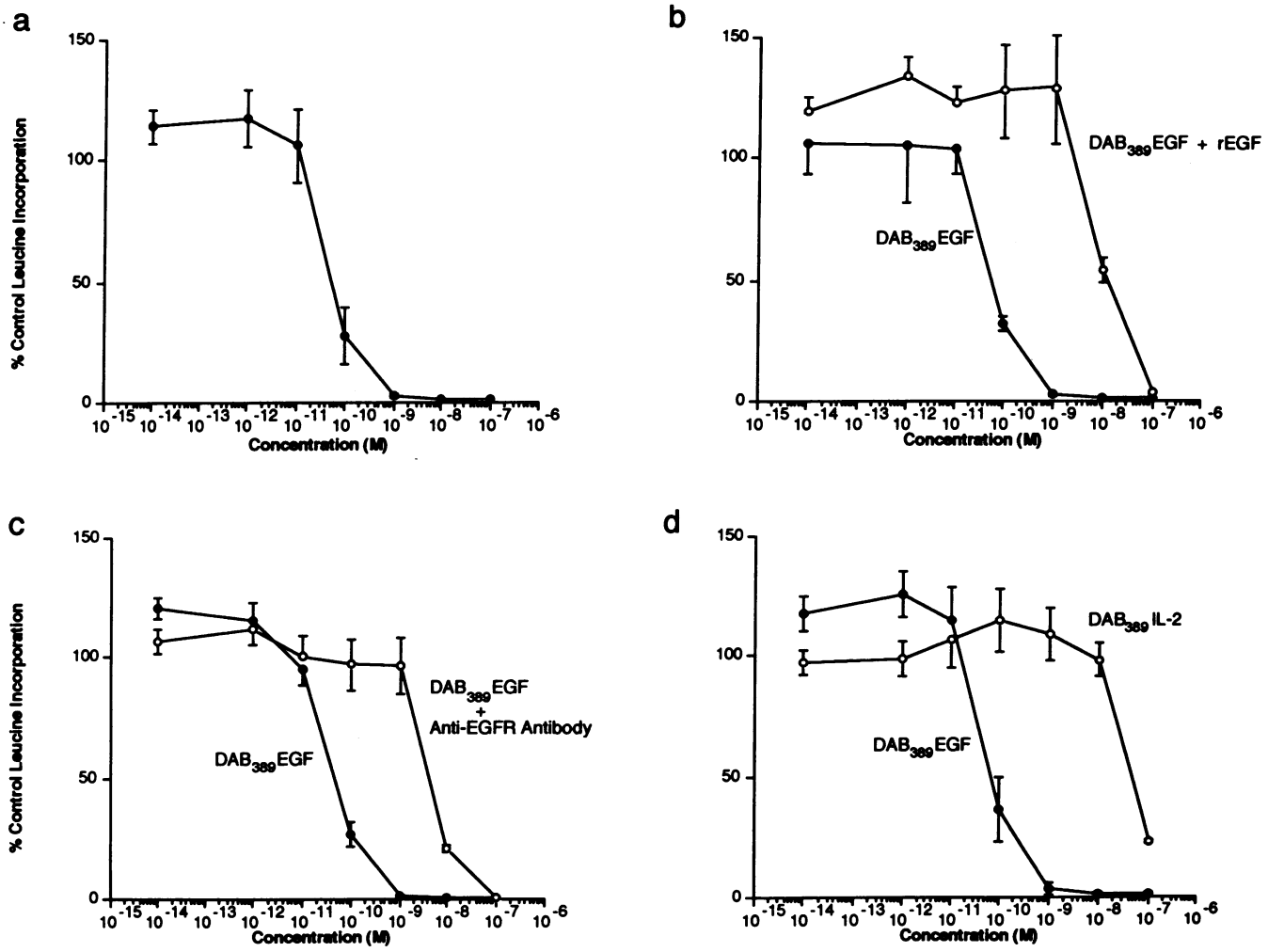

Figure 1. Cytotoxicity of $DAB_{389}EGF$. (*a*) Primary cultures of human vascular smooth muscle cells were incubated for 24 h with the indicated concentrations of the fusion protein. Leucine incorporation is expressed relative to that of cells incubated in media without cytotoxin. The data represent the mean±SD of three experiments with cells originating from three patients. (*b*) Cytotoxic effect of $DAB_{389}EGF$ on primary cultures of human vascular smooth muscle cells in the presence or absence of competition with 1.6 μM human recombinant EGF; and (*c*) in the presence or absence of 10 nM monoclonal antibody to the human EGF receptor. (*d*) Comparison of cytotoxic effects of $DAB_{389}EGF$ and $DAB_{389}IL\text{-}2$ on human vascular smooth muscle cells. The data in *b*, *c*, and *d* represent the mean±SD of triplicate samples.

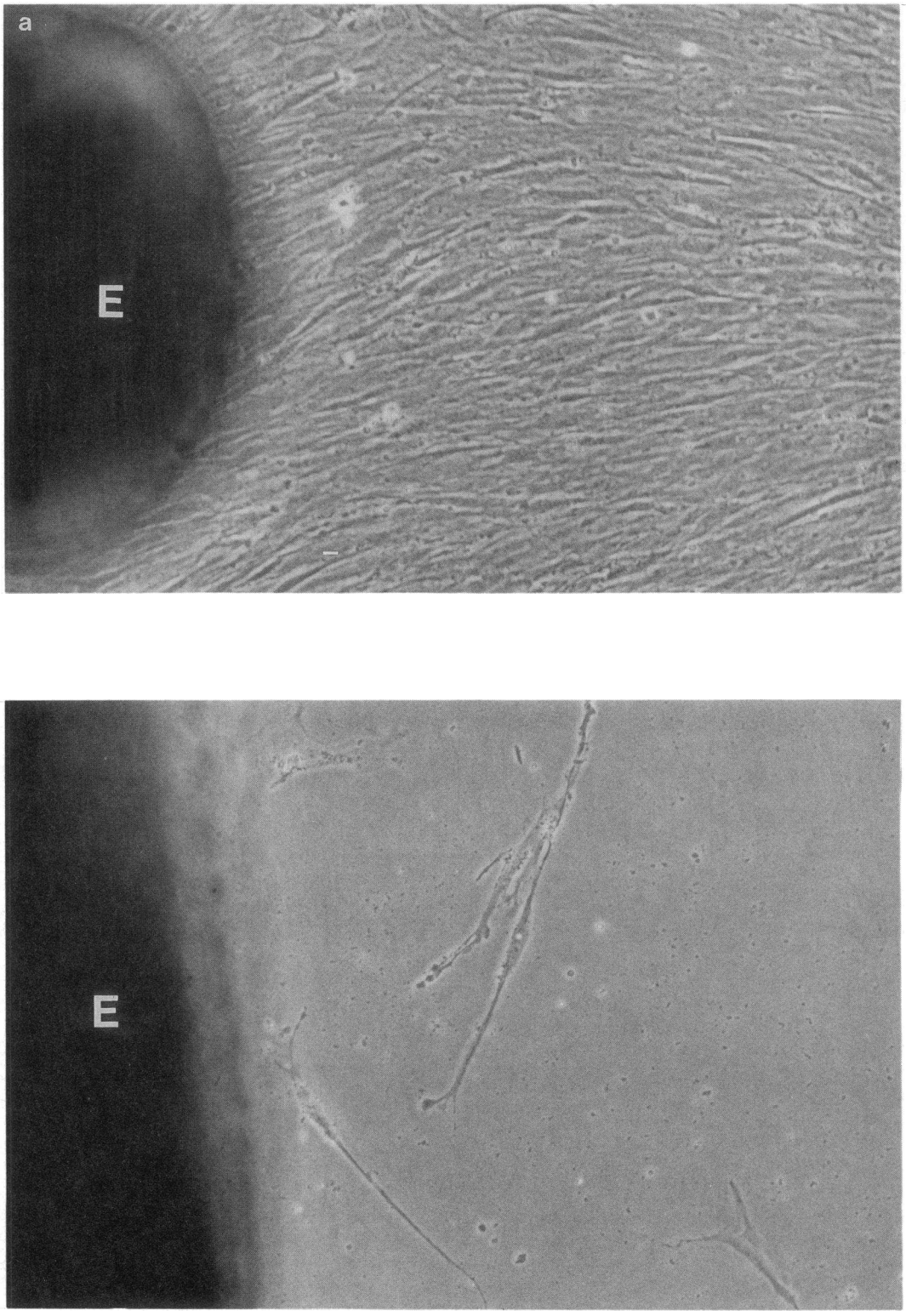

Figure 2. Effect of $DAB_{389}EGF$ on the outgrowth of smooth muscle cells from fragments of explanted human atherosclerotic plaque. (*a*) Representative phase-contrast photomicrographs illustrating abundant 15-d outgrowth from control fragment of human atherosclerotic plaque (*top*); minimal outgrowth from similar fragment exposed to 10^{-11} M $DAB_{389}EGF$ (*bottom*). *E,* explant. (*b*) Dose-response of human smooth muscle cell outgrowth to $DAB_{389}EGF$. Data points indicate the proportion of atherosclerotic explant fragments yielding any outgrowth of smooth muscle cells. (*c*) Results of outgrowth assay after atherosclerotic fragments were washed free of 10^{-8} M $DAB_{389}EGF$ at designated intervals. Outgrowth was inhibited after as little as 1 h of incubation with fusion toxin.

incubated in serum-supplemented media for 48 h. Cells in triplicate wells were then exposed to serial dilutions of fusion toxin for 24 h. They were then incubated for 4 h with L[3,4,5-^{3}H]leucine (8 μCi/ml, 180 Ci/mmol; New England Nuclear, Boston, MA) in leucine-free medium (minimal essential media; Gibco Laboratories). Cells were washed extensively, air dried, and solubilized in 0.25 M sodium hydroxide. Radioactivity was determined by liquid scintillation counting.

To determine if the cytotoxic effect was mediated by the EGF receptor, experiments were performed in the presence of either excess EGF (Gibco Laboratories) or monoclonal antibody to the human EGF receptor (Upstate Biotechnology, Inc., Lake Placid, NY). As a negative control, the effect of a second fusion protein, $DAB_{389}IL$-2, was evaluated. This cytotoxin does not undergo receptor-mediated binding to smooth muscle cells, and any effect would be nonspecific.

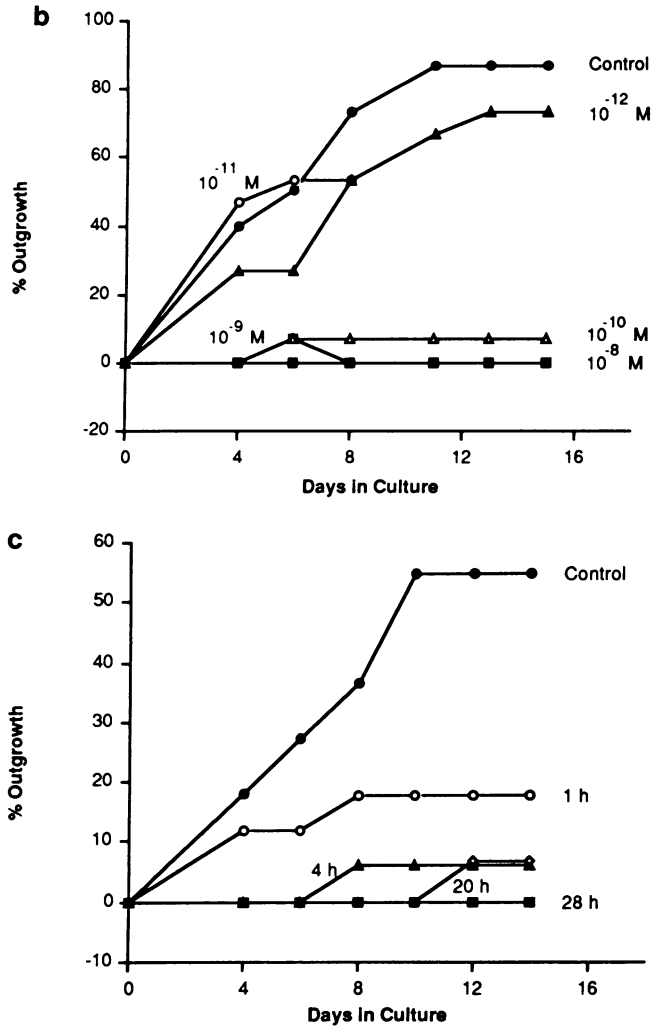

Figure 2 (Continued)

Smooth muscle cell outgrowth assay. Smooth muscle cell outgrowth from explants of human atherosclerotic plaque was evaluated as previously described (18). Briefly, atherosclerotic plaques were retrieved from patients percutaneously by directional atherectomy (Devices for Vascular Intervention, Redwood City, CA). The lumen surface was gently scraped to dislodge endothelial cells. Lesions were then divided into 75–90 fragments, each measuring ~ 1 mm^3. These were allowed to adhere to the surface of fibronectin-coated culture wells (five fragments per 9.4 cm^2 well) in media (M199; Gibco Laboratories) supplemented with 15% fetal bovine serum. Under these conditions, cells migrate from the lesion and proliferate, yielding an outgrowth of smooth muscle cells, the identity of which were confirmed by positive immunostaining for smooth muscle α actin (clone 1A4; Sigma Chemical Co.). 24 h after placing the explant fragments in culture, $DAB_{389}EGF$ was added to the media at concentrations ranging from 10^{-14} M to 10^{-7} M. The proportion of explant fragments yielding an outgrowth of at least one smooth muscle cell was determined every 2–3 d. In these experiments the media was not exchanged during the observation period (14–15 d). To assess the kinetics of cytotoxicity, the media containing $DAB_{389}EGF$ was removed at designated time points (1–28 h) and the explant fragments were washed and refed with toxin-free, serum-supplemented media.

Cell proliferation within atherosclerotic plaque. For these experiments, explanted fragments of human plaque were incubated, free floating in media. $DAB_{389}EGF$ (10^{-8} M) was added to wells, in the presence or absence of excess EGF, 24 h after the tissue was retrieved and placed in culture. 3 d later [^{3}H]thymidine (6 μCi/ml, 6.7 Ci/mmol; New England Nuclear) was added to all wells and fragments were incubated for a further 24 h. Tissues were washed extensively, fixed in formalin, and paraffin-embedded sections were cut onto glass slides. Slides were dipped in radiographic emulsion (NTB-2; Kodak), air dried, and incubated at 4°C in the dark for 7 d. After developing, sections were counterstained with hematoxylin and eosin and the proportion of labeled cells determined. All cells in a given tissue section were counted (range, 93–306 cells/section).

Results

Exponentially growing human vascular smooth muscle cells harvested from operatively excised internal mammary arteries were exposed to varying doses of $DAB_{389}EGF$ for 24 h. There was a potent, dose-dependent inhibition of protein synthesis, as measured by the incorporation of radiolabeled leucine (Fig. 1 *a*). The concentration at which protein synthesis was inhibited by 50% (IC_{50}) was 40 pM (1.9 ng/ml). Three separate experiments demonstrated that the cytotoxic effect of $DAB_{389}EGF$ was mediated through the EGF receptor. First, incubation of cells in the presence of 1.6 μM human recombinant EGF resulted in a dose-dependent inhibition of cytotoxicity, as measured by incorporation of radiolabeled leucine (Fig. 1 *b*). Second, incubation of cells in the presence of 10^{-8} M monoclonal antibody to the human EGF receptor (Upstate Biotechnology, Inc.) led to a similar inhibition of cytotoxicity (Fig. 1 *c*). Finally, cells were exposed to either $DAB_{389}EGF$ or a second fusion protein ($DAB_{389}IL$-2) comprised of the identical truncated diphtheria molecule but fused to the IL-2 molecule. Mesenchymal cells are not known to express a receptor for IL-2 and, unlike the EGF fusion toxin, this protein was not cytotoxic, up to a dose of 10^{-8} M (Fig. 1 *d*). At very high concentrations (10^{-7} M) of $DAB_{389}IL$-2 a cytotoxic effect was evident. This is consistent, however, with previous findings (19) in which a toxic effect of $DAB_{389}IL$-2 on IL-2 receptor–deficient cells was seen at this high dose.

We subsequently evaluated the effect of $DAB_{389}EGF$ on the in vitro outgrowth of smooth muscle cells from intact segments of human atheromata. The outgrowth of cells from atherosclerotic plaque explanted into a growth-stimulating culture environment represents the integrated consequences of both migration and proliferation of smooth muscle cells (18). It can thus be considered to be a similar response to that which occurs in vivo, following trauma to the atherosclerotic vessel wall. Fig. 2 depicts the effect of $DAB_{389}EGF$ on smooth muscle cell outgrowth from one of the lesions studied. Of the fragments not exposed to the fusion toxin, 90% yielded an outgrowth of smooth muscle cells in the ensuing 15 d. Outgrowth at this time typically appeared as a confluent monolayer of cells emanating from all borders of the fragment. In contrast, outgrowth was completely prevented among fragments incubated with 10^{-8} M $DAB_{389}EGF$ and < 10% of fragments exposed to either 10^{-9} or 10^{-10} M $DAB_{389}EGF$ yielded any outgrowth. At a concentration of 10^{-11} M the proportion of outgrowing explants was similar to controls; the number of smooth muscle cells that accumulated around a given explant fragment, however, was distinctly lower with only isolated cells appearing at the edges of the explant. These dose-dependent findings were confirmed by studying atherosclerotic lesions, retrieved by atherectomy, from two other patients (data not shown). When explants were incubated with 10^{-8} M $DAB_{389}EGF$ and an excess of human

EGF (10^{-6} M), the ability to yield an outgrowth of smooth muscle cells was retained, although the outgrowth from individual explants was less than that of untreated controls. When explants were incubated with 10^{-9} M $DAB_{389}EGF$ and 10^{-6} M EGF both the proportion of outgrowing explants and the number of smooth muscle cells that accumulated around the individual explants was similar to that of controls.

The length of time that human atherosclerotic plaque must be exposed to $DAB_{389}EGF$ to induce inhibition of smooth muscle cell outgrowth is depicted in Fig. 2 *c*. The proportion of explants yielding an outgrowth of smooth muscle cells was markedly reduced after as little as 1 h of incubation. Again, among those fragments that did yield an outgrowth there was only a scant accumulation of cells.

To determine if the fusion toxin was interacting with cells still within the body of the plaque explant, versus cells that had recently migrated out of the tissue (and consequently been exposed to a higher concentration of fusion toxin), explants were incubated free floating in serum-supplemented culture medium in the presence or absence of 10^{-8} M $DAB_{389}EGF$, and proliferation was measured based on the incorporation of radiolabeled thymidine. Under these conditions the atherosclerotic tissue explants received a growth stimulus, but outgrowth of smooth muscle cells would not occur. 6% of cells in control tissue were labeled, as assessed by autoradiography; in contrast, no cells in lesions exposed to the fusion toxin were labeled. Thus, incubation with $DAB_{389}EGF$ prevented cell proliferation within the human atherosclerotic tissue.

Discussion

EGF receptor–targeted fusion proteins have attracted interest as a potential therapy for solid tumors (20–22). $DAB_{389}EGF$ in particular has been shown to kill tumor cells in culture at very low concentrations and with rapid kinetics (12). Furthermore, studies with animal tumor models have indicated that $DAB_{389}EGF$ can have a therapeutic effect at nontoxic doses (Shaw, J. P., manuscript in preparation). Targeted cytotoxic therapy may also be of value in certain vascular disorders, characterized by the rapid accumulation of cells. Restenosis after angioplasty may be particularly amenable to cytotoxic therapy since the location of disease is predictable (i.e., the site of a previous catheter-based intervention) and the cytotoxic agent could be delivered to a localized, isolated region of the vessel (23, 24) thereby minimizing damage to normal tissues.

Our results demonstrate that $DAB_{389}EGF$ is highly cytotoxic to human vascular smooth muscle cells proliferating in culture. The fusion protein can prevent the outgrowth of smooth muscle cells from human atherosclerotic plaque explanted into a growth-promoting environment and can prevent proliferation within the plaque itself. We have shown that the effect is mediated specifically by the EGF receptor. It has been previously demonstrated that the intracellular events leading to cell death are identical to those associated with native diphtheria toxin (12). Specifically, receptor-mediated endocytosis leads to the appearance of the molecule within endosomes. This is followed by translocation of fragment A of diphtheria toxin into the cytosol, where it catalyzes the covalent linkage of ADP-ribose to elongation factor-2. This reaction arrests protein synthesis.

Assessing the outgrowth of smooth muscle cells from explants of human atherosclerotic plaque provides a unique and valuable means of assessing the effect of agents designed to inhibit intimal proliferation (18). In particular, potentially unique features of smooth muscle cells specifically within atherosclerotic tissue may be preserved. Furthermore, the influence of the extracellular matrix and hormonal milieux of the atherosclerotic plaque on cell behavior may also be preserved. Finally, the approach eliminates potential species variations in receptor expression, a potentially important factor when studying agents with receptor-binding properties. To our knowledge, the present study represents the first report of successful inhibition of the accumulation of human vascular smooth muscle cells.

Studies using a different EGF receptor–targeted toxin, comprised of pseudomonas exotoxin A and transforming growth factor alpha, have demonstrated selective toxicity to rat and rabbit aortic vascular smooth muscle cells in culture (11). The effect on nonproliferating smooth muscle cells was dramatically (30-fold) lower than in proliferating cells, supporting the rationale for the use of this approach in the treatment of vascular lesions associated with smooth muscle cell proliferation. The potency of its effect on protein synthesis, however, was two to ten times lower than that found in the present study and its kinetics of action are unknown.

The rapid formation of vascular lesions containing abundant smooth muscle cells is responsible for considerable morbidity and mortality in patients requiring balloon angioplasty, coronary artery bypass surgery, peripheral vascular surgery, and cardiac transplantation. The dramatic cytotoxic effect shown here in human vascular smooth muscle cells suggests the efficacy of a novel therapy for human vascular disease associated with smooth muscle cell accumulation.

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