Journal of Cell Science 107, 2599-2608 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

The heparin-binding domain of heparin-binding EGF-like growth factor can target *Pseudomonas* exotoxin to kill cells exclusively through heparan sulfate proteoglycans

Enrique A. Mesri[1,*], Minoru Ono[2], Robert J. Kreitman[1], Michael Klagsbrun[2] and Ira Pastan[1,†]

[1]Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bldg 37, Rm 4E16, Bethesda, MD 20892, USA
[2]Department of Biological Chemistry and Molecular Pharmacology Children's Hospital and Harvard Medical School, Boston, MA 02115, USA

*Present address: Diabetes Research Center, Department of Medicine, Division of Endocrinology, Albert Einstein College of Medicine, Bronx, NY 10461, USA
[†]Author for correspondence

SUMMARY

Heparin-binding EGF-like growth factor (HB-EGF) is a smooth muscle cell mitogen composed of both EGF receptor and heparin-binding domains. To better understand the function of its domains, intact HB-EGF or its heparin-binding (HB) domain (amino acids 1-45) were fused to a mutant *Pseudomonas* exotoxin with an inactivated cell-binding domain. The resulting chimeric toxins, HB-EGF-PE* and HB-PE*, were tested on tumor cells, proliferating smooth muscle cells and a mutant Chinese hamster ovary cell line deficient in heparan sulfate proteoglycans (HSPGs). Two targets were found for HB-EGF-PE*. Cells were killed mainly through EGF receptors, but the HB domain was responsible for killing via HSPGs. HB-PE* did not bind to the EGF receptor and thus was cytotoxic by interacting exclusively with HSPGs. We conclude that the HB domain of HB-EGF is able to mediate internalization through HSPGs, without requiring the EGF receptor.

Key words: immunotoxins, *Pseudomonas* exotoxin A, smooth muscle cells, cancer cells, antitumor agents, heparin

INTRODUCTION

Heparin-binding EGF-like growth factor (HB-EGF) is a potent smooth muscle cell (SMC) mitogen that is capable of binding to the EGF receptor (EGFR) and heparin (Higashiyama et al., 1991). Proliferation of vascular SMCs is characteristic of pathological states such as atherosclerosis or restenosis (Austin et al., 1987; Woolf, 1990; Ross, 1993). Since vascular SMCs can secrete HB-EGF (Temizer et al., 1992; Dluz et al., 1993) and proliferating SMCs have elevated levels of EGFR (Epstein et al., 1991), an HB-EGF autocrine loop could be a major feature in SMC proliferation associated with those disorders. The mature HB-EGF polypeptide is composed of a Cys-rich EGF-like domain and an N-terminal region rich in basic amino acids. The heparin-binding (HB) site has been recently identified and lies mostly within the N-terminal region, although it encompasses some residues within the EGF-like domain (Higashiyama et al., 1991, 1993; Mesri et al., 1993; Thompson et al., 1994). Like other HB growth factors such as the members of the fibroblast growth factor (FGF) family (Kjellen and Lindahl, 1991; Ruoslahti and Yamaguchi, 1991; Klagsbrun and Baird, 1991; Yanagishita and Hascall, 1992), HB-EGF binds to heparan sulfate proteoglycans (HSPGs) on the cell surface (Besner et al., 1992; Higashiyama et al., 1992; Thompson et al., 1994). Binding of HB-EGF to HSPGs on the surface of smooth muscle cells is important for optimal binding to the EGFR, and therefore for optimal biologic activity (Higashiyama et al., 1993). Interestingly, HSPGs undergo endocytosis and recycling (Yanagishita and Hascall, 1992) and HSPGs have recently been shown to internalize HB growth factors such as basic FGF (Gannoun-Zaki et al., 1991; Roghani and Moscatelli, 1992; Rusnati et al., 1993). This latter finding is important since heparan sulfate molecules and HB growth factors are both thought to be active intracellularly (Baldin et al., 1990; Imamura et al., 1990; Wight et al., 1992). We have chosen to study the internalization of HB-EGF by connecting it to a toxin.

Chimeric toxins are molecules composed of a toxin fused to a growth factor or antibody that kill target cells with specific surface properties (Pastan et al., 1986; Vitetta et al., 1987; Pastan et al., 1992). We have designed several different chimeric toxins based on *Pseudomonas* exotoxin (PE) (Pastan and FitzGerald, 1989; Pastan et al., 1992). To kill a cell, the toxin is internalized by a specific receptor and is translocated to the cytosol where it arrests protein synthesis and causes cell death (FitzGerald et al., 1980). Each function is performed by a different domain (Hwang et al., 1987). The domain responsible for cell binding can be either deleted or mutated, and

replaced by a growth factor capable of specifically targeting the toxin (Pastan and FitzGerald, 1989; Pastan et al., 1992).

In a previous report, we fused HB, the 45 N-terminal amino acids of HB-EGF (Mesri et al., 1993), to the amino terminus of TGFα-PE*. In TGFα-PE*, transforming growth factor α is fused to the N terminus of PE^{4E}KDEL (abbreviated PE*). PE* is a full-length form of PE containing inactivating mutations in the cell-binding domain (Chaudhary et al., 1990b; Lorberboum-Galski, 1990; Kreitman et al., 1991; Siegall et al., 1991), and the KDEL carboxyl terminal mutation which increases activity (Chaudhary et al., 1990a; Lorberboum-Galski, 1990; Seetharam et al., 1991). The resulting molecule, HB-TGFα-PE*, contained as a ligand HB-TGFα. Since TGFα binds to the EGFR, HB-TGFα was thought to be functionally similar to HB-EGF (Mesri et al., 1993). HB-TGFα-PE* specifically killed cells bearing EGFRs. Furthermore, the addition of HB conferred on the chimeric TGFα toxins the ability to bind to heparin and surface HSPGs, suggesting the HB sequence as a functional HB domain of HB-EGF. The addition of the HB domain of HB-EGF to TGFα-PE* also increased the cytotoxicity to smooth muscle cells, and to cancer cell lines expressing low numbers of EGFRs, by a mechanism involving binding to cell surface HSPGs.

Our previous experiments using HB-TGFα-PE* were limited in their ability to characterize the binding and uptake of HB-EGF for several reasons. First, HB-TGFα was a hybrid protein that may be functionally similar but not identical to HB-EGF. Secondly, it was impossible to establish the exclusive importance of the HB domain to the binding and internalization of HB-EGF, since on nearly all target cells the TGFα domain was much more important than the HB domain in the cytotoxic activity of HB-TGFα-PE* (Mesri et al., 1993). Most importantly, we could not exclude the possibility that the HB domain requires interaction with a specific receptor, such as those for FGF or EGF, to induce internalization via HSPGs.

In the present report, we design new experiments to analyze directly the role of the interaction between the HB domain and HSPGs in cell killing. We constructed and characterized two new chimeric toxins derived from HB-EGF. First, we used the complete HB-EGF ligand fused to PE* to generate HB-EGF-PE*. We then fused just the HB domain (amino acids 1-45) to PE* to generate HB-PE*, a HB-toxin lacking an EGFR-binding site. By comparing the cytotoxicity and the specificity of HB-EGF-PE*, HB-PE* and PE* towards human cancer cell lines, bovine aortic smooth muscle cells and transfected Chinese hamster ovary (CHO) cells either expressing or not expressing HSPGs, we were able to determine the contribution of the HB domain to the cytotoxicity. Our results showed that although HB-EGF-PE* kills cells via the EGFR, the HB domain has an important contribution to the cytotoxicity by mediating killing through HSPGs. This was confirmed by the demonstration that HB-PE* does not bind to the EGFR and kills cells by specifically interacting with HSPGs, indicating that the HB domain of HB-EGF is sufficient to promote internalization of the chimeric toxins via HSPGs.

MATERIALS AND METHODS

Human cancer cell lines

Most cell lines were from the American Type Culture Collection (Rockville, MD). HUT-102 cells were a gift from Dr T. Waldmann (NCI, NIH). NCI 417D was generously provided by Dr D. Bigner (Department of Pathology, Duke University Medical Center, Durham, NC). CHO 667 (Esko et al., 1988) cells were kindly provided by Dr J. Esko (Department of Biochemistry, Schools of Medicine and Dentistry, University of Birmingham, AL). BASMC were obtained as described (Higashiyama et al., 1993).

Plasmid construction

pEM4EK encodes OmpA-PE^{4E}KDEL. OmpA is a signal peptide that mediates the secretion of PE^{4E}KDEL into the periplasm. To clone HB-EGF-PE* and HB-PE*, DNA fragments encoding either the mature HB-EGF polypeptide, or the heparin-binding domain (amino acids 1-45) (Mesri et al., 1993) were generated by PCR. The synthetic oligonucleotides EA-15 (5′-TTG-CAA-GAG-GCA-GAT-CTG-GAC-CAT-ATG-GTC-ACT-TTA-TCC-TCC-3′) and EA-16 (5′-TGT-GTG-GTC-ATA-GGT-ATA-TAA-GCA-AGC-TTC-CAC-TGG-GAG-GCT-CAG-CCC-3′) were used to amplify the sequence encoding the mature HB-EGF from a plasmid carrying a full-length HB-EGF cDNA (pJMU2-1) (a gift from Dr Judith Abraham, Scios Nova Inc., Mountain View, CA). These PCR primers introduce an *Nde*I site 5′ and a *Hin*dIII site 3′ into the coding region of HB-EGF, which allows both the subsequent cloning and the introduction of a methionine as first amino acid for expression in *Escherichia coli*. To amplify the HB domain from the plasmid pEMHBT4EK (Mesri et al., 1993) we used a synthetic oligonucleotide corresponding to the T7 promoter and the oligonucleotide EA-18 (5′-TT-GTC-CTC-CTG-CAC-CAA-AAA-AGC-TTC-TTC-GGC-ATG-GAT-ACA-CAA-ATC-TTT-G-3′), which allows the introduction of a *Hin*dIII site. The PCR fragments were cut by *Nde*I and *Hin*dIII, gel purified, and ligated ‘in gel’ (Sea Plaque GTG, and Nu Sieve GTG agarose, FMC, Rockland, ME) with a gel-purified 3.9 kb fragment of the plasmid pEMHBT4EK (Mesri et al., 1993) cut with *Nde*I and *Hin*dIII. The resulting plasmids pEMHBE4EK and pEMHB4EK encoded HB-EGF-PE^{4E}KDEL (HB-EGF-PE*) and HB-PE^{4E}KDEL (HB-PE*), respectively.

Expression and purification of the chimeric toxins

Expression, refolding and purification of the HB-TGFα-PE* and HB-EGF-PE* chimeric toxins was accomplished as previously described (Mesri et al., 1993). Briefly, *E. coli* BL21 transformed with the corresponding plasmids was cultured, induced at an A_{660}=1 by 1 mM IPTG and harvested after 90 minutes of induction. Spheroplasts were prepared by osmotic shock. Inclusion bodies were obtained from the spheroplasts by sonication and were then denatured, refolded and loaded onto a Econo-Pak heparin column (Bio-Rad, Richmond, CA) as described (Mesri et al., 1993). HB-PE* was refolded as follows. The inclusion bodies were denatured with 7 M guanidine-HCl, and refolded by dilution in PBS. The refolding solution was clarified and loaded onto an Econo-Pak heparin column. The Econo-Pak columns were eluted with a 0.15-1 M NaCl gradient. The fractions eluting from 0.4 to 0.9 M NaCl were pooled, diluted and loaded onto a TSK-heparin column (Toso-Haas, Philadelphia, PA), and eluted with a 0.15-1 M NaCl gradient. Each fraction was characterized by cytotoxic activity towards A431 cells, SDS-PAGE (Laemmli, 1970), and analytical size exclusion chromatography (TSK-G3000, Toso-Haas, Philadelphia, PA). For the expression and purification of PE*, we obtained the periplasm from induced bacteria by osmotic shock, added 1 M Tris-HCl, pH 7.4, up to 20 mM, and purified the protein sequentially by Q-Sepharose, Mono-Q and TSK-G3000 chromatography. TGFα-PE* was obtained in the same way as described previously (Kreitman et al., 1991).

Cytotoxicity assays

Cytotoxicity assays were performed as described (Kreitman et al., 1991; Mesri et al., 1993). Briefly, adherent tumor cells were plated 24 hours prior to toxin addition in 96-well plates at a density of 1×10^5

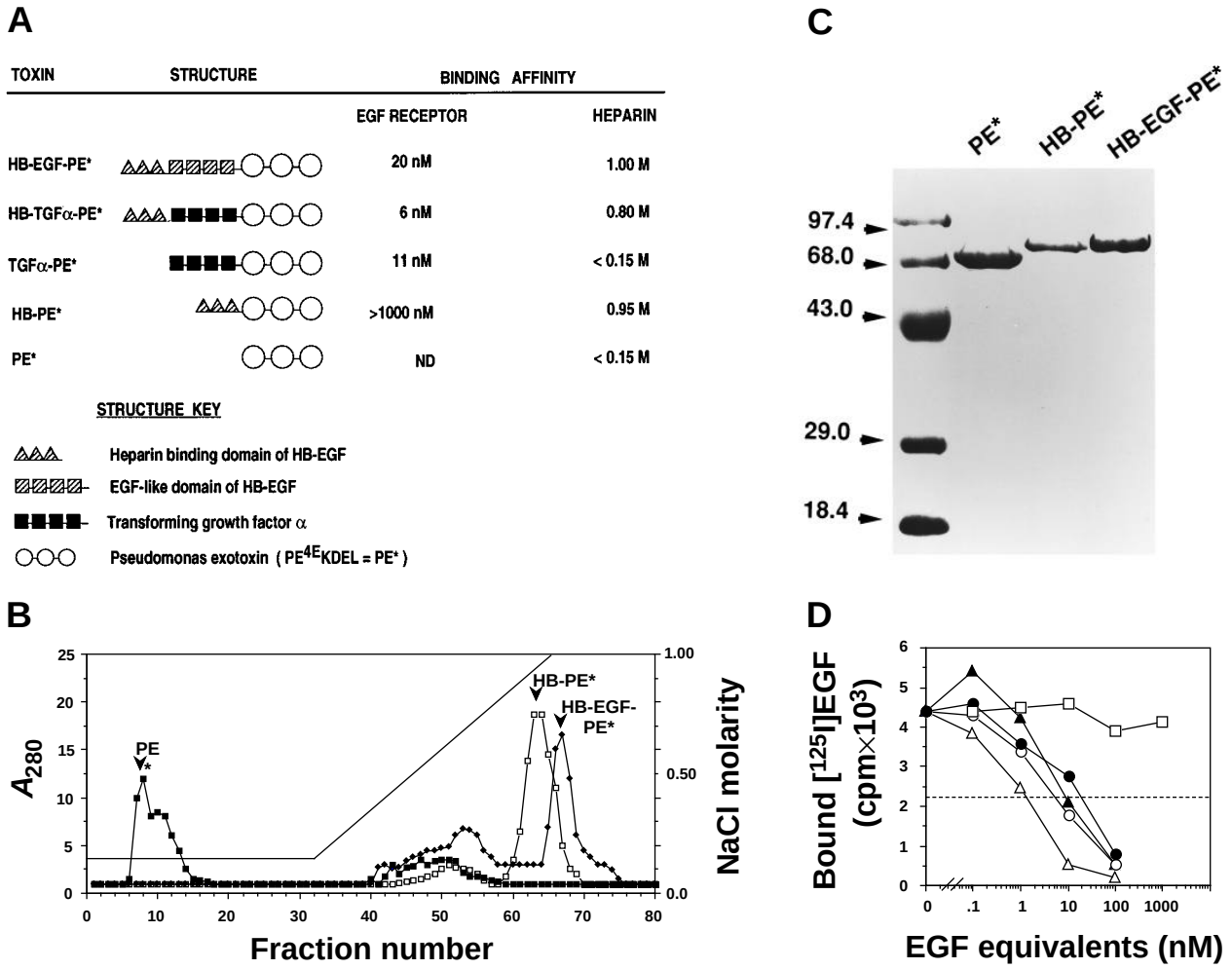


Fig. 1. Structure, purification and binding of the recombinant toxins. (A) Detail of chimeric toxin structure: The HB-EGF ligand is separated into the HB domain (amino acids 1-45) and the EGF-like domain. $PE^{4E}KDEL$ is a mutant of PE that contains the mutations K57E, H246E, R247E, and H249E in the binding domain and KDEL replacing the amino acids REDLK at the carboxyl teminus. The EGF receptor column indicates the concentration of agent required to displace [^{125}I]EGF binding to A431 cells by 50%. The heparin column denotes the concentration of NaCl necessary to elute the protein from a TSK-heparin column. (B) Heparin affinity chromatography profiles on TSK-heparin columns of the recombinant toxins. The arrow indicates the fraction of each protein with the highest cytotoxicity to A431 cells. (C) SDS-PAGE gel of purified recombinant toxins. HB-EGF-PE* and HB-PE*, pool of TSK-heparin fractions eluting at 0.9-1 M NaCl. PE* ($PE^{4E}KDEL$) Pool of TSK-3000 monomeric fractions. Size standards (in kDa) are shown at the left. (D) Displacement of the binding of [^{125}I]EGF to A431 cells by the different chimeric toxins. (△) EGF, (▲) TGFα-PE*, (○) HB-TGFα-PE*, (●) HB-EGF-PE*, (□) HB-PE*.

cells per ml. After incubating with toxins at 37°C for 16-18 hours the cells were pulsed with 1 μCi of [^{3}H]leucine for 4 additional hours and counted. Bovine aortic SMCs (BASMCs) were cultured in DMEM as described previously (Higashiyama et al., 1991). CHO cells were cultured as described previously (Yayon et al., 1991). BASMCs and CHO cells were plated subconfluently in 96-well plates 24 to 48 hours prior to toxin addition. After incubating with toxins at 37°C for 16 hours, 5 μCi of [^{3}H]leucine was added to each well, and the cells were incubated for an additional 2 hours at 37°C, harvested and counted. The cpm data reported from cytotoxicity assays were the medians of triplicate experiments, each of which generally deviated from their means by less than 10%. ID_{50} values and cytotoxicity profiles reported are representative of several confirmatory assays.

Effect of heparin EGF and heparitinase on cytotoxicity

Heparin (10,000 U/ml, 150 U/mg; UpJohn, Kalamazoo, MI) was added to the cells with the toxin. For competition experiments, two hours before adding the toxin, the plates were cooled on ice, and EGF (Gibco-BRL, Gaithersburg, MD or Collaborative Biomedical Products, Bedford, MA) was added up to 4 μg/ml. Plates were incubated at 4°C for 90 minutes with gentle rocking. Toxins with or without heparin were then added and the assay was continued as described above. For heparitinase digestion, the cells were washed once in heparitinase buffer (DMEM, 20 mM HEPES, pH 7.4, 5 mg/ml BSA) and heparitinase (Linhardt et al., 1990; heparitinase III, heparan sulfate lysate EC 4.2.2.8; Seikagaku America, Rockville, MD) was added to the cells in 24-well plates up to 1 mU/ml for three hours. The cells were cooled prior to replacement of the heparitinase solution with medium containing the toxin at 4°C. The toxin was incubated for 2 hours at 4°C, then the toxin was aspirated, and the cells were washed twice with medium-FBS and incubated with 1 ml of fresh medium overnight. The assay was then continued as described above.

Binding assays

Displacement assays were performed as described (Mesri et al., 1993). A431 cells were plated overnight at 8×10^3 cells/ml per well in 24-

well plates, washed twice with ice-cold binding buffer (DMEM containing 1 μg/ml BSA and 50 mM BES, pH 6.8). Then 200 μl of binding buffer with 0.5 ng of [^{125}I]EGF (0.1 μCi/ng; NEN, Wilmington, DE) and toxins or controls were added. After incubation at 4°C with gentle rocking, the binding solution was aspirated and the cells washed three times with binding buffer, lysed with 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 0.5% SDS, EDTA 1 mM) and counted with a gamma detector.

RESULTS

Construction, expression and purification of the HB-EGF chimeric toxin

To construct the HB-EGF-PE* chimeric toxins we fused a DNA fragment encoding either the mature HB-EGF polypeptide (Higashiyama et al., 1991) or the 45 N-terminal residues encompassing the HB domain (Mesri et al., 1993) to PE^{4E}KDEL (PE*), a mutant form of PE bearing four mutations in domain Ia that impairs its ability to interact with the PE receptor (Chaudhary et al., 1990b; Kreitman et al., 1991). The toxin also carries a strong ER localization signal (KDEL) to enhance its cytotoxicity (Chaudhary et al., 1990a; Seetharam et al., 1991), and has previously been fused to acidic FGF (Siegall et al., 1991) and HB-TGFα (Mesri et al., 1993). In this way, we obtained HB-EGF-PE* and HB-PE*.

Fig. 1 describes the structure of the toxins constructed in the present study, as well as those that were used as controls. TGFα-PE* (Mesri et al., 1993) is a toxin targeted by TGFα, an EGFR ligand. HB-TGFα-PE* is a toxin targeted by a fusion ligand HB-TGFα thought to be functionally similar to HB-EGF, constructed by fusing the HB domain of HB-EGF (amino acids 1-45) to the amino terminus of TGFα (Mesri et al., 1993). PE* is the mutated toxin used in all the fusions.

The fusion proteins were expressed and purified as described in Materials and Methods. TSK-heparin affinity chromatography was used both as a final purification step and as a method to measure heparin affinity. An EGF displacement assay was used to measure affinity for the EGFR. As indicated in Fig. 1A and B, HB-EGF-PE* eluted from the TSK-heparin column at 1.0 M NaCl, the same salt concentration necessary to elute HB-EGF secreted by U937 cells (Higashiyama et al., 1991). In contrast, PE* eluted at low salt concentration (Fig. 1A,B). This indicates that the HB domain of HB-EGF was able to confer to the mutant PE toxin the ability to bind heparin with almost the same affinity as that of HB-EGF. Both HB-EGF-PE* and HB-PE* eluted from the TSK-heparin column in a highly purified form as shown by SDS-PAGE (Fig. 1C). HB-EGF-PE* can bind to the EGFR of A431 cells as shown by a binding displacement assay (Fig. 1A and D). In contrast, HB-PE*, in which almost all the EGFR-binding domain has been deleted, lacks binding to the EGFR.

The binding of HB-TGFα-PE* to heparin and the EGFR was compared to that of HB-EGF-PE*, to determine how well HB-TGFα of the previous study (Mesri et al., 1993) approximated HB-EGF. Fig. 1A shows that the NaCl concentration required to elute HB-EGF-PE* from TSK-heparin was higher than that required to elute HB-TGFα-PE*, indicating that HB-EGF has a higher affinity for heparin than HB-TGFα. In contrast, Fig. 1D shows that HB-TGFα-PE* has a higher affinity for the EGFR than HB-EGF-PE*.

Cytotoxic activities of the heparin-binding toxins

To determine whether the difference in binding affinity between HB-TGFα-PE* and HB-EGF-PE* would be associated with differences in cytotoxicity, we compared the cytotoxicity of HB-TGFα-PE* and HB-EGF-PE* towards cell lines expressing different levels of EGFR (Table 1). HB-TGFα-PE*, which has a higher affinity for the EGFR (Fig. 1D), was more cytotoxic than HB-EGF-PE* towards cell lines expressing high numbers of EGFRs. HB-EGF-PE*, which has higher affinity for heparin than HB-TGFα-PE* (Fig. 1A), was either just as or more cytotoxic than HB-TGFα-PE* towards cells with lower numbers of EGFR such as MCF-7 and HEP-G2 (Table 1). HB-EGF-PE* and HB-TGFα-PE* were either just as much or more cytotoxic than TGFα-PE* towards these cells (Table 1). These results indicate a contribution of the HB domain of HB-EGF to the cytotoxicity of HB-EGF-PE* towards these types.

In order to test precisely the contribution of the HB domain of HB-EGF to the cytotoxicity, we used HB-PE* (Fig. 1A) and compared the activity of HB-EGF-PE*, HB-PE*, and PE* towards several human cancer cell lines (Table 2 and Fig. 2). HB-PE* was more cytotoxic than PE* on all cell lines (Table 2 and Fig. 2). Since HB-PE* cannot bind to the EGFR (Fig. 1D), this result indicates that the HB domain allows the toxin to target cells exclusively via HSPGs. In all the cell lines, HB-EGF-PE* was more toxic (20- to 100-fold) than HB-PE*, indicating that the HB-EGF ligand was primarily targeted by the EGF-like domain. However, in a cell line with few or no EGFR

Table 1. Cytotoxicity of HB-EGF-PE*, HB-TGFα-PE* and TGFα-PE*

	ID_{50} (ng/nl)		
Cell line (*EGFR/cell*)	TGFα-PE*	HB-TGFα-PE*	HB-EGF-PE*
A431 (*10^6*)	0.03	0.12	0.4
KB (*10^5*)	0.35	0.7	2.5
HEP-G2 (*10^4*)	0.5	0.1	0.05
MCF-7 (*10^3*)	0.5	0.5	0.5

ID_{50}: concentration of toxin necessary to achieve 50% inhibition of [^{3}H]leucine incorporation

Table 2. Cytotoxicity of HB toxins to cancer cell lines: effect of heparin on the cytotoxicity

	ID_{50}*/(ID_{50} with Heparin†)		
Cell line (*cancer type*)	HB-EGF-PE*	HB-PE*	PE*
A431 (*epidermoid*)	0.2/0.2	8/400	300/300
KB (*epidermoid*)	2.5/9	300/>1000	>1000/>1000
MCF-7 (*breast*)	0.6/60	30/300	100/100
CRL 1739 (*gastric*)	0.15/15	10/500	300/300
SW-13 (*adrenal*)	2/200	50/1000	1000/1000
HEP-G2 (*hepatic*)	0.3/30	10/500	50/50
HTB-2 (*bladder*)	0.3/3	200/1000	1000/1000
J-82 (*bladder*)	30/100	700/>1000	>1000/>1000
T 98 MG (*glioma*)	0.3/5	20/>1000	700/1000
U-251(*glioma*)	0.03/0.5	5/500	250/300
NCID471 (*lung*)	0.6/32	32/1000	1000/1000
HUT 102 (*ATL*)	30/400	100/1000	1000/1000

*ID_{50}: concentration of toxin necessary to achieve 50% inhibition of [^{3}H]leu incorporation.

†ID_{50} determined in the presence of 10 U/ml of heparin.

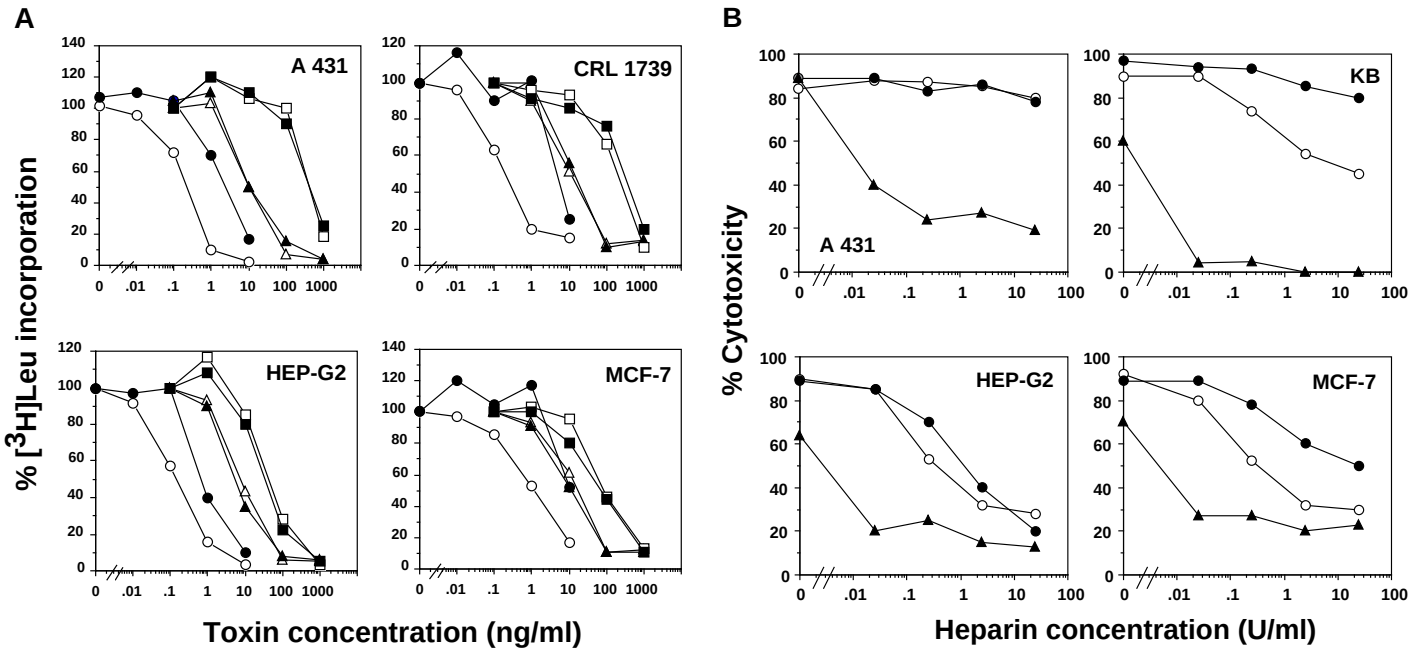


Fig. 2. Cytotoxicity of HB-containing toxins. (A) Protein synthesis inhibition towards target cell lines (A431, CRL 1739, HEP-G2 and MCF-7) of (○) HB-EGF-PE*, (●) HB-EGF-PE* + EGF, (△) HB-PE*, (▲) HB-PE* + EGF, (□) PE, and (■) PE* + EGF. The final EGF concentration, when added, was 3 μg/ml. (B) Effect of heparin in the cytotoxicity of HB toxins. A fixed amount of toxin was incubated with the cells with the addition of increasing amounts of heparin (A431, 1 ng/ml; KB, 10 ng/ml; HEP-G2, 10 ng/ml; MCF-7, 10 ng/ml). (○) HB-EGF-PE*, (●) HB-TGFα-PE*, (▲) HB-PE*.

such as the adult T-cell leukemia line HUT-102, HB-EGF-PE* was only three times more cytotoxic than HB-PE* (Table 1). Thus, HB contributes to the binding and internalization of HB-EGF, and this contribution is most important on cells with fewer EGFRs.

Study of the specificity of the cell killing

To study the specificity of the chimeric toxins we performed competition experiments with EGF and heparin. EGF can compete for the cytotoxicity of HB-EGF-PE* but not HB-PE* or PE (Fig. 2). Heparin blocks the toxicity of HB-EGF-PE* on all the cell lines except A431 (Table 2), and to a minimal extent on KB cells (Table 2). On cells with lower numbers of EGFRs, such as HEP-G2 and MCF-7 (Fig. 2B), most of the protein synthesis inhibition by HB-EGF-PE* could be reversed by heparin. Moreover, heparin can completely block the cytotoxicity of HB-PE* to all the cell lines including A431 (Fig. 2 and Table 2). In contrast, heparin does not affect the cytotoxicity of PE* (Table 2). Thus, all the cell lines contain heparin-like receptors that can bind and internalize the HB domain of HB-EGF. A dose-response experiment on several cell lines showed that HB-EGF-PE* is more sensitive than HB-TGFα-PE* to competition by heparin (Fig. 2B). Thus, compared to HB-TGFα-PE*, a greater component of the internalization of HB-EGF-PE* is due to the HB domain. Taken together, these results indicate that HB-TGFα and HB-EGF toxins kill cells by a pathway involving both binding to the EGFR and heparin-like molecules. In contrast, HB-PE* appears to kill cells only by the latter pathway.

To identify further HSPGs as the target of the chimeric toxins, we performed experiments with heparitinase, an enzyme that specifically degrades heparan sulfate (Linhardt et al., 1990; Rapraeger et al., 1991) and does not affect other cellular targets of the chimeric toxins such as EGFRs (Mesri et al., 1993). As shown in Fig. 3, the cytotoxicity of HB-EGF-PE* to all the cell lines except A431 was decreased by treatment with heparitinase, indicating that toxicity was mediated in part by interaction with HSPGs. The toxicity of HB-PE* to all cell lines including A431 was decreased by heparitinase addition, indicating that in these cell lines, HB-PE* killed by a mechanism involving binding to HSPGs (Fig. 3). In particular, for MCF-7 cells, the cytotoxicity of the HB-PE* could be completely suppressed by high doses of heparitinase (Fig. 3B) indicating that towards such cells the cytotoxicity of HB-PE* is entirely mediated through HSPGs.

Cytotoxicity of HB-EGF chimeric toxins to smooth muscle cells

HB-EGF has been characterized primarily as a very potent smooth muscle cell (SMC) mitogen. SMC proliferation is implicated in various pathological states such as atherosclerosis and restenosis (Austin et al., 1987; Woolf, 1990; Ross, 1993) and targeting of the EGFR of proliferating SMCs has potential for the treatment of these maladies (Epstein et al., 1991; Pickering et al., 1993; Fu et al., 1993). Therefore, it was of interest to study the ability of the HB-EGF chimeric toxins to kill SMC. As shown in Fig. 4A, HB-EGF-PE* is highly cytotoxic to proliferating bovine aortic SMCs (BASMCs), with an ID_{50} of 0.4 ng/ml. HB-PE* is 200-fold less cytotoxic than HB-EGF-PE* (ID_{50} 80 vs 0.4 ng/ml), indicating a major contribution of the EGFR-binding domain in the cytotoxicity of HB-EGF-PE* towards BASMCs. Since HB-EGF-PE* is more cytotoxic than TGFα-PE* (ID_{50} 0.4 vs 2 ng/ml) and since HB-PE* is more cytotoxic than PE* (ID_{50} 80 vs >1000 ng/ml) the

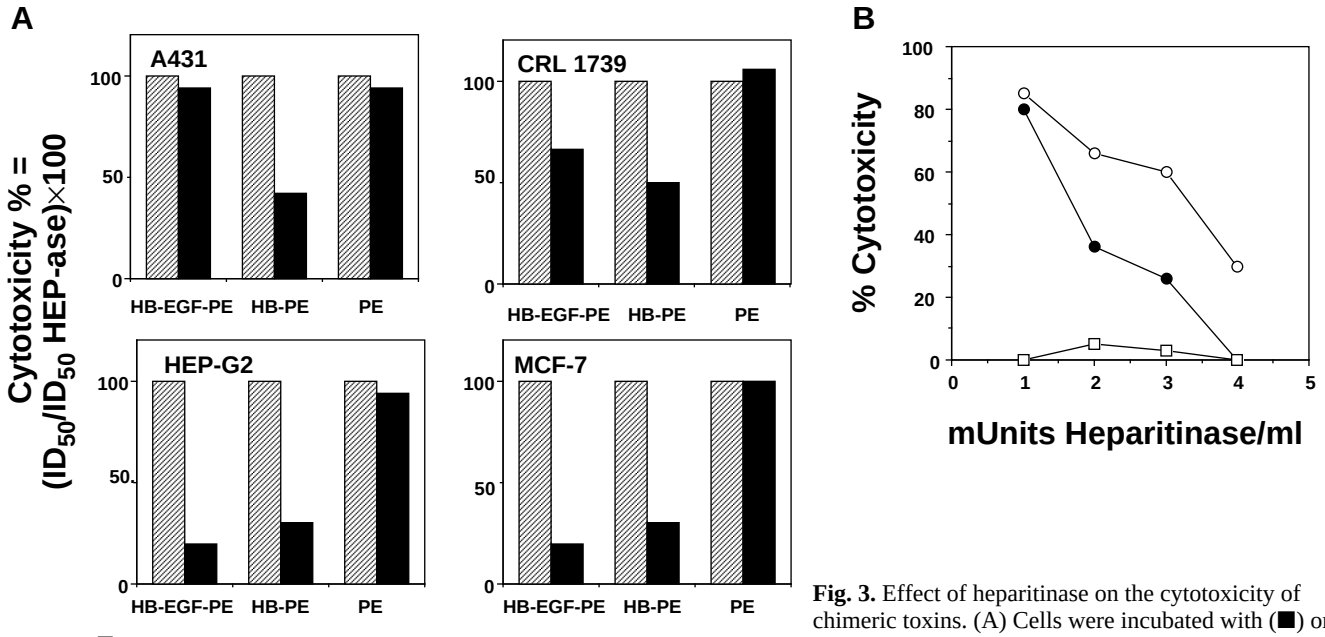


Fig. 3. Effect of heparitinase on the cytotoxicity of chimeric toxins. (A) Cells were incubated with (■) or without (▨) the addition of heparitinase (1 mU/ml) before the addition of the toxins as described in Materials and Methods. (B) Effect of incubating MCF-7 cells with increasing amounts of heparitinase. (○) HB-EGF-PE*, (●) HB-PE*, (□) PE*.

HB domain contributes to BASMC cytotoxicity as it does for cancer cells. Preincubation of the BASMCs with EGF reduced the toxicity of HB-EGF-PE* and TGFα-PE* whereas it had no effect on HB-PE* (Fig. 4B). On the other hand, heparin produced a dose-dependent inhibition of both HB-EGF-PE* and HB-PE* (Fig. 4C) and not TGFα-PE*. Taken together, these results indicate that, as in tumor cells, HB-EGF-PE* is primarily targeting the EGFR of BASMCs, and that the HB domain significantly contributes to the cell binding that is required for cytotoxicity.

Toxicity for cells deficient in HSPG biosynthesis

Our data indicated that binding of the HB domain of the chimeric toxins to the cell surface HSPGs is sufficient by itself to promote cell binding and internalization. The specificity of the cytotoxicity of HB-EGF containing toxins for surface HSPGs was further tested using Chinese hamster ovary cells (CHO) with different levels of surface HSPGs expression. CHO-K1 is the wild-type cell and CHO 667 is a mutant that expresses 1-10% of the normal surface HSPGs (Esko et al., 1988). Results shown in Fig. 5A show that HB-EGF-PE* was more cytotoxic than TGFα-PE* towards CHO-K1 cells (HSPG+). The cytotoxicity of HB-EGF-PE* to CHO 667 (HSPG–) is significantly decreased and reaches almost the same value as TGFα-PE* (Fig. 5). Furthermore, HB-PE* is cytotoxic to wild-type CHO cells but not to CHO HSPG– cells. Competition experiments (Fig. 5) showed a strong inhibitory effect of heparin on the cytotoxicity of HB-EGF-PE* and HB-PE* to CHO-K1, but only a slight effect on the cytotoxicity of HB-EGF-PE* to CHO 667 cells and as expected, no effect on the cytotoxicity of TGFα-PE*. These results using CHO mutants firmly establish that part of the binding and internalization of HB-EGF-PE* is mediated by binding to HSPGs. The absence of cytotoxicity of HB-PE* to HSPG– cells indicates that HB-PE* specifically targets cells through HSPGs.

Table 3. Summary of the results for the recombinant toxins in different types of cells

	High EGFR				Low EGFR				CHO HSPG+		CHO HSPG–	
Toxin	Tx	H	E	He	Tx	H	E	He	Tx	H	Tx	H
HG-EGF-PE*	++	++	–	++	+++	+	++	–	++	–	+	+
TGFα-PE*	+++	+++	–	+++	++	++	–	++	+	+	+	+
HB-PE*	+	–	+	–	+	–	+	–	+	–	–	–
PE*	–	–	–	–	–	–	–	–	–	–	–	–

Results expressed in cytotoxicity comparative units from (+++) very cytotoxic, to (–) no cytotoxicity. Tx, cytotoxicity; H, competition with heparin; E, EGF competition; He, treatment with heparitinase.

DISCUSSION

HB-EGF chimeric toxins: two targets and two targeting domains

To kill cells, chimeric *Pseudomonas* toxins must be internalized by specific receptors. Thus, cytotoxicity data supplies important information about the receptors involved in ligand binding and internalization (Pastan et al., 1992). In this study, we analyzed a bifunctional ligand, HB-EGF, capable of binding both to the EGFR and to HSPGs on the cell surface. To carry out this study, we used HB-EGF-PE* and HB-TGFα-PE*, chimeric toxins with bifunctional ligands, and TGFα-PE* and HB-PE*, which are chimeric toxins with monofunctional ligands (Fig. 1). We characterized their cytotoxicity towards a panel of cells expressing various levels of EGFR and HSPGs. Our results, summarized in Table 3, clearly show the existence of two alternative pathways for internalization of HB-EGF and HB-TGFα chimeric toxins, defined by two different targets (Fig. 6). Cells are killed predominantly by binding of the EGF-like domain of HB-EGF to the EGFR. However, the heparin-binding domain has an important contribution to the cytotox-

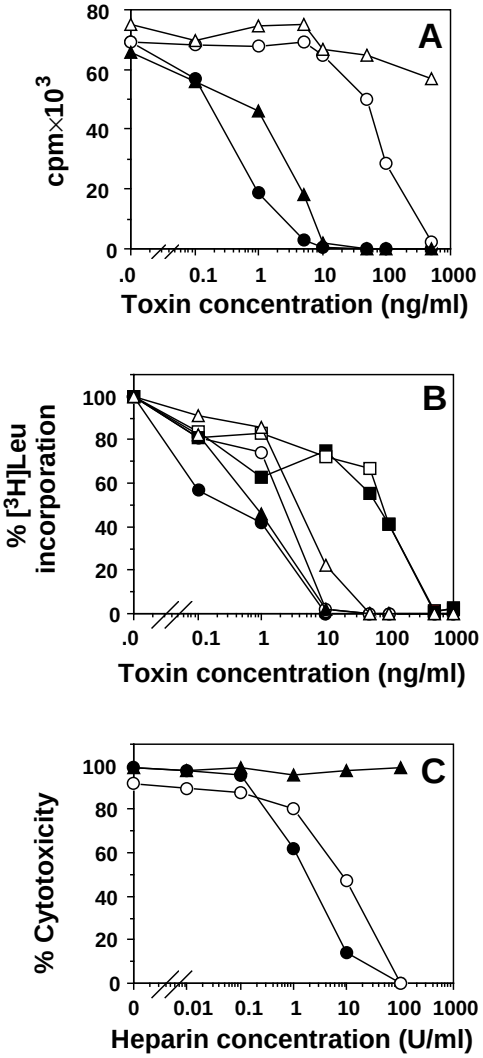


Fig. 4. Cytotoxicity of chimeric toxins towards BASMCs. (A) Without competition. (●) HB-EGF-PE*, (○) HB-PE*, (▲) TGFα-PE* (△) PE*. (B) Effect of EGF competition (4 μg/ml) on the cytotoxicity towards BASMC. (●) HB-EGF-PE*, (■) HB-PE*, (▲) TGFα-PE*, (○) HB-EGF-PE* + EGF, (□) HB-PE* + EGF, (△) TGFα-PE + EGF. (C) Effect of heparin in the cytotoxicity of chimeric toxins to BASMC. A fixed amount of toxin was incubated with the cells with the addition of increasing amounts of heparin (●) HB-EGF-PE* 10 ng/ml (▲) TGFα-PE* 10 ng/ml (○) HB-PE* 500 ng/ml.

icity by allowing the toxin to bind and to be internalized by HSPGs.

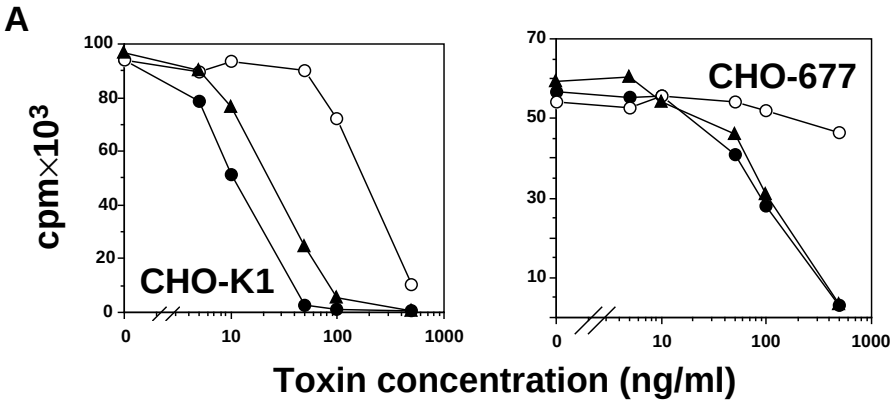
The contribution of the HB domain to the cytotoxicity of the HB-EGF and HB-TGFα chimeric toxins is more clearly manifested in cells with intermediate or low levels of EGFR expression (MCF-7, HEP-G2, smooth muscle cells and CHO cells). In these cells we found that HB-EGF-PE* and HB-TGFα-PE* are usually more cytotoxic than TGFα-PE* (Table 1, Figs 4 and 5; Mesri et al., 1993). HB-EGF-PE* is more cytotoxic than HB-TGFα-PE* towards some of these cells, probably because HB-EGF-PE* has a higher affinity for heparin than HB-TGFα-PE*. On such cells, the increased cytotoxicity of HB-TGFα-PE* and HB-EGF-PE* relative to TGFα-PE* is due to the HB domain, since they all have similar affinities for the EGFR (Fig. 1D), and the increased cytotoxicity can be blocked by either heparin or heparitinase treatment (see Table 2 and Figs 2, 3, 4 and 5). Finally, we confirm that the HB domain facilitates cytotoxicity by specifically binding to HSPGs, showing that HB-EGF-PE* is more cytotoxic to CHO cells than TGFα-PE*, only if these cells express surface HSPGs (Fig. 5A,B).

The HB domain is a new targeting ligand for HSPGs

The results of the present study further confirm that the binding of the heparin-binding domain to surface HSPGs contributes to the cytotoxicity. One way by which this interaction may facilitate the entry of the toxin is by facilitating the binding to the EGFR (Ruoslahti and Yamaguchi, 1991; Mesri et al., 1993). However, since HSPGs are internalized during recycling, binding of HB-EGF to HSPGs could be sufficient by itself to promote toxin internalization and cell killing. To analyze this possibility we used HB-PE*, a recombinant toxin carrying, as a unique targeting ligand, the N-terminal portion of HB-EGF, which contains the heparin-binding site (Higashiyama et al., 1993; Thompson et al., 1994). The cytotoxicity exhibited by HB-PE* towards all the cells tested indicates that the HB domain alone is able to mediate the internalization of PE*. This indicates that the HB domain of HB-EGF-PE* is also able to target the toxin. The cytotoxic effect of HB-PE* can be entirely blocked by heparin and is sensitive to treatment with heparitinase, indicating that the cytotoxicity of HB-PE* involves a HB domain-HSPGs interaction. This conclusion is also supported by the fact that those fractions of HB-PE* that after refolding achieved the highest heparin affinity were also the most cytotoxic (data not shown). Finally, the specificity of HB-PE* for HSPGs was strictly tested by using a mutant CHO cell line deficient in HSPG biosynthesis. The results with CHO cells (Fig. 5) clearly indicate that HB-PE* can kill cells only by specifically binding HSPGs. These observations demonstrate that the heparin-binding domain is enough to mediate the internalization of the toxins through HSPGs, a fact that reinforces the role of HSPGs in endocytosis of heparin-binding growth factors.

Although the rate of HSPGs internalization is much slower than EGF, with a half-life of 3-8 hours (Yanagishita and Hascall, 1992), our data indicate that it could be significant in cells with intermediate levels of EGFR expression and high levels of surface HSPGs, such as the proliferating smooth muscle cells tested in Fig. 4. Our results are consistent with HSPG-mediated internalization of basic FGF, which like HB-EGF is a heparin-binding growth factor that binds to HSPGs and a specific receptor (Gannoun-Zaki et al., 1991; Roghani and Moscatelli, 1992; Rusnati et al., 1993). In a recent interesting study, it was found that the toxin saporin chemically conjugated to basic FGF could internalize via HSPGs alone, but required internalization by a complex of FGF receptor and HSPG to be cytotoxic (Reiland and Rapraeger, 1993). In contrast, our results using HB-EGF-PE* and HB-PE* indicate that either route of internalization of HB-EGF (HSPG or EGFR) leads to cytotoxicity. This is probably because by either route our toxin PE^{4E}KDEL (PE*), can be delivered to the cytosol of the target cell (Chaudhary et al., 1990a; Seetharam et al., 1991).

HSPGs and heparin-binding growth factors play important roles in cell growth and proliferation (Burgess and Maciag,



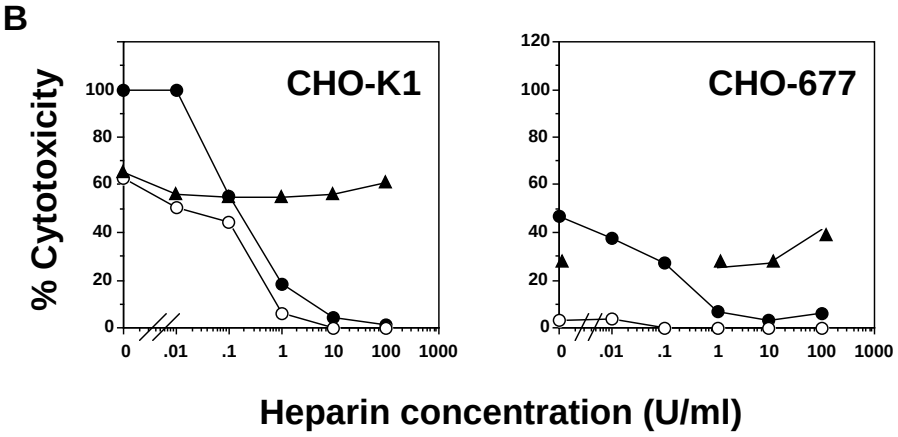
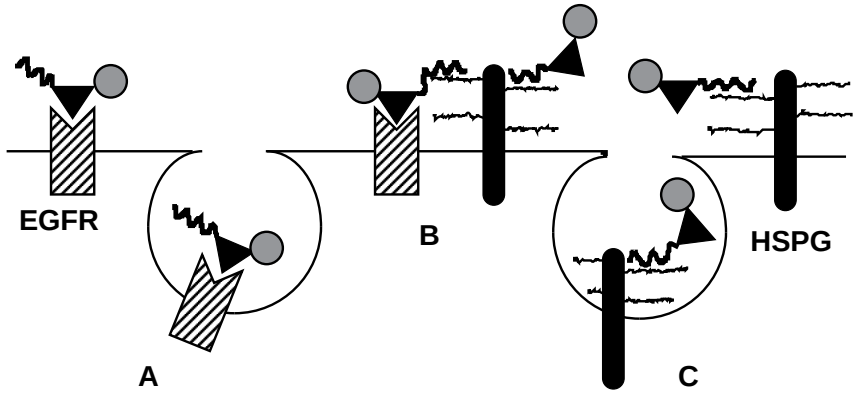


Fig. 5. Cytotoxicity of chimeric toxins towards CHO cells. (A) Without competition. (●) HB-EGF-PE*, (○) HB-PE*, (▲) TGFα-PE*. (B) Effect of heparin on the cytotoxicity of chimeric toxins to CHO cells. A fixed amount of toxin was incubated with the cells with the addition of increasing amounts of heparin. (●) HB-EGF-PE*, 10 ng/ml; (○) HB-PE*, 500 ng/ml; (▲) TGFα-PE*, 10 ng/ml.



	PATHWAYS INVOLVED IN CELL KILLING			
TOXIN	HIGH EGFR	LOW EGFR	CHO K1, HSPG+	CHO 667, HSPG-
HB-EGF-PE*	A >>B, C	A , B, C	A, B, C.	A
TGF-PE*	A	A	A	A
HB-PE*	C	C	C	-
PE*	-	-	-	-

Fig. 6. Initial steps in the pathways involved in the internalization of HB-EGF-PE*. (A) EGFR-mediated endocytosis. (B) HSPGs-facilitated EGFR-mediated internalization. (C) HSPG-mediated internalization. Schematic of toxin domains: (〰) heparin-binding domain, (▲) EGFR-binding domain, (●) toxin domains.

1989; Karnovsky et al., 1989; Kjellen and Lindahl, 1991; Wight et al., 1992), both at the extracellular and intracellular level (Baldin et al., 1990; Imamura et al., 1990; Wight et al., 1992). Therefore, the existence of a pathway that links them intracellularly could be of biological significance. The heparin-binding domain of HB-EGF contains a consensus sequence for bipartite nuclear targeting containing the putative nuclear targeting signal KRKKK that may be functional intracellularly

(Dingwall and Laskey, 1991) as has been shown for the fibroblast growth factor family (Imamura et al., 1990) and for amphiregulin, another heparin-binding EGFR-binding growth factor (Shoyab et al., 1989; Johnson et al., 1991; Modrell et al., 1992). Both heparin-binding growth factors and heparan sulfates have been shown recently to directly affect transcriptional regulation in several systems (Nakanishi et al., 1992; Busch et al., 1992). Interestingly, along the stretch of basic amino acids of HB-EGF that can promote HSPG-mediated internalization are included the sequences that may promote nuclear localization, suggesting that these biological activities could be somehow connected.

New recombinant toxins derived from HB-EGF and *Pseudomonas* exotoxin

This study describes chimeric toxins capable of targeting both HSPGs and/or the EGFR. HB-EGF-PE* is highly cytotoxic to malignant cells with low numbers of EGFRs, and to vascular smooth muscle cells. Specific targeting of proliferating SMC may be useful for the treatment of important vasculopathies such as atherosclerosis and restenosis (Epstein et al., 1991; Cascells et al., 1992; Pickering et al., 1993). We have recently shown that HB-TGFα-PE* can preferentially target proliferating SMCs while preserving quiescent SMCs (Mesri et al., 1993; Fu et al., 1993). Our data indicates that this could also be the case for HB-EGF-PE* (Fig. 4 and unpublished data). Cell surface HSPGs are important molecules in cell adhesion, migration and proliferation (Wight et al., 1992). Abnormal expression and/or internalization of heparan sulfate has been associated with pathological conditions such as diabetic retinopathy and nephropathy (Hanneken et al., 1991; Makino et al., 1992), and atherosclerosis (Karnovsky et al., 1989; Ross, 1993). The availability of HB-PE*, which specifically targets surface HSPGs, may facilitate studies on the involvement of HSPGs in normal and pathological cell growth.

We appreciate the assistance of E. Lovelace and A. Harris in cell culture and Althea Jackson for editorial assistance. We thank Dr Judith Abraham, Scios Nova Inc., Mountain View, CA, for the gift of plasmid pJMU-2 and for her useful comments on the manuscript. Enrique A. Mesri dedicates this paper to his mother Frida Schlaien (1937-1982).

REFERENCES

Austin, G. E., Ratliff, N. B., Hollman, J., Tabeil, S. and Phillips, D. (1987). Intimal proliferation of smooth muscle cells as an explanation for recurrent coronary artery restenosis after coronary angioplasty. *J. Amer. Coll. Cardiol.* **6**, 369-375.

Baldin, V., Roman, A. M., Bosc-Bierne, I., Amalric, F. and Bouche, G. (1990). Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. *EMBO J.* **9**, 1511-1517.

Besner, G. A., Whelton, D., Crissman-Combs, M. A., Steffen, C. L., Kim, G. Y. and Brigstock, D. R. (1992). Interaction of heparin-binding EGF-like growth factor (HB-EGF) with the epidermal growth factor receptor: modulation by heparin, heparinase or synthetic heparin-binding HB-EGF fragments. *Growth Factors* **7**, 289-296.

Burgess, W. H. and Maciag, T. (1989). The heparin-binding (fibroblast) growth factor family of proteins. *Annu. Rev. Biochem.* **58**, 575-606.

Busch, S. J., Martin, G. A., Barnhart, R. L., Mano, M., Cardin, A. D. and Jackson, R. L. (1992). Trans repressor activity of nuclear glycosaminoglycans on Fos and Jun/AP-1 oncoprotein mediated transcription. *J. Cell Biol.* **116**, 31-42.

Cascells, W., Lappi, D. A., Olwin, B. B., Wai, C., Siegman, M., Speir, E. H., Sasse, J. and Baird, A.. (1992). Elimination of smooth muscle cells in experimental restenosis: Targeting of fibroblast growth factor receptors. *Proc. Nat. Acad. Sci. USA* **89**, 7159-7163.

Chaudhary, V. K., Jinno, Y., FitzGerald, D. and Pastan, I. (1990a). *Pseudomonas* exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Nat. Acad. Sci. USA* **87**, 308-312.

Chaudhary, V. K., Jinno, Y., Gallo, M. G., FitzGerald, D. and Pastan, I. (1990b). Mutagenesis of *Pseudomonas* exotoxin in identification of sequences responsible for the animal toxicity. *J. Biol. Chem.* **265**, 16306-16310.

Dingwall, C. and Laskey, R. A. (1991). Nuclear targeting sequences - a consensus. *Trends Biochem. Sci.* **16**, 478-481.

Dluz, S. M., Higashiyama, S., Damm, D., Abraham, J. and Klagsbrun, M. (1993). Heparin-binding epidermal growth factor-like growth factor expression in cultured fetal human vascular smooth muscle cells. Induction of mRNA levels and secretion of active mitogen. *J. Biol. Chem.* **268**, 18330-18334.

Epstein, S. E., Siegall, C. B., Biro, S., Fu, Y., FitzGerald, D. and Pastan, I. (1991). Cytotoxic effects of a recombinant chimeric toxin on rapidly proliferating vascular smooth muscle cells. *Circulation* **84**, 778-787.

Esko, J. D., Rostand, K. S. and Weinke, J. L. (1988). Tumor formation dependent on proteoglycan biosynthesis. *Science* **241**, 1092-1096.

FitzGerald, D. J. P., Morris, R. E. and Saelinger, C. B. (1980). Receptor-mediated internalization of *Pseudomonas* toxin by mouse fibroblasts. *Cell* **21**, 867-873.

Fu, Y.-M., Mesri, E. A., Yu, Z.-Y., Pastan, I. and Epstein, S. E. (1993). Cytotoxic effects on vascular smooth muscle cells of the chimeric toxin heparin-binding transforming growth factor alpha-*Pseudomonas* exotoxin. *Cardiovasc. Res.* **27**, 1691-1697.

Gannoun-Zaki, L., Pierri,, I., Badet, J., Moenner, M. and Barritault, D. (1991). Internalization of basic fibroblast growth factor by Chinese hamster lung fibroblast cells: Involvement of several pathways. *Exp. Cell. Res.* **197**, 272-279.

Hanneken, A., de Juan, E. J., Lutty, G., Fox, G., Schiffer, S. and Hjemmeland, L. (1991). Altered distribution of basic fibroblast growth factor in diabetic retinopathy. *Arch. Ophtalmol.* **109**, 1005-1011.

Higashiyama, S., Abraham, J., Miller, J., Fiddes, J. and Klagsbrun, M. (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936-938.

Higashiyama, S., Lau, K., Besner, G. E., Abraham, J. A. and Klagsbrun, M. (1992). Structure of heparin-binding EGF like growth factor. *J. Biol. Chem.* **267**, 6205-6212.

Higashiyama, S., Abraham, J. and Klagsbrun, M. (1993). Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: Dependence on interaction with cell surface heparan sulfate. *J. Cell. Biol.* **122**, 933-940.

Hwang, J., FitzGerald, D. J., Adhya, S. and Pastan, I. (1987). Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli. Cell* **48**, 129-136.

Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A. J., Maier, J. A. M., Hla, T. and Maciag, T. (1990). Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. *Science* **249**, 1567-1570.

Johnson, G. R., Saeki, T., Auersperg, N., Gordon, A. W., Shoyab, M., Salomon, D. S. and Stromberg, K. (1991). Response to and expression of amphiregulin by ovarian carcinoma and normal ovarian surface epithelial cells: Nuclear localization of endogenous amphiregulin. *Biochem. Biophys. Res. Commun.* **180**, 481-488.

Karnovsky, M. J., Wright, T. C. J., Castellot, J. J. J., Choay, J., Lormeau, J.-C. and Petitou, M. (1989). Heparin, heparan sulfate, smooth muscle cells, and atherosclerosis. *Ann. NY Acad. Sci.* **556**, 268-281.

Kjellen, L. and Lindahl, U. (1991). Proteoglycans: Structures and interactions. *Annu. Rev. Biochem.* **80**, 443-475.

Klagsbrun, M. and Baird, A. (1991). A dual receptor system is required for basic fibroblast growth factor activity. *Cell* **67**, 229-231.

Kreitman, R. J., Chaudhary, V. K., Siegall, C., FitzGerald, D. J. and Pastan, I. (1991). Rational design of a chimeric toxin: An intramolecular location for the insertion of transforming growth factor alpha within *Pseudomonas* exotoxin as a targeting ligand. *Bioconjug. Chem.* **3**, 58-62.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D. and Gallagher, J. T. (1990). Examination of the substrate specificity of heparin and heparan sulphate lyases. *Biochemistry* **29**, 2611-2617.

Lorberboum-Galski, H., Garsia, R. J., Gately, M., Brown, P. S., Clark, R. E., Waldmann, T. A., Chaudhary, V. K., FitzGerald, D. J. and Pastan, I.

(1990). IL2-PE66^{4Glu}, a new chimeric protein cytotoxic to human-activated T lymphocytes. *J. Biol. Chem.* **265**, 16311-7.

Makino, H., Ikeda, S., Haramoto, T. and Ota, Z. (1992). Heparan sulfate proteoglycans are lost in patients with diabetic nephropathy. *Nephron* **61**, 415-421.

Mesri, E. A., Kreitman, R. J., Fu, Y.-M., Epstein, S. E. and Pastan, I. (1993). Heparin binding transforming growth factor alpha-*Pseudomonas* exotoxin A: A heparan sulfate proteoglycan modulated chimeric toxin cytotoxic to cancer and proliferating smooth muscle cells. *J. Biol. Chem.* **268**, 4853-4862.

Modrell, B., McDonald, V. and Shoyab, M. (1992). The interaction of amphiregulin with nuclei and putative nuclear localization sequence binding protein. *Growth Factors* **7**, 305-314.

Nakanishi, Y., Kihara, K., Mizuno, K., Masamune, Y., Yoshitake, Y. and Nishikawa, K. (1992). Direct effect of basic fibroblast growth factor on gene transcription in a cell free system. *Proc. Nat. Acad. Sci USA* **89**, 5216-5220.

Pastan, I., Willingham, M. C. and FitzGerald, D. J. P. (1986). Immunotoxins. *Cell* **47**, 641-648.

Pastan, I. and FitzGerald, D. (1989). *Pseudomonas* exotoxin: chimeric toxins. *J. Biol. Chem.* **264**, 15157-15160.

Pastan, I., Chaudhary, V. K. and FitzGerald, D. P. (1992). Recombinant toxins, novel therapeutic agents. *Annu. Rev. Biochem.* **61**, 331-354.

Pickering, J. G., Bacha, P. A., Weir, L., Jakanowski, J., Nichols, J. C. and Isner, J. M. (1993). Prevention of smooth muscle cell outgrowth from human atherosclerotic plaque by a recombinant cytotoxin specific for the epidermal growth factor receptor. *J. Clin. Invest.* **91**, 724-729.

Rapraeger, A. C., Krufka, A. and Olwin, B. B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**, 1705-1708.

Reiland, J. and Rapraeger, A. C. (1993). Heparin sulfate proteoglycan and FGF target basic FGF to different intracellular destinations. *J. Cell Sci.* **105**, 1085-1093.

Roghani, M. and Moscatelli, D. (1992). Basic fibroblast growth factor is internalized through both receptor-mediated and heparan sulfate proteoglycan-mediated mechanisms. *J. Biol. Chem.* **267**, 22156-22162.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 90's. *Nature* **362**, 801-809.

Ruoslahti, E. and Yamaguchi, Y. (1991). Proteoglycans as modulators of growth factor activities. *Cell* **64**, 867-869.

Rusnati, M., Urbinati, C. and Presta, M. (1993). Internalization of basic fibroblast growth factor (bFGF) in cultured endothelial cells: Role of the low affinity heparin-like bFGF receptors. *J. Cell. Physiol.* **154**, 154-162.

Seetharam, S., Chaudhary, V. K., FitzGerald, D. and Pastan, I. (1991). Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. *J. Biol. Chem.* **266**, 17376-17381.

Shoyab, M., Plowman, G., McDonald, V., Bradley, J. G. and Todaro, G. J. (1989). Structure and function of human amphiregulin: A member of the epidermal growth factor family. *Science* **243**, 1074-1076.

Siegall, C. B., Epstein, S., Speir, E., Hla, T., Forough, R., Maciag, T., FitzGerald, D. J. and Pastan, I. (1991). Cytotoxic activity of chimeric proteins composed of acidic fibroblast growth factor and *Pseudomonas* exotoxin on a variety of cell types. *FASEB J.* **5**, 2843-2849.

Temizer, D. H., Yoshizumi, M., Perrella, M., Sussani, E., Quertermous, T. and Lee, M.-E. (1992). Induction of heparin-binding epidermal growth factor mRNA by phorbol ester and angiotensin II in rat aortic smooth muscle cells. *J. Biol. Chem.* **267**, 24892-24896.

Thompson, S. A., Higashiyama, S., Wood, K., Pollit, N. S., Damm, D., McEnroe, G., Garrick, B., Ashton, N., Lau, K., Hancock, N., Klagsbrun, M. and Abraham, J. A. (1994). Characterization of sequences within heparin-binding EGF-like growth factor that mediate interaction with heparin. *J. Biol. Chem.* **269**, 2541-2549.

Vitetta, E. S., Fulton, R. J., May, R. D., Till, M. and Uhr, J. W. (1987). Redesigning nature's poisons to create anti-tumor reagents. *Science* **238**, 1098-1104.

Wight, T. N., Kinsella, M. G. and Qwarnstrom, E. E. (1992). The role of proteoglycans in cell adhesion, migration and proliferation. *Curr. Opin. Cell Biol.* **4**, 793-801.

Woolf, N. (1990). Pathology of atherosclerosis. *Br. Med. Bull.* **46**, 960-985.

Yanagishita, M. and Hascall, V. C. (1992). Cell surface heparan sulphate proteoglycans. *J. Biol. Chem.* **267**, 9451-9454.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841-848.

(Received 11 April 1994 - Accepted 27 May 1994)