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European Journal of Pharmacology 351 (1998) 79–83

ejp

Heparinase III limits rat arterial smooth muscle cell proliferation in vitro and in vivo

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Received 10 February 1998; accepted 7 April 1998

Abstract

Heparinase III degrades heparan sulfate proteoglycans, which are co-receptors for growth factors that stimulate arterial proliferation. We assessed the ability of locally-delivered heparinase III to limit medial vascular smooth muscle cell proliferation induced by balloon catheter injury in rat carotid arteries. Whereas vehicle-treated arteries showed 12% of smooth muscle cells proliferating after 2 days, heparinase III (0.022–5.7 mg/kg) treated arteries showed 0.8–4%. Chemically-inactivated heparinase III did not limit proliferation. In isolated rat A10 vascular smooth muscle cells, heparinase III (1 IU/ml) inhibited both PDGF-BB and bFGF mediated increases in proliferation and migration. These results suggest that heparinase III can limit proliferation by affecting heparan sulfate proteoglycan binding growth factors following arterial injury.

Keywords: Restenosis; Smooth muscle, vascular; Growth factor; Heparan sulfate proteoglycan

1. Introduction

The vascular smooth muscle cell proliferative response of arteries is thought to contribute to restenosis following balloon catheter injury, and any drug which inhibits this process may have therapeutic value in the treatment of conditions which arise from smooth muscle cell replication (e.g., restenosis following coronary angioplasty or accelerated atherosclerosis in cardiac allograft recipients). Following injury, proliferation and subsequent neo-intima formation depend upon the early proliferation (within 48 h) and migration (within 4 days) of smooth muscle cells from the media to the intima. Early stimuli that are thought to mediate migration and proliferation are platelet and non-platelet derived growth factors and cytokines, including platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and other growth factors, such as transforming growth factor-β (see Ross (1987) and Fagin and Forrester (1992) for some reviews).

Heparan sulfate proteoglycans are present at the cell membrane and are co-receptors for many growth factors. Heparan sulfate proteoglycan-binding growth factors include bFGF (Yayon et al., 1991), heparin binding EGF (Higashiyama et al., 1993) and vascular endothelial growth factor (Goren et al., 1992); two heparin-binding domains have also been found in PDGF (Khachigian et al., 1992). Certain heparin and heparan sulfate fragments (Castellot et al., 1986; Schmidt et al., 1992), as well as anionic aromatic compounds, such as RG-13577 (Benezra et al., 1994), act as heparan sulfate proteoglycan mimetics, which bind some of these growth factors and limit vascular smooth muscle cell proliferation.

We have explored another way to modulate heparan sulfate proteoglycan-growth factor binding as a mechanism to limit vascular proliferation. Heparinase III (EC 4.2.2.8) is a bacterial glycosaminoglycan lyase which only has

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0014-2999/98/$19.00

affinity for heparan sulfate (Ernst et al., 1995). Previous studies with isolated vascular smooth muscle cells in vitro have shown that treatment with heparinase III can limit bFGF-mediated proliferation by removing heparan sulfates (Castellot et al., 1981; Vlodavsky et al., 1995). Since heparan sulfate proteoglycans are co-receptors for many of the growth factors involved in vascular proliferation, and since heparinase III can degrade heparan sulfates, we sought in the present study to examine whether immediate, local administration of heparinase III following balloon catheter injury can influence the vascular proliferative response in vivo. In addition, we have quantified the inhibitory effects of heparinase III on PDGF-BB and bFGF dependent increases in proliferation and migration in vitro in the same species by using isolated rat A10 vascular smooth muscle cells.

2. Materials and methods

2.1. Materials

A stock solution of heparinase III (Specific activity = 53 IU/mg; IBEX Technologies, Montreal, Canada) purified from *Flavobacterium heparinum* was stored at 4°C pending preparation of the dosing solution. The dosing solution was prepared by dilution of the stock. Inactivated heparinase III was prepared by incubation with diethyl pyrocarbonate. Enzymatic analysis showed that activity was reduced to $< 0.1\%$ of the original activity by this procedure.

The diluent used for preparation of dosing solutions was phosphate buffered saline, containing 0.05 M sodium phosphate and 0.150 M sodium chloride, pH 7.0. This diluent was also used as the vehicle control.

2.2. In vivo studies

Young adult, male (300–360 g) Sprague–Dawley rats (*Rattus norvegicus*) were used in this study. All procedures were reviewed by the Institutional Animal Care and Use Committee (Phoenix International Life Sciences).

On Day 1, all animals were anesthetized by intramuscular injection of ketamine (120 mg/kg) in combination with xylazine (12 mg/kg) and subjected to a balloon catheter injury procedure in the left carotid artery. Surgery was carried out under aseptic conditions. Following arterial injury using a balloon catheter, ca. 400 μl of the appropriate vehicle or heparinase III solution was delivered locally following removal of the balloon catheter. Delivery was achieved over approximately 15 s and the dosing solution was maintained in the vessel for approximately 30 s. The catheter used for delivery was then removed and the vehicle or heparinase III solution was allowed to enter the systemic circulation. The left external carotid artery was ligated after removal of the delivery catheter and the wound was closed using surgical clips.

In order to avoid dehydration, animals received a subcutaneous injection (2 ml) of sterile saline on each side at the level of the quadriceps muscle (total of 4 ml per rat). This procedure was carried out following dosing.

On Day 2, all animals were anesthetized by intramuscular injection of ketamine (75 mg/kg) in combination with xylazine (7.5 mg/kg). Bromodeoxyuridine tablets (Boehringer Mannhein) were implanted subcutaneously in the abdomen where they remained for approximately 24 h. Bromodeoxyuridine is a thymidine analogue that is incorporated into the DNA of S-phase proliferating cells (Clowes et al., 1983).

On Day 3, all rats were anaesthetized with ketamine (150 mg/kg) in combination with xylazine (15 mg/kg). Each rat received an intravenous injection (0.4 ml) of Evans Blue Dye (6.0%) prior to killing by a lethal dose of Somnotol®. Post mortem perfusion with Lactated Ringer's solution followed by 4% paraformaldehyde solution was performed via the abdominal aorta.

Arteries were fixed, dehydrated and infiltrated with paraffin wax in a programmed sequence using a Jung TP 1050 Tissue Processor (LEICA) and were then embedded using a Jung Histoembedder (LEICA). Slides were prepared using a Jung 2035 BIOCUT rotary microtome (LEICA), flotation bath and slide warmer. Slides were stained using a bromodeoxyuridine immunohistochemical staining method; slides were incubated with anti-bromodeoxyuridine mouse monoclonal antibody (clone BMC 9318, 1gG1). The monoclonal antibody binds to bromodeoxyuridine incorporated into cellular DNA. This antibody preparation also contains specific nucleases which allows access to bromodeoxyuridine after fixation in ethanol. Hematoxylin-counterstained nuclei and replicating bromodeoxyuridine-stained nuclei were quantified by an observer blinded to treatment. The ratio of bromodeoxyuridine-stained nuclei/total cells was calculated as index of proliferation.

Analysis of variance (ANOVA) was performed for comparing percent medial proliferation of all groups. Duncan's multiple comparison test was used for comparing the means of all groups; a p-value < 0.05 was used for significance.

2.3. In vitro studies

The migration of rat A10 vascular smooth muscle cells (American Type Tissue Culture Collection) was assessed using a 48-well micro-Boyden chamber apparatus from Neuroprobe (Cabin John, MD). Briefly, cells were serum-starved in DMEM for 24 h. They were then exposed to either heparinase III (1 IU/ml) or saline for 1 h at 37°C, and then tyrpsinized for 2 min. They were washed with DMEM containing 20% FBS and resuspended in DMEM medium at a density of 1×10^5 cells/ml. DMEM (25 μl) containing growth factors was first loaded into the lower chamber. The wells were subsequently covered with a

polyvinylpyrroidone-free filter membrane with 8 μm pores (Neuroprobe) pre-coated with type I collagen (Cell System Kirkand, WA). The chamber was assembled and 50 μl of the cellular suspension were then added to the upper wells of the chamber. The chamber was incubated at 37°C under 5% CO_2 in humidified air. After 4 h, the filter membrane was gently removed and smooth muscle cells adhering to the upper side of the filter were removed by scraping 8 times with a rubber blade. Cells that had migrated to the lower side of the filter were fixed and stained in Diff-Quik staining solution (Baxter). Migrated cells were counted for three randomly chosen fields per well under a microscope at a magnification of 400 × in a total of 3–5 experiments. For measuring effects on proliferation, rat A10 cells were seeded at a density of 20 000 cells per 60 mm-dish. The following day they were serum-starved in DMEM for 48 h. Heparinase III (1 IU/ml) or saline was added for 1 h at 37°C in fresh DMEM. Growth factors were added to the enzyme-containing medium for 5 h and replaced with serum-starved medium for 16 h. A second treatment/stimulation was undertaken, and cells were trypsinized and counted with a hemocytometer 2 days later.

3. Results

In injured carotid arteries treated with phosphate buffered saline ($n = 8$), the percent medial proliferation, as evaluated by the anti-bromodeoxyuridine immunohistochemical technique represented 13.73 ± 1.2, 13.98 ± 1.64 and 12.64 ± 1.4% at different sites in the carotid artery (Fig. 1). In comparison, the percentage of vascular smooth muscle cells proliferating in the media of carotid arteries treated with heparinase III (0.22 mg/kg) was 3.75 ± 0.72, 2.90 ± 0.50 and 0.76 ± 0.14% at similar sites (Fig. 1).

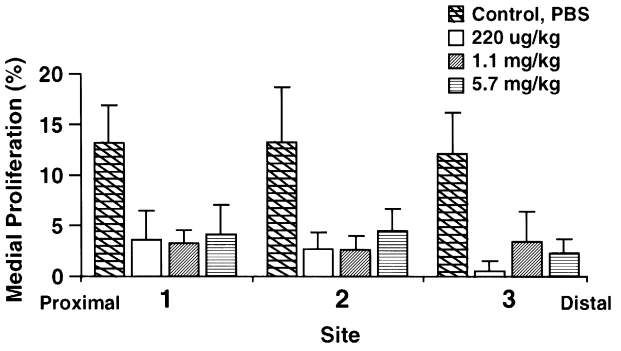

Fig. 1. Effect of heparinase III (0.22, 1.1, 5.7 mg/kg) on medial vascular smooth muscle cell proliferation at different sites in injured left carotid arteries of rats. Values are the mean ± S.E. for 8 rats/group. Arteries were subjected to balloon catheter injury and treatment with heparinase III or phosphate buffered saline (PBS) vehicle as described in the text. Three different sites within the lesion were sampled and are indicated proximal to distal relative to the base of the balloon. The percentage of proliferating medial cells for all groups was calculated by the ratio of bromodeoxyuridine immunochemically-stained vascular smooth muscle cell nuclei/hematoxylin-stained vascular smooth muscle cell nuclei as described in the text. ANOVA showed that all heparinase III-treated sites were similar and significantly ($p < 0.05$) less than the three phosphate buffered saline sites.

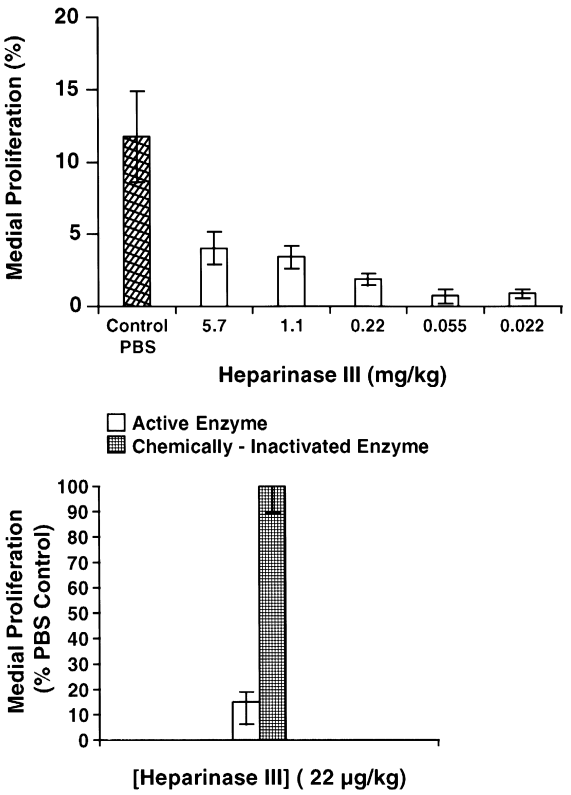

Fig. 2. Top panel. Effects of heparinase III (dose range of 0.022 mg/kg to 5.7 mg/kg) on medial proliferation in injured left carotid arteries. All procedures and quantification were as described in Fig. 1, except that all determinations at 3–4 sites within the arteries were used to generate a single percentage of medial proliferation/artery. Values represent the mean ± S.E. for 6–16 animals/per group. All heparinase III-treated groups were significantly less ($p < 0.05$) than the control phosphate buffered saline group (PBS). Bottom panel. Comparison of heparinase III(active enzyme) with a chemically-inactivated form of heparinase III. Both forms of heparinase III were tested at 22 μg/kg. Values are expressed as % of the phosphate buffered saline control; active enzyme inhibited proliferation by approximately 86% while the inactive enzyme did not affect proliferation.

Percent medial proliferation, expressed as a mean of the three sites, was approximately 12 ± 3% in the control arteries treated with phosphate buffered saline compared to values of 0.8 to 4.2% in the carotid arteries treated with heparinase III (dose range of 22 μg/kg to 5.7 mg/kg, Fig. 2A). In further studies, chemically-inactivated heparinase III was compared with active enzyme (Fig. 2B). Whereas active enzyme inhibited proliferation at all sites by approximately 86%, the inactive enzyme did not produce any significant effect on proliferation.

To further probe the mechanism of action of heparinase III, in vitro studies with isolated rat A10 vascular smooth muscle cells quantifying inhibition of growth factor-dependent proliferation or migration by heparinase III were performed (Table 1). Heparinase III decreased both PDGF-

Table 1
Effect of heparinase III on bFGF or PDGF-BB dependent rat A10 vascular smooth muscle cell proliferation or migration

	Heparinase III (1 IU/ml)		% inhibition
	−	+	
Proliferation (Cells/dish, $\times 10^4$)[a]			
bFGF	4.4 ± 0.4	2.8 ± 0.2	36
PDGF-BB	8.9 ± 0.6	5.3 ± 0.1	40
Migration (Cells/field)[a]			
bFGF	95 ± 5	70 ± 3	27
PDGF-BB	140 ± 20	80 ± 10	43

[a]Cell numbers (mean ± S.E.) are given for bFGF-(1 ng/ml) or PDGF-BB-(10 ng/ml) stimulated rat A10 vascular smooth muscle cell proliferation or migration ($n = 3–5$ experiments/group). Cells were incubated with vehicle (cell medium) or heparinase III (1 IU/ml) for 1 h and then washed to remove heparinase III prior to testing as described in the text.

BB and bFGF mediated increases in proliferation and migration without affecting basal proliferation or migration.

4. Discussion

This study shows that local administration of heparinase III can limit vascular smooth muscle cell proliferation that occurs following vascular injury during balloon catheter injury in vivo. Doses as low as 22 μg/kg were effective, suggesting that discreet application at the time of injury can profoundly affect early proliferation. Consistent with this observation is the additional finding that inactivation of the enzyme completely eliminated the anti-proliferative effect, suggesting that this was not related to the mere presence of the protein, but to enzymatic activity directed against heparan sulfate proteoglycans.

These data further corroborate the finding that heparan sulfate proteoglycans play a central role in modulating growth factor-dependent vascular proliferation (Vlodavsky et al., 1995). Previous studies showed that adding heparan sulfate mimetics or heparin/heparan sulfate fragments, which presumably bind growth factors, prevented vascular proliferation (Castellot et al., 1986; Schmidt et al., 1992; Benezra et al., 1994). The current data show that enzymatic removal of binding sites on heparan sulfates can also produce the same effect in vivo. It is not known at this time which specific growth factors may be affected by heparinase III in vivo, but, since both bFGF and PDGF-BB mediated responses were affected by heparinase III treatment in isolated rat vascular smooth muscle cells, it seems likely that these two growth factors may have been inhibited in vivo. However, it is also possible that other heparin binding growth factors may also be involved.

It is not known if this singular effect on early vascular proliferation would ultimately affect neo-intimal growth/formation which occurs several weeks following arterial injury. Early studies in a similar model of rat arterial injury with antibodies directed against bFGF showed inhibition of early proliferation with no ultimate effect on neo-intima formation (Lindner and Reidy, 1991). Antibodies against PDGF had no great effect on early proliferation, but did affect neointima formation, possibly by affecting later migration (Ferns et al., 1991). It seems unlikely that a single, early heparinase III treatment would be effective. However, future studies should focus on developing methodology to continuously release heparinase III or other anti-growth factor compounds at the site of vascular injury, so that the multiple waves of growth factor-dependent proliferative and migratory responses might be affected.

Acknowledgements

The authors wish to express their appreciation to Ms. Jocelyne Daoust and Ms. Lise Remillard for their technical assistance during the conduct of these studies.

References

Benezra, M., Ben-Sasson, S.A., Regan, J., Chang, M., Bar-Shavit, R., Vlodavsky, I., 1994. Antiproliferative activity to vascular smooth muscle cells and receptor binding of heparin-mimicking polyaromatic anionic compounds. Arterioscler. Thromb. 14, 1992–1999.

Castellot, J.J., Addonizio, M.L., Rosenberg, R.D., Karnovsky, M.J., 1981. Cultured endothelial cells produce a heparin-like inhibitor of cell growth. J. Cell Biol. 90, 372–379.

Castellot, J.J., Choay, J., Lormeau, J.-C., Petitou, M., Sache, E., Karnovsky, M.J., 1986. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells: II. Evidence for a pentasaccharide sequence that contains a 3-*O*-sulfate group. J. Cell Biol. 102, 1979–1984.

Clowes, A.W., Reidy, M.A., Clowes, M.M., 1983. Mechanisms of stenosis after arterial injury. Lab. Invest. 49, 208–215.

Ernst, S., Langer, R., Cooney, C.L., Sasisekharan, R., 1995. Enzymatic degradation of glycosaminoglycans. Crit. Rev. Biochem. Mol. Biol. 30, 387–444.

Fagin, J.A., Forrester, J.S., 1992. Growth factors, cytokines and vascular injury. Trends Cardiovasc. Med. 2, 90–94.

Ferns, G.A., Raines, E.W., Sprugel, K.H., Montani, A.S., Reidy, M.A., Ross, R.S., 1991. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 253, 1129–1132.

Goren, H., Soker, S., Vlodavsky, I., Neufel, G., 1992. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. J. Biol. Chem. 267, 6093–6098.

Higashiyama, S., Abraham, J.A., Klagsbrun, M., 1993. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. J. Cell Biol. 122, 933–940.

Khachigian, L.M., Owensby, D.A., Chesterman, C.N., 1992. A tyrosinated peptide representing the alternatively spliced exon of the

platelet-derived growth factor A-chain binds specifically to cultured cells and interferes with binding of several growth factors. J. Biol. Chem. 267, 1660–1666.

Lindner, V., Reidy, M.A., 1991. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA 88, 3739–3743.

Ross, R., 1987. Platelet-derived growth factor. Annu. Rev. Med. 38, 71–79.

Schmidt, A., Yoshida, K., Buddecke, E., 1992. The antiproliferative activity of arterial heparan sulfate resides in domains enriched with 2-*O*-sulfated uronic acid residues. J. Biol. Chem. 267, 19242–19247.

Vlodavsky, I., Miao, H.Q., Atzmon, R., Levi, E., Zimmerman, J., Bar-Shavit, R., Peretz, T., Ben-Sasson, S.A., 1995. Control of cell proliferation by heparan sulfate and heparin-binding growth factors. Thromb. Haemost. 74, 534–540.

Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., 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.