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Induction of vascular smooth muscle cell growth by selective activation of the thrombin receptor

Effect of heparin

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The synthetic peptide, SFLLRNPNDKYEPF, has been recently described as a peptide mimicking the new amino-terminus created by cleavage of the thrombin receptor, therefore acting as an agonist of the thrombin receptor. This peptide was a potent mitogen for rabbit arterial smooth muscle cells (SMC) and exhibited the same activity as that of native α-thrombin. Both compounds stimulated the proliferation of growth-arrested SMCs with half-maximum mitogenic responses at 1 nM. NAPAP, a synthetic inhibitor of the enzymatic activity of thrombin, specifically inhibited thrombin-induced SMC growth (IC_{50} = 0.35 ± 0.04 μM) but was without effect on the mitogenic effect of the agonist peptide. These results therefore demonstrate that the mitogenic effect of α-thrombin for SMCs is intimately linked to its esterolytic activity. Heparin, which inhibited fetal calf serum-induced SMC growth, was without effect on thrombin-induced SMC growth but strongly reduced the mitogenic effect of the agonist peptide (IC_{50} = 32 ± 5 μg/ml). This effect was not related to the anti-coagulant activity of heparin but was highly dependent on molecular mass and on the global charge of the molecule and was also observed for other sulphated polysaccharides such as pentosan polysulphate.

Thrombin; Vascular smooth muscle cell; Mitogen

1. INTRODUCTION

Over the past several years, a series of observations have demonstrated that the proliferation of vascular smooth muscle cells (SMC) is a key step in the pathogenesis of atherosclerosis [1]. Although much attention has been focused on factors that stimulate SMC proliferation, very little is known about the mechanisms involved in maintaining SMCs in a quiescent growth state or re-establishing the normally quiescent growth state of SMCs following their proliferative response to intimal injury.

Thrombin has central functions in hemostasis but has also been shown to elicit a wide range of cellular responses, and multiple thrombin-mediated effects on vascular cells have been described. In cultured SMCs, α-thrombin stimulates many growth-related signals, including activation of phospholipase C with generation of inositol triphosphate and increase in $[Ca^{2+}]_i$, stimulation of Na^+/H^+ exchange and intracellular alkalinization or induction of the protooncogene, c-*fos* [2–4]. Thrombin exhibits direct mitogenic effect on neonatal rat vascular SMC [2] and bovine aortic SMCs [5]. Whereas it is now recognized that the enzymatically active form of α-thrombin is capable of initiating proliferation of quiescent fibroblasts [6,7], it was recently suggested that α-thrombin was a potent mitogen for SMCs through a distinct non-enzymatic domain [5]. However, since Bar Shavit et al. [5] showed that thrombin immobilized to a naturally produced extracellular matrix retained potent mitogenic activity toward SMCs, other authors have not been able to demonstrate thrombin-induced DNA synthesis or mitogenesis [8].

Recently, molecular cloning of a functional thrombin receptor on platelets and vascular endothelial cells revealed a novel proteolytic mechanism of receptor activation [9,10]. These works revealed a new signalling mechanism in which α-thrombin cleaved its receptor's amino-terminal extension to create a new receptor amino-terminus that functioned as a tethered ligand and activated the receptor. In these studies, a synthetic peptide of 14 residues (SFLLRNPNDKYEPF) (hereafter refered to as thrombin agonist peptide: TRA) corresponding to the new receptor N-terminal was shown to activate the cloned thrombin receptor and induce platelet activation. Since this ligand can only be generated by catalytic action of the enzyme, our work therefore consisted of studying the effect of this synthetic agonist peptide on the growth of vascular SMCs in culture. We also evaluated the activity of sulphated polysaccharides on thrombin- or the TRA-induced SMC proliferation.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), glutamine, collagenase (type II), streptokinase and penicillin were purchased from Boehringer-Mannheim (France). Standard heparin (from porcine intestinal mucosa, 168 IU/mg) was from Sigma Chemicals Co. (France). Pentosan polysulphate (PPS), pentosan, *N*-desulphated heparin, low molecular weight heparin (LMWH) (CY 216–Fraxiparin), *N*α-(2-naphtyl-sulphonyl-glycyl)-DL-*p*-amidinophenylalanyl-piperidine (NAPAP) [11] and the synthetic peptide (TRA)(SFLLRNPNDKYEPF) were from Sanofi Recherche (Toulouse, France). The heparin fraction with no affinity for anti-thrombin III (non-affine heparin) was a kind gift from J.C. Lormeau (Sanofi Recherche, Gentilly, France). Human α-thrombin (3,000 NIH U/mg) was purchased from Centre Regional de Transfusion Sanguine (Strasbourg, France).

2.2. *Culture of vascular smooth muscle cells*

Smooth muscle cells (SMC) were isolated from the rabbit aorta as described previously [12]. Briefly, media fragments from the thoracic aorta from New-Zealand rabbits (2.0–2.5 kg, Charles River, France) were incubated for 16 h at 37°C in DMEM containing collagenase (0.15%), FCS (5%), penicillin (100 IU/ml), streptomycin (100 μg/ml) and glutamine (4 mM). After incubation, SMCs were sedimented by gentle centrifugation (400 × g, 10 min), resuspended in DMEM + 10% FCS and grown at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium (DMEM + 10% FCS) was changed every 3 days and a confluent SMC monolayer was obtained after about 7 days. Cells were routinely used from the third to the sixth passage.

2.3. *Cell proliferation assays*

Cells were plated sparsely (10^3 cells/well) in 96 well cluster plates (Nunc, Denmark) in DMEM + 0.5% FCS. After 3 days, cells in representative dishes were counted with a coulter counter (Coultronics, France) and fresh medium was added to the remaining dishes (DMEM + 0.5% FCS, α-thrombin or TRA and the different concentrations of the drugs to be tested). For growth rate determinations, after 3 days in culture, cells were detached from triplicate wells by trypsin treatment (0.05% trypsin, 0.02% EDTA) and counted in a coulter counter. IC_{50} values were calculated on the basis of the linear regression lines established for each compound tested using a regression program [17].

3. RESULTS AND DISCUSSION

3.1. *Effects of α-thrombin and TRA on the proliferation of SMC in culture*

Addition of either native α-thrombin or TRA to quiescent rabbit aortic SMCs resulted in stimulation of their growth. As indicated in Fig. 1, both α-thrombin and TRA induced a 14–18-fold increase in cell number over a period of 1–4 days. The concentration of α-thrombin required to get optimal cell proliferation (A_{max}) was 1 μM with a half-maximal response (ED_{50}) at 1 nM (Fig. 2). This observation is consistent with already published results [5]. The TRA, when tested at concentrations ranging from 0.1 nM to 10 μM, exhibited a significant mitogenic effect for SMCs, the A_{max} as well as the ED_{50} of TRA being highly similar to those observed for native α-thrombin.

Since the peptide we used has already been shown by Vu et al. [9] to mimick the new amino-terminus created by cleavage of the thrombin receptor, our results suggest that enzymatic activity of α-thrombin is necessary for the triggering of its mitogenic effect on SMCs. Hence, our results create a discrepancy with those of Bar Shavit et al. who recently suggested that α-thrombin was a potent mitogen for vascular SMCs through a distinct non-enzymatic domain [5]. In order to confirm our hypothesis, the specificity of thrombin-induced proliferation was demonstrated by growing SMCs in the presence of NAPAP. This direct thrombin inhibitor [11] did not affect the mitogenic effect of FCS or TRA but blocked the α-thrombin-induced proliferation of SMCs with an IC_{50} value (concentration which inhibits 50% of cell growth) of 0.35 ± 0.04 μM (n = 3) (Fig. 3). At this concentration, NAPAP exhibited a ~60% inhibition of the amidolytic activity of α-thrombin, a 100% inhibitory effect being attained after incubation of α-thrombin with 1 μM of NAPAP. Therefore, all of our observations indicate that α-thrombin functions as a potent mitogen toward vascular SMCs through its catalytic activity.

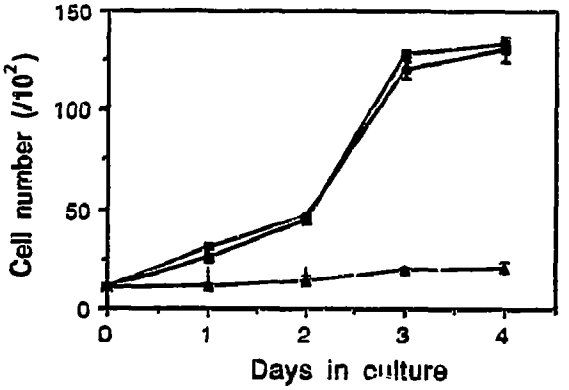

Fig. 1. Time-course of SMC growth in the presence of α-thrombin or TRA. Rabbit aortic SMCs were seeded (10^3 cells/well) in culture medium containing 0.5% FCS alone (▲), 1 μM α-thrombin (●) or 1 μM TRA (■). Cells were trypsinized and counted at the indicated times. Data are reported as mean cell density ± S.D. from 3 replicate cultures.

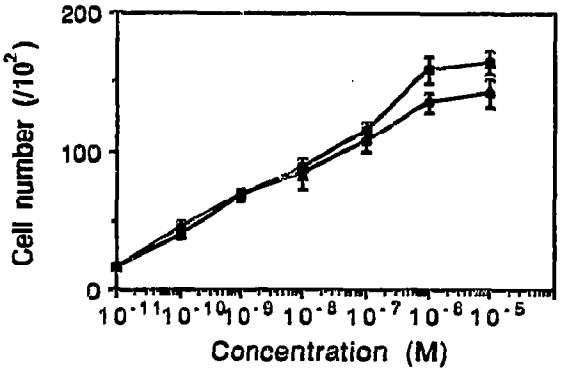

Fig. 2. Proliferative effects of α-thrombin and TRA on SMC. Sparse cultures of SMCs (10^3 cells/well) were grown in DMEM + 0.5% FCS with the indicated concentrations of α-thrombin (●) or TRA (■). After 3 days in culture, cells were trypsinized and counted. Data are reported as mean cell density ± S.D. from 3 replicate cultures.

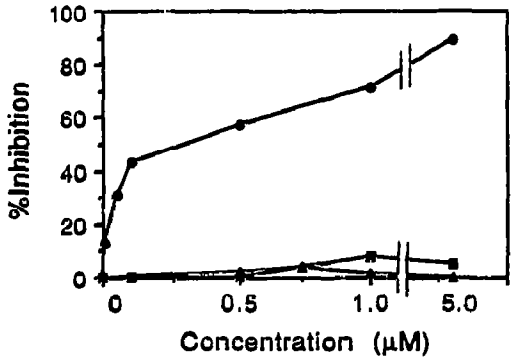

Fig. 3. Effect of NAPAP on SMC growth. SMCs (10^3 cells/well) were allowed to grow in the presence of DMEM + 0.5% FCS supplemented with α-thrombin (1 μM) (●), TRA (1 μM) (■) or 5% FCS (▲). Increasing concentrations of NAPAP were added simultaneously. After 3 days in culture, triplicate wells were trypsinized and cells were counted. In the controls, cell numbers were (cells/well): thrombin 13,500 ± 700, TRA 15,900 ± 1,000, FCS 14,300 ± 550. Data are expressed as mean % inhibition of proliferation compared with replicate cultures grown without the inhibitor (n = 3).

3.2. *Effect of heparin and related compounds on α-thrombin or TRA-induced SMC growth*

In recent years, glycosaminoglycans, such as heparin, have been shown to be potent inhibitors of the proliferation of vascular SMCs in culture and in vivo [12–14]. Indeed, in the blood vessel wall, heparan sulphate may play a role in the regulation of cell proliferation, since endothelial cells, but also SMC themselves, produce heparan sulphate with high anti-proliferative activity for SMC [15,16]. We therefore evaluated the effect of heparin on thrombin- or TRA-induced SMC growth. When different concentrations of standard heparin were added to sparse cultures of SMCs in combination with TRA, a strong anti-proliferative effect was observed (Fig. 4). Standard heparin, at a concentration as low as

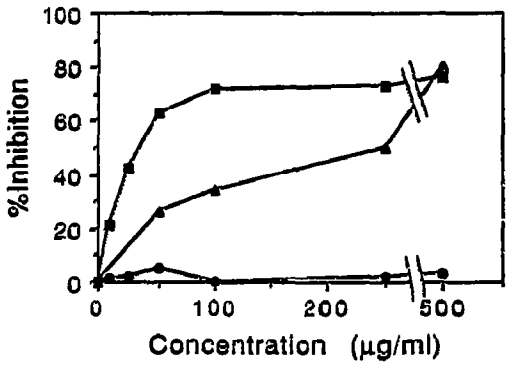

Fig. 4. Effect of standard heparin on SMC growth. SMCs (10^3 cells/well) were allowed to grow in the presence of DMEM + 0.5% FCS supplemented with α-thrombin (1 μM) (●), TRA (1 μM) (■) or 5% FCS (▲). Increasing concentrations of standard heparin were added simultaneously. After 3 days in culture, triplicate wells were trypsinized and cells were counted. Cell number in the controls were the same as that determined in Fig. 3. Data are expressed as mean % inhibition of proliferation compared with replicate cultures grown without heparin (n = 3).

Table I

Comparative inhibitory effects of various sulphated polysaccharides on serum- and TRA-induced SMC growth

Compounds	IC_{50} (μg/ml)	
	FCS	TRA
Standard heparin	250 ± 13	32 ± 4.5
Low mol. wt. heparin	465 ± 31	312 ± 27
Non-affine heparin	280 ± 29	28 ± 3
N-Desulphated heparin	> 500	> 500
Pentosan polysulphate	41 ± 9	6 ± 1
Pentosan	> 500	> 500

SMCs were allowed to grow in the presence of TRA (1 μM) or 5% FCS. Increasing concentrations of the various compounds were added simultaneously. After 3 days in culture, triplicate wells were trypsinized and cells were counted. Data are expressed as means ± S.D. (n = 3).

10 μg/ml, significantly reduced the proliferative response to an optimal concentration of TRA (1 μM), the IC_{50} value being 32 ± 4.5 μg/ml. Under the same experimental conditions, even at high concentrations, standard heparin did not affect the α-thrombin-stimulated SMC growth, whereas, when added in combination with 5% FCS, standard heparin reduced SMC growth with an IC_{50} value of 250 ± 13 μg/ml. This latter figure is in agreement with already published results [12]. The fact that TRA induced SMC growth without altering thrombin mitogenic response may indicate that heparin interacts directly with TRA, but further characterization of the binding properties of TRA to SMCs will be necessary to resolve this issue.

As indicated in Table I, heparin, which showed no affinity for anti-thrombin III, exhibited almost the same effect as standard heparin on both TRA- and FCS-induced proliferation of SMCs. Therefore, as already shown in vivo or with respect to the mitogenic effect of FCS [13,14], our observations indicate that the effect of heparin on the mitogenic activity of TRA is not related to its anti-coagulant activity. Molecular mass and global charge of the molecule appeared to be important for the anti-proliferative effect of heparin since decrease in the mean molecular weight (from 12.5 kDa for standard heparin to 4.7 kDa for the low molecular weight heparin fraction) or N-desulphation of standard heparin resulted in a strong loss of its anti-proliferative effect with regard to both mitogens.

Since pentosan polysulphate (PPS) has been described as a potent inhibitor or SMC proliferation [12], we investigated it for its ability to reduce TRA-induced SMC proliferation. In our experimental conditions, PPS exhibited a 5-fold higher anti-proliferative effect than standard heparin and showed no effect on the mitogenic effect of native thrombin. Such an activity has already been reported with regard to FCS-induced SMC growth [12]. As already observed for heparin, pentosan was devoid of any effect, showing that the anti-proliferative

activity of PPS was highly dependent on the charge of the molecule.

In summary, the present study demonstrates that enzymatic activity of α-thrombin is necessary for its mitogenic activity with regard to SMCs. Furthermore, our work reports for the first time the mitogenic effect of a peptide mimicking the amino-terminus created by enzymatic cleavage of the thrombin receptor. We also focused on the effect of heparin and related molecules on this mitogenic effect and work is now underway to determine if these effects may be considered part of their mechanism of action on SMC proliferation.

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