Nucleic acids potentiate Factor VII-activating protease (FSAP)-mediated cleavage of platelet-derived growth factor-BB and inhibition of vascular smooth muscle cell proliferation

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FSAP (Factor VII-activating protease) can cleave and inactivate PDGF-BB (platelet-derived growth factor-BB) and thereby inhibits VSMC (vascular smooth-muscle cell) proliferation. The auto-activation of FSAP is facilitated by negatively charged polyanions such as heparin, dextran sulfate or extracellular ribonucleic acids. Since auto-activation is essential for the anti-proliferative function of FSAP, the influence of nucleic acids as cofactors for the FSAP-mediated inhibition of PDGF-BB was investigated. Natural or artificial RNA was an effective cofactor for FSAP mediated PDGF-BB degradation, whereas the effect of DNA was weak. RNA-induced cleavage of PDGF-BB was inhibited by serine protease inhibitors. The pattern of PDGF-BB cleavage was identical with either heparin or RNA as a cofactor. One of the cleavage sites in PDGF-BB was at the positions 160–162 (R¹⁶⁰KK¹⁶²), which

is an important region for receptor binding and activation. In VSMCs, PDGF-BB-stimulated DNA synthesis was inhibited by FSAP in the presence of RNA. RNA was more effective than DNA and the cofactor activity of RNA was neutralized after pre-treatment with RNase. FSAP binding to RNA protected the nucleic acid from degradation by RNase. These data are relevant to situations where extracellular nucleic acids released from necrotic or apoptotic cells could activate local FSAP, leading to inhibition of PDGF-BB.

Key words: atherosclerosis, cofactor, Factor VII-activating protease (FSAP), nucleic acid, platelet-derived growth factor-BB (PDGF-BB), serine protease.

INTRODUCTION

FSAP (Factor VII-activating protease) was originally isolated as plasma hyaluronan-binding protein [1]. This serine protease is present in human plasma at a concentration of 12 µg/ml and has multiple roles in haemostasis, such as activation of Factor VII [2], high molecular mass kininogen [3] and uPA (pro-urokinase) [4]. FSAP also inhibits PDGF-BB (platelet-derived growth factor-BB)-mediated proliferation and migration of VSMCs (vascular smooth muscle cells) through specific cleavage of the growth factor [5]. The Marburg I polymorphism of FSAP, resulting in a single amino acid substitution (G534E), was reported to be associated with late complications of carotid stenosis [6] and venous thromboembolism [7]. This variant of FSAP exhibits diminished proteolytic activity towards PDGF-BB [8], which could explain its association with enhanced cardiovascular risk.

FSAP has a strong affinity for negatively charged substances such as hyaluronic acid, dextran sulfate, heparin or nucleic acids which potentiate auto-activation [9]. More specifically, RNA was found to be a much more effective activator of FSAP compared with DNA [9]. The binding of such charged molecules via the EGF3 (epidermal growth factor-like) domain of FSAP promotes the auto-activation of the proenzyme into the two-chain form [10]. Since the autocatalytic activation of FSAP by heparin is necessary for the inhibition of PDGF-BB-mediated proliferation by FSAP [5], we have investigated whether nucleic acids are cofactors for this FSAP-mediated inhibition of PDGF-BB as well. RNA was found to be a potent activator of FSAP-mediated cleavage of PDGF-BB, whereas DNA had a much weaker effect. Our data indicate that nucleic acids released from dying cells of necrotic

tissue could augment activation of FSAP and thereby inhibit mesenchymal cell proliferation and migration through FSAP-mediated inactivation of PDGF-BB.

MATERIALS AND METHODS

Materials

FSAP was prepared from human plasma as described previously [5]. Artificial RNA analogue poly(I)·(C) and artificial DNA analogue poly(dI-dC)·(dI-dC) were from Amersham Pharmacia. Heparin was from Ratiopharm (Ulm, Germany).

Isolation of nucleic acids

Total cellular RNA and genomic DNA were isolated from CHO (Chinese hamster ovary) cells using an RNeasy kit and a Genomic DNA kit respectively (Qiagen) following the manufacturer's instructions. Before use, the concentrations of nucleic acids were determined photometrically by Gene Quant (Amersham Pharmacia) and their quality was checked by agarose gel electrophoresis followed by ethidium bromide staining. Total cellular RNA was pre-treated by RNase A (Fermentas, St. Leon-Rot, Germany) where indicated.

BrdU (bromodeoxyuridine) incorporation assay and MAPK (mitogen-activated protein kinase) 44/42 phosphorylation in VSMC

Human and mouse VSMCs were cultured as described previously [5]. Cells were grown in 96-well plates for 48 h and then cultured

Abbreviations used: BrdU, bromodeoxyuridine; CHO, Chinese hamster ovary; FSAP, Factor VII-activating protease; MAPK, mitogen-activated protein kinase; PDGF-BB, platelet-derived growth factor-BB; VSMC, vascular smooth-muscle cell.

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Figure 1 RNA is a cofactor for FSAP-mediated cleavage of PDGF-BB

(A) [¹²⁵I]-PDGF-BB was incubated with 1 μg/ml of FSAP in the presence of 10 μg/ml of heparin, natural RNA or natural DNA for 60 min at 37 °C. (B) [¹²⁵I]-PDGF-BB was incubated as described in (A), except that poly(I) · (C) or poly(dI/dC) · (dI/dC) were used as cofactors. (C) Similarly, FSAP was compared with inactivated PPACK (Phe-Pro-Arg-CH₂Cl)-FSAP, or FSAP in the presence of 2 μg/ml of the protease inhibitor aprotinin or 8 μg/ml of the protease nexin-1 (PN-1), in the presence of poly(I) · (C) or heparin (each 10 μg/ml). After SDS/PAGE under reducing conditions, autoradiography was performed.

in serum-free medium overnight before stimulation with agonists for 24 h. BrdU was added during the last 4 h of the DNA synthesis phase and the binding of a monoclonal anti-BrdU antibody (Roche Diagnostics) was used to quantify the incorporation of BrdU into DNA. Experiments were performed in triplicate and results are shown as means ± S.D. Serum-starved VSMCs were incubated for 15 min with test substances and thereafter the cells were lysed in SDS buffer [62.5 mM Tris/HCl, pH 6.8, 25 % (w/v) SDS, 10 % (v/v) glycerol and 0.05 % Bromophenol Blue] containing 1 mM orthovanadate. Western blot analysis was performed with antibodies against total and phosphorylated MAPK 44/42 (New England Biolabs) to measure activation of this signal transduction pathway.

PDGF-BB cleavage by FSAP

Cleavage experiments were performed with [¹²⁵I]-PDGF-BB (Amersham Biosciences, GE Healthcare) and cleavage was followed by SDS/PAGE (15 % gels) under reducing conditions

[10 % (v/v) 2-mercaptoethanol] and autoradiography. Additionally, recombinant PDGF-BB (R&D Systems) was incubated with single-chain FSAP in the absence or presence of cofactors in 20 mM Tris/HCl (pH 7.4) containing 100 mM NaCl. To determine the cleavage sites in the PDGF-B chain, the gels were blotted on to PVDF membranes and the bands cut out for further analysis. The N-terminal sequences of the fragments were determined by automated Edman degradation using an Applied Biosystems 492 pulsed liquid phase sequencer equipped with an on-line 785A phenylthiohydantoin derivative analyser. Edman degradation was performed through 10 cycles, and the amino acids detected at each cycle were aligned with the PDGF-B chain sequence, thereby allowing for the detection of multiple N-termini in the preparation.

RNA protection assay

Cellular total RNA (60 μg/ml) was incubated with RNase A (Fermentas) at different concentrations (0, 3, 10 and 30 ng/ml)

Figure 2 Analysis of the cleavage sites in PDGF-BB

PDGF-BB (20 μ g/ml) was incubated with FSAP (200 μ g/ml) in the presence of heparin or poly(I:C) · (I/C) (10 μ g/ml each), as indicated, for 60 min at 37 °C. SDS/PAGE was performed under reducing conditions and the protein fragments were detected by silver-staining. From a similar experiment, proteins were blotted on to a PVDF membrane and stained with Coomassie Blue and bands were analysed by N-terminal sequencing. Molecular mass standards are indicated in kDa. The results of the N-terminal sequencing are shown and the complete sequence of the PDGF-B chain is marked with the FSAP-cleavage sites.

in HEPES-buffered saline (pH 7.4) for 5 min in the absence or presence of 60 μ g/ml FSAP, control buffer (5 mM sodium citrate, pH 4.5, 20 mM NaCl and 200 mM arginine) or 2000 units/ml RNase inhibitor (SUPERaseIn; Ambion). Analysis of RNA was performed by 1% (w/v) agarose gel electrophoresis, followed by ethidium bromide staining.

RESULTS

Nucleic acids are cofactors for FSAP-mediated cleavage of PDGF-BB

We have shown previously [8] that FSAP cleaves PDGF-BB in a dose- and time-dependent manner in the presence of the cofactor heparin, and that the cleavage was only discernable under reducing conditions, and not under non-reducing conditions. This indicates that, after limited proteolysis, the PDGF fragments were held together through disulfide bridges and that the antibody-binding site was intact. [125 I]-PDGF-BB cleavage was determined in the presence of natural RNA, DNA as well as synthetic poly(I) · (C) or synthetic poly(dI-dC) · (dI-dC). Natural or artificial RNA was a more effective cofactor for the cleavage of PDGF-BB than DNA (Figures 1A and 1B). This effect was dependent on the concentration of RNA (Figures 1A and B) and could be inhibited by protease inhibitors (Figure 1C). These results suggest that RNA is an effective cofactor for the limited proteolysis of PDGF-BB by FSAP. Fragments of cleaved PDGF-BB (Figure 2) were resolved by SDS/PAGE and blotted on to PVDF membrane, and were analysed by N-terminal sequencing (Figure 2). This led to the determination of preferential proteolysis sites after amino acid Arg¹¹³, Arg¹⁶⁰ and Lys¹⁶¹. Arg¹¹³ is a known cleavage site in PDGF-BB and natural fragments starting at this point are found in platelets; however, the responsible protease was unknown [11]. The last two cleavage sites are in loop III of PDGF-BB, which is important for receptor binding and activation [12].

Nucleic acids are cofactors for FSAP-mediated inhibition of PDGF-BB-induced cell proliferation

In the absence of any cofactor, there was only a moderate inhibition of PDGF-BB-mediated proliferation by FSAP, whereas heparin augmented this activity as described previously (Figure 3A) [10]. In the presence of cellular RNA, a complete inhibition of proliferation was observed (Figure 3A). For comparison, in the absence of FSAP, RNA and heparin were ineffective in this function (Figure 3A). Pre-treatment of RNA with RNase A completely abolished the cofactor activity of RNA, whereas RNase alone did not influence proliferation (Figure 3B).

The influence of RNA on FSAP anti-proliferative activity was dose-dependent from 0.1 to 100 μ g/ml, with a significant inhibition already observed with the lowest concentration of RNA tested (Figure 3C). In contrast, inhibition of proliferation was also observed with DNA between 0.1 and 10 μ g/ml, but this inhibition was not dose-dependent. Maximal inhibition to basal levels was observed with 100 μ g/ml DNA in the presence of FSAP (Figure 3C). Similarly, synthetic RNA inhibited proliferation dose-dependently between 0.1 and 100 μ g/ml, whereas synthetic DNA-mediated inhibition was not effective (Figure 3D). Thus cellular or synthetic nucleic acids were effective cofactors for mediating FSAP-dependent PDGF-BB inhibition at concentrations from as low as 0.1 μ g/ml, and RNA was a more potent cofactor than DNA.

Nucleic acids are cofactors for FSAP-mediated inhibition of PDGF-BB-induced MAPK phosphorylation

We have shown previously that PDGF-BB-mediated MAPK 44/42 phosphorylation was inhibited by FSAP in the presence of the cofactor heparin. Similarly, phosphorylation of MAPK 44/42 induced by PDGF-BB was significantly reduced by FSAP in the presence of 10 μ g/ml of extracellular RNA or poly(I) · (C). No effect was observed with genomic DNA or poly(dI/dC) · (dI/dC) in either mouse or human VSMCs (Figure 4). Hence only RNA is effective in the rapid MAPK phosphorylation assay.

Protection of RNA from RNase by FSAP

Since RNA binds very strongly to FSAP [10], we hypothesized that FSAP might protect RNA from the actions of RNase. Indeed, a co-incubation of RNA with FSAP delayed the cleavage of RNA by RNase in comparison with controls (Figure 5). No RNA degradation was observed in the presence of RNase inhibitor, which served as a positive control. Hence RNA is not only a cofactor for FSAP, but FSAP also protects RNA from degradation by RNases.

DISCUSSION

PDGF-BB is a key mediator regulating mesenchymal cell proliferation and migration in restenosis and atherosclerosis [13] and other fibrosis-related diseases of the liver, kidney and the lung [14–17]. FSAP is a potent inhibitor of PDGF-BB-mediated VSMC activation in the presence of negatively charged polyanions such as heparin [5]. We have also shown that wild-type FSAP, but not the natural variant Marburg I-FSAP, can inhibit neointima formation in a mouse vascular injury model [8]. Hence the regulation of the enzymatic activity of FSAP is critical and nucleic acids, particularly RNA, can contribute to this regulation.

We have shown previously that FSAP enzymatic activity is potentiated by RNA, whereas DNA was far less efficient [9,10]. Moreover, the size of the nucleic acid molecule is also an important factor with respect to FSAP activation, with fragments larger

Figure 3 Nucleic acids are cofactors for FSAP-mediated inhibition of PDGF-BB-induced DNA synthesis in VSMC

(A) DNA synthesis was measured by BrdU incorporation in the absence (open bars) or presence (closed bars) of 20 ng/ml PDGF-BB. PDGF-BB (10 ng/ml) was pre-incubated for 1 h in the presence of FSAP (12 μg/ml) with or without CHO cell-derived RNA (10 μg/ml) and heparin (10 μg/ml). (B) The cofactor activity of RNA upon pre-treatment by RNase A. The inset panel shows an agarose gel electrophoresis analysis of intact RNA and the influence of RNase treatment on RNA. PDGF-BB-mediated DNA synthesis in the presence of FSAP as well as RNA, RNase or RNase-treated RNA was measured as described above. (C) Increasing concentrations of cellular total RNA or genomic DNA (0.1–100 μg/ml) were tested for cofactor activity as described above. (D) The cofactor activity of different concentrations of artificial RNA analogue poly(I) · (C) and artificial DNA analogue poly(dI/dC) · (dI/dC) (0.1–100 μg/ml) were measured as described above. The results are shown means ± S.D. of absorbance measured at 405 nm for triplicate wells.

that 100 bp being generally more effective. In the present study, we demonstrate that the cofactor function of nucleic acids, with respect to FSAP auto-activation, is also relevant for PDGF-BB cleavage and inhibition of mesenchymal cell proliferation. The effect of nucleic acids was observed in mouse and human VSMCs at the level of PDGF-BB cleavage, MAPK phosphorylation and cell proliferation. Experiments with artificial RNA and DNA indicate that nucleic acids are indeed the active principle and these effects cannot be due to any contaminants that may have been co-purified with the nucleic acids. The effect on proliferation was observed with nucleic acid concentrations of 0.1 μg/ml. The probable reason why DNA was an effective cofactor for FSAP in the proliferation assay, but not in the MAPK phosphorylation assay, is likely to be due to the different time spans of these responses. Although PDGF-BB degradation by FSAP is slower/weaker in the presence of DNA compared with RNA, because of the longer incubation times in the proliferation assay there is a significant effect, but this is not observed in the short-term MAPK phosphorylation assay. This indicates that RNA is a more potent cofactor for FSAP activation than DNA with respect to PDGF-BB degradation.

PDGF-BB is a dimer of anti-parallel chains held together by disulfide bridges. The structure of PDGF has been elucidated [18] and a large number of studies have mapped the regions involved in receptor binding and activation [12]. In loop III, the cationic residues Arg¹⁶⁰, Lys¹⁶¹ and Lys¹⁶² are important for binding of PDGF-B, as well as the A-chain, to its respective receptor [12]. Peptides from this region are antagonists of PDGF-receptor binding and cellular activation. FSAP in the presence of RNA or heparin cleaves PDGF-BB at Arg¹⁶⁰ and Lys¹⁶¹ and this is a plausible explanation for the reduction in the activity of PDGF-BB by FSAP. It is known that PDGF-B chain isolated from human platelets contains up to 30 % of a cleaved product starting at Thr¹¹⁴ [8]. This was another site where FSAP cleaved PDGF, indicating that such naturally cleaved forms are also found *in vivo*.

Since atherosclerotic plaques contain a varying degree of damaged cells due to cholesterol accumulation [19], coupled with apoptosis/necrosis [20] and an overactive inflammatory response, nucleic acids are certainly likely to be present in the plaque [21]. These are likely to be degraded by endothelial cell derived-RNases [22], plasma RNases [23] or DNases, so that substantial concentrations may not be attained. From a stability point of view,

Figure 4 Nucleic acids are cofactors for FSAP-mediated inhibition of PDGF-BB-induced MAPK phosphorylation

(A) The effect of nucleic acids and FSAP on phosphorylation of MAPK 44/42 induced by PDGF-BB in mouse or human VSMCs. PDGF-BB (10 ng/ml) was pre-incubated with FSAP (12 μ g/ml) in the presence or absence of total cellular RNA, poly(I)·(C), genomic DNA or poly(dI/dC)·(dI/dC) (each at 10 μ g/ml). Western blot analysis was used to determine the levels of phosphorylated MAPK, and total MAPK analysis was performed on stripped blots to confirm equal loading of the wells. (B) Densitometric analysis was performed to quantify the changes in the phosphorylation levels.

Figure 5 FSAP protects RNA from degradation by RNase

Total cellular RNA (60 μ g/ml) was incubated with RNase A in dose-dependent manner (0, 3, 10 and 30 ng/ml) in Hepes-buffered saline (pH 7.4) for 5 min, as well as in the presence of FSAP (60 μ g/ml) or FSAP control buffer or RNase inhibitor (2000 units/ml). RNA was then loaded on an agarose gel and visualized by Ethidium Bromide staining.

DNA is more likely to be the relevant polyanion than RNA, since it is more likely to be stable in the plaque even though it is less effective with respect to FSAP activation. There are indications that nucleic acids, particularly oxidatively modified ones, are found in atherosclerotic plaques as well [24]. The fact that FSAP protects RNA against degradation by RNase suggests that RNA may also be a significant factor regulating FSAP activity.

We propose that nucleic acids released from dying cells would activate local FSAP, which, in turn, could cleave and inactivate PDGF-BB. These results provide a novel insight into the role of FSAP and nucleic acids in atherothrombosis. Moreover, extra-

cellular nucleic acids can also regulate complement activation [25] as well as the innate immune system [26], both of which are also likely to be involved in atherosclerotic plaque development [27].

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