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Prothrombin Cleavage by Human Vascular Smooth Muscle Cells: A Potential Alternative Pathway to the Coagulation Cascade

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Abstract Thrombin is a potent mitogen for human vascular smooth muscle cells (HVSMC) and its enzymatic activity is required for this function. The present study demonstrates that prothrombin is also mitogenic for HVSMC due to the generation of enzymatically active thrombin which occurs upon incubation of prothrombin with the cells. Analysis by SDS-PAGE, immunoblotting, and amino acid sequencing revealed that prothrombin incubated with HVSMC undergoes limited proteolysis. Prethrombin 1 was formed through cleavage at R^{155}-S^{156}. Cleavage at R^{271}-T^{272} generated fragment 1.2 and prethrombin 2 whilst cleavage at R^{284}-T^{285} yielded truncated prothrombin 2 (prethrombin 2'). However, cleavage at R^{320}-I^{321} which, during prothrombin activation produces two-chain α-thrombin, was not detectable. Studies on HVSMC-conditioned medium revealed that a similar pattern of prothrombin cleavage occurred by a cell-secreted factor(s). Amidolytic activity analysis indicated that 1–3% catalytically active thrombin-like activity was generated upon incubation of prothrombin with HVSMC-conditioned medium. By treating conditioned medium with various classes of proteinase inhibitors or hirudin, it was determined that prothrombin is cleaved by a cell-derived serine proteinase-like factor(s) at R^{271}-S^{272} and by α-thrombin at R^{155}-S^{156} and R^{284}-T^{285} . Antibodies neutralising the activity of either urokinase, tissue plasminogen activator, or factor Xa failed to alter the prothrombin cleaving activity of conditioned medium. This activity which may catalyse an alternative pathway for the generation of thrombin, was eluted from a gel filtration column as a single peak with apparent molecular mass of 30–40 kDa.

Key words: prothrombin, prethrombin 2, fragment 1.2, α-thrombin, prothrombin activation, serine proteinase, human vascular smooth muscle cells, mitogenic activity, enzymatic activity

In the final stages of blood coagulation, α-thrombin is generated from prothrombin as a result of two successive cleavages at R^{271}-T^{272} and R^{320}-I^{321} bonds [reviewed by Mann, 1994]. Cleavage of these bonds is catalysed by activated factor X (Xa) in the presence of factor Va and Ca^{2+} assembled on a phospholipid surface (the prothrombinase complex). Since the conversion of prothrombin to α-thrombin requires two successive cleavages, two reaction pathways involving different transient intermediates are possible (Fig. 1) [Mann et al., 1990; Doyle and Haley, 1993]. Prethrombin 2 is generated if the R^{271}-T^{272} bond is cleaved first. If, however, R^{320}-I^{321} cleavage occurs first, then meizothrombin is generated. Both prethrombin 2 and meizothrombin are further cleaved at R^{320}-I^{321} and R^{271}-T^{272}, respectively, to yield the two-chain disulphide-linked α-thrombin. Once formed, α-thrombin cleaves an additional 13 amino acids from its A-chain N-terminus at R^{284}-T^{285} generating a stable form of α-thrombin [Butkowski et al., 1977].

The zymogen prothrombin, a 72 kDa glycoprotein, is synthesized in the liver and at 0.07–0.1 mg/mL is the most abundant of the coagulation factors present in the plasma [Mann, 1994]. The main function of α-thrombin in haemostasis is

Abbreviations used: CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; DMEM, Dulbecco's Modified Eagle's Medium; DTT, 1,4-dithiothreitol; ECL, enhanced chemiluminescence; FCS, foetal calf serum; HVSMC, human vascular smooth muscle cells; PBS, phosphate buffered saline; PMSF, phenylmethanesulphonyl fluoride; PVDF, polyvinylidene difluoride; SMC, smooth muscle cells; TBS, Tris buffered saline; TBS-T, TBS-tween; [^{3}H]-TdR, tritiated thymidine; uPA, urokinase type plasminogen activator; VSMC, vascular smooth muscle cells; TPA, tissue plasminogen activator.

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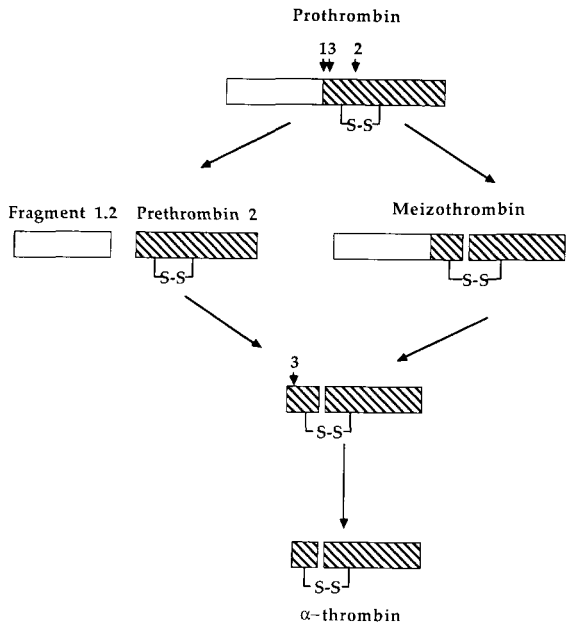

Fig. 1. Schematic diagram of prothrombin activation. Factor Xa cleaves prothrombin at R^{271}-T^{272} (site 1) and R^{320}-I^{321} (site 2) bonds. If cleavage at site 1 occurs first, then prethrombin 2 is formed as an intermediate product. If site 2 is cleaved before site 1, then meizothrombin is generated as an intermediate. α-Thrombin is formed from both intermediates by cleavage of site 2 in prethrombin 2 or site 1 in meizothrombin. Thrombin generated possesses autocatalytic activity which removes 13 amino acids of its own A-chain by cleavage at R^{284}-T^{285} (site 3) generating a stable form of α-thrombin.

to convert soluble fibrinogen to fibrin monomers which aggregate to form the insoluble fibrin clot. This enzyme also activates factors V, VIII, XI, and XIII and, via a negative feedback mechanism, inhibits its own formation by activating protein C [Mann et al., 1990; Esmon, 1993]. In addition to its role in coagulation, thrombin is a potent vascular smooth muscle cell (VSMC) mitogen [Huang et al., 1987; Bar-Shavit et al., 1990; Kanthou et al., 1992; McNamara et al., 1993] and exerts a wide spectrum of effects on the cells of the vessel wall and the circulation. These effects include platelet activation [Harmon and Jamieson, 1986], release of vasoactive and growth promoting factors from the endothelium [Garcia et al., 1992], induction of the directional migration of monocytes, macrophages, and neutrophils [Bar-Shavit et al., 1992; Bizios et al., 1986], and mitogenesis in endothelial cells, macrophages, and fibroblasts as well as VSMC [Belloni et al., 1992; Obberghen-Schilling and Pouyssegur, 1993].

The presence of thrombin receptors on VSMC and their elevated expression in atherosclerotic and restenotic lesions [McNamara et al., 1993; Nelken et al., 1992; Wilcox et al., 1994], together with data indicating reduced neointimal growth in balloon catheterised animals infused with thrombin inhibitors [Sarembock et al., 1991; Walters et al., 1994], suggest that this proteinase may play a key role in the development of vascular wall lesions. Indeed, the contribution of thrombosis to atherosclerotic plaque formation is well documented [Fuster et al., 1992; Wilcox et al., 1994]. Several other enzymes and cofactors of the coagulation and fibrinolytic cascades such as tPA [Herbert et al., 1994], protein S, and factors X and Xa [Gasic et al., 1992] may contribute to the mitogenic stimulation of quiescent VSMC in the arterial wall. According to the response-to-injury hypothesis for the development of atherosclerosis, the initial smooth muscle cell (SMC) proliferative event occurs once the damaged endothelium ceases to provide a non-thrombogenic barrier [Ross, 1993]. The cellular components of the vascular wall are then exposed to factors secreted by infiltrating cells, circulating plasma proteins and products resulting from the activation of the coagulation cascade. Prothrombin as a major protein of blood plasma is likely to be in contact with VSMC. Although thrombin generation via the prothrombinase complex has been well documented using either synthetic vesicles or cells, the interactions of prothrombin with cultured VSMC have remained largely uninvestigated.

In the present study, we examine the interaction of prothrombin with cultured HVSMC. We report that upon incubation with HVSMC, prothrombin is cleaved by a cell secreted factor(s) to specific fragments with some generation of enzymatically and mitogenically active α-thrombin in a factor Xa-independent manner. These findings are important in that they demonstrate that prothrombin may be at least partially activated by HVSMC via an alternative mechanism to that of the coagulation cascade. The biological significance and implications of prothrombin cleavage by HVSMC and the question as to whether this process represents either an alternative mechanism of prothrombin activation or a defence reaction, are discussed.

MATERIALS AND METHODS

Reagents

Purified human prothrombin was purchased from Enzyme Research Laboratories (Swansea, U.K.). Highly purified human α-thrombin (spe-

cific activity >3,000 U/mg) and leech-derived hirudin (specific activity 8,930 U/mg) were obtained from Sigma (Poole, U.K.). The purity of prothrombin, thrombin, and hirudin was further ascertained by SDS-PAGE and silver staining. Single chain human tissue plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) were from Sigma. Rabbit polyclonal anti-human prothrombin antibody which recognises both intact prothrombin and its proteolytically cleaved fragments [Benezra et al., 1993] was from Dako (High Wycombe, U.K.). The following neutralising antibodies: anti-human tPA monoclonal antibodies (ESP 4 and ESP 5), anti-human uPA goat polyclonal antibody, and anti-human factor Xa monoclonal antibody (clone 5224) were from American Diagnostica (Greenwich, CT). Factor Xa, tPA, and uPA neutralising antibodies were used at concentrations (20, 100, and 20 μg/mL, respectively) which are far in excess of those stated by the manufacturer to be able to fully neutralise the activities of these factors at amounts likely to be present in HVSMC-conditioned media. The neutralising activities of the above antibodies were further ascertained by chromogenic assays for tPA and uPA and clotting assay for factor Xa antibodies. Anti-human smooth muscle cell α-actin monoclonal antibody, FITC-coupled anti-mouse IgG, and Tween-20 were from Sigma. Peroxidase-conjugated anti-rabbit IgG, non-immune mouse IgG, and non-immune goat IgG were from Jackson Immuno-Research Laboratories (Philadelphia, PA). Proteinase inhibitors PMSF, pepstatin, E64, amastatin, and soya-bean trypsin inhibitor were from Calbiochem-Novabiochem (Nottingham, U.K.). Cell culture media and supplements were from Gibco-BRL Life Technologies (Paisley, U.K.). Tritiated thymidine ([^{3}H]-TdR), specific activity 5 Ci/mmol, and protein electrophoresis molecular weight markers were from Amersham (Amersham, U.K.). Electrophoresis reagents, nitrocellulose membrane (0.45 μm pore size), and Bio-Gel P60 gel filtration medium were from Bio-Rad Laboratories (Hemel Hempstead, U.K.). Sephadex G-25 columns were from Pharmacia Biotech (St. Albans, U.K.). "Immobilon" PVDF membrane was purchased from Millipore (Watford, U.K.). S-2238 chromogenic substrate was purchased from Chromogenix (Mölndal, Sweden). Other reagents not specified above were of the highest grade commercially available.

HVSMC Isolation and Culture

HVSMC were isolated from sections of normal abdominal aorta obtained from kidney transplant donors (10–45 years of age) as described previously [Kanthou et al., 1992]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 100 IU/mL penicillin, 100 μg/mL streptomycin sulphate, 2.5 μg/mL amphotericine B, 4 mM L-glutamine, and 15 mM HEPES buffer, pH 7.4. Cultures were kept in a humidified atmosphere at 37°C and 10% CO_2, were subcultured at a split ratio of 1:4, and were used between passages 2–8. HVSMC were identified as such by their characteristic "hills and valleys" pattern of growth and by their positive immunostaining with an anti-human smooth muscle cell α-actin antibody [Chamley-Campbell et al., 1977].

DNA Synthesis Assay

DNA synthesis assays were performed as described previously [Kanthou et al., 1992]. Briefly, HVSMC in suspension were plated out (96-well plates; 1×10^4 cells per well) in DMEM/10% FCS. At confluence, cells were growth arrested for 72 h in DMEM supplemented with 0.5% (v/v) FCS, treated with the indicated factors for 16 h in serum-free medium, and labelled with 1 μCi/mL [^{3}H]-TdR for 24 h (16 to 40 h post-stimulation). At the end of the labelling period, cells were dislodged by trypsinisation and harvested onto glass fibre GF/B (Whatman, Maidstone, UK) filters using an automatic cell harvester. [^{3}H]-TdR incorporated into the DNA was quantified by liquid scintillation counting.

Conditioned Medium Preparation and Column Chromatography

Confluent HVSMC cultures (4×10^4 cells/cm^2) were rinsed three times in phosphate-buffered saline (PBS) and incubated in serum-free medium for 24 h to remove serum. HVSMC were then incubated in serum-free medium at a ratio of 2 mL medium/10^6 cells for specified periods of time. Conditioned medium was collected and cells and debris removed by centrifugation (2,500g, 10 min). Membranes generated by cell lysis were eliminated by ultracentrifugation (100,000g, 1 h, 4°C) and medium was stored

at −70°C. Prior to subjecting conditioned medium to column chromatography, this was first concentrated 20-fold by Aquacide (Calbiochem-Novabiochem, Nottingham, UK) and dialysed against PBS. Five milliliter 20-fold-concentrated conditioned medium was loaded onto a Bio-Gel P-60 column (1 × 40 cm) previously calibrated using a Pharmacia gel filtration kit (Pharmacia Biotech, St. Albans, U.K.). Samples (0.5 mL fractions) were eluted in PBS at a flow rate of 0.5 mL/min and used for analysis of prothrombin cleavage (as for conditioned medium below) with PBS as a control.

Analysis of Prothrombin Cleavage

Confluent HVSMC monolayers in 24-well plates (8×10^4 cells/well) were rinsed three times in PBS and incubated in serum-free medium for 24 h. Prothrombin, at a final concentration of 6 to 12 μM, was then added to each well in 0.5 mL serum-free DMEM. Incubations were carried out at 37°C in 10% CO_2 for specified times after which the medium was collected resuspended in Laemmli sample buffer and analysed by SDS-PAGE under reducing conditions [Laemmli, 1970]. In these experiments, the controls consisted of adding prothrombin (6 to 12 μM) to 0.5 ml DMEM in separate wells free of HVSMC.

The analysis of prothrombin cleavage by HVSMC-conditioned medium was assessed by incubating prothrombin at a final concentration of 12 μM with HVSMC-conditioned medium or serum-free medium (control) at 37°C for specified times. The reactions were stopped by the addition of sample buffer and samples were analysed by SDS-PAGE. The sensitivity of HVSMC-conditioned medium prothrombin converting activity to different agents was analysed by subjecting such medium to various treatments prior to the addition of prothrombin and analysis of cleavage products as before. Aliquots of conditioned medium were treated with either trypsin (10 μg/mL, 15 min 37°C followed by addition of 100 μg/mL soya bean trypsin inhibitor), heat (5 min, 95°C), acidification (decreasing the pH to 2.0 with HCl for 20 min followed by neutralisation with 1N NaOH), DTT (10 mM, 1 h, 4°C; DTT subsequently removed by filtration on a G-25 column). The sensitivity of prothrombin converting activity to proteinase inhibitors (pepstatin, 1 μM; EDTA, 10 mM; amastatin, 10 μM; E64, 10 μM; PMSF, 1 mM; aprotinin, 2 μg/mL; hirudin, 2–20 U/mL) was examined by incubating aliquots of HVSMC-conditioned medium with each inhibitor for 10 min prior to the addition of prothrombin. The analysis of prothrombin cleavage in the presence of uPA, tPA, or factor Xa was carried out in serum-free DMEM. Prothrombin (12 μM) was incubated in the presence of either 1–5 U/mL uPA or 100–400 U/mL tPA or 2 μg factor Xa at 37°C for 12 h after which aliquots were removed and subjected to SDS-PAGE and immunoblotting. The effects of the specific anti-tPA, anti-uPA, and anti-factor Xa antibodies on the prothrombin converting activity were studied by incubating conditioned medium with each antibody or a similar amount of non-immune IgG control, for 20 min at 37°C just prior to the addition of the prothrombin. Fresh antibody or non-immune IgG control were added to each sample every 2 h until the end of the 12 h incubation period after which time prothrombin cleavage was analysed by SDS-PAGE and immunoblotting.

For Western blotting analysis, proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes (0.8 m A/cm^2 of membrane, 45 min) using an LKB-Pharmacia semi-dry blotter. The blot buffer consisted of 48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS, and 20% methanol, pH 8.9 [Kyhse-Andersen, 1984]. The nitrocellulose membrane was blocked with 5% (w/v) dry milk in Tris-buffered saline (pH 7.2) (TBS) for 1 h at 20°C and then incubated for 1 h with the prothrombin antibody diluted 1:200 in TBS. Membranes were washed three times with TBS containing 0.1% (v/v) Tween-20 (TBS-T) and further incubated with an anti-rabbit antibody conjugated with horseradish peroxidase. After extensive washings of the membrane in TBS-T, protein bands were visualised by the Enhanced Chemiluminescence technique using an ECL kit (Amersham, Amersham, U.K.). Alternatively, for N-terminal amino acid sequencing, prothrombin cleavage products were separated by SDS-PAGE and electrotransferred as described above onto PVDF membranes using 10 mM 3-[cyclohexylamino]-1-propanesulphonic acid (CAPS) transfer buffer pH 11. Protein bands were visualised by staining with Coomassie blue and N-terminal amino acid analysis was carried out using the Edman degradation method on an Applied Biosystems Inc. Model 473A amino acid sequencer.

Assay for Thrombin Amidolytic Activity

Thrombin amidolytic activity was assayed with the chromogenic substrate S-2238 [Gaffney et al., 1977]. Samples of prothrombin or thrombin were incubated with HVSMC-conditioned medium or DMEM (control) for 12 h at 37°C. Serial dilutions of the samples were performed in 0.1 M $NaPO_4$, 0.2 M NaCl, 0.5% PEG, and 0.02% azide, pH 7.5, buffer [Kettner and Shaw, 1981], and 50 μl was added to 150 μl 0.13 mM S-2238 in the same buffer in the wells of a 96-well plate in a temperature controlled automatic microplate reader (Molecular Devices, Menlo Park, CA). Changes in absorbance (405 nM) at 37°C were monitored every 18 s and standard curves were performed using a thrombin preparation of known activity from which values of unknown samples were extrapolated.

RESULTS

Mitogenic Activity of Human Prothrombin on HVSMC

Several studies have previously demonstrated that α-thrombin is a potent SMC mitogen and that the integrity of thrombin's catalytic site is required for this effect [Huang et al., 1987; Kanthou et al., 1992; McNamara et al., 1993]. The effect of prothrombin, which is enzymatically inactive, on HVSMC mitogenesis was investigated and compared to that of α-thrombin. Growth-arrested HVSMC were treated with either prothrombin, human α-thrombin, or control serum-free DMEM, and the level of DNA synthesis was determined by measuring [^{3}H]-TdR incorporation 16–40 h post-stimulation. Table I (A) shows that prothrombin in contact with HVSMC induced an increase in the level of DNA synthesis in a dose dependent manner. Concentrations of either 1,200 nM prothrombin, which is within the physiological range of prothrombin in the circulation (1 to 2 μM), or 4 nM thrombin induced a half-maximal increase in the level of [^{3}H]-TdR incorporation by HVSMC. These data suggested that either prothrombin was mitogenic for HVSMC, or that the prothrombin preparation was contaminated with active thrombin, or an enzymatically active and mitogenic form of thrombin was generated during incubation of prothrombin with HVSMC. Serial dilutions of the authentic prothrombin preparation were examined for amidolytic activity. No amidolytic activity was detectable in samples of 0.06–6 μM prothrombin, indicating that no active thrombin was present in the prothrombin preparation. Pre-incubation of both α-thrombin and prothrombin with the thrombin-specific inhibitor, hirudin, for 5 min at 37°C prior to their addition to the cells completely abolished their mitogenic effect (Table I, B). These data indicate that the inhibition of prothrombin-mediated mitogenesis by hirudin was due to the inactivation of enzymatically active thrombin generated during the incubation of prothrombin with the cells.

TABLE I. Effects of Prothrombin and Thrombin on DNA Synthesis in Aortic HVSMC*

Treatment	Concentration (nM)	cpm ± sem
A		
Control DMEM	0	3,302 ± 402
Prothrombin	1.2	4,750 ± 138
	12	6,301 ± 224
	120	8,716 ± 654
	1,200	9,957 ± 935
	6,000	17,314 ± 1,821
α-Thrombin	0.8	3,570 ± 117
	4.0	9,516 ± 326
	8.0	16,841 ± 1,527
	40.0	12,970 ± 1,035
B		
Control DMEM	0	4,359 ± 321
Prothrombin	120	13,379 ± 1,021
α-Thrombin	8	17,564 ± 1,447
Hirudin	(5 U/mL)	4,694 ± 232
Prothrombin + hirudin	120 + (5 U/mL)	5,365 ± 108
α-Thrombin + hirudin	8 + (5 U/mL)	5,722 ± 225

*Growth arrested HVSMC (4×10^4 cells/cm^2) in 96-well plates were treated with the indicated amounts of human prothrombin or α-thrombin (A) or prothrombin or α-thrombin in the presence or absence of hirudin (B). Incubations were carried out for 40 h and cells were labelled with 1 μCi/mL [^{3}H]-TdR during the last 24 h of incubation. Cells were harvested onto glass fibre filters and incorporated radioactivity was determined by liquid scintillation counting.

Analysis of Prothrombin Cleavage by HVSMC

Prothrombin (6 μM) was incubated with HVSMC in serum-free medium for various durations and then the cell culture medium was analysed by a combination of SDS-PAGE and immunoblotting using a polyclonal anti-human prothrombin antibody, which recognises both intact prothrombin and its cleavage products.

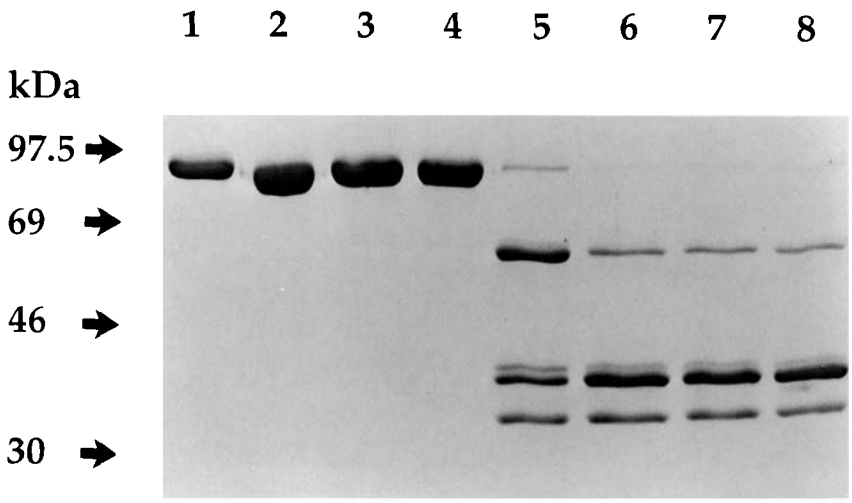

Fig. 2. Analysis of HVSMC-mediated prothrombin cleavage by SDS-PAGE and Western blotting. Prothrombin (6 μM) was incubated with cultured HVSMC (4×10^4 cells/cm^2) in 24-well plates in serum-free medium for either 1 h (*lane 3*), 4 h (*lane 4*), 8 h (*lane 5*), 12 h (*lane 6*), 24 h (*lane 7*), 48 h (*lane 8*), or in serum-free medium in the absence of cells for 24 h (*lane 2*). *Lane 1* represents native prothrombin (6 μg). Aliquots were removed and subjected to SDS-PAGE (10% acrylamide) under reducing conditions followed by immunoblotting using a polyclonal anti-human prothrombin antibody which recognises prothrombin and its cleaved products. Immunodetection was performed by ECL as indicated in Materials and Methods.

As seen in Figure 2 (lane 5), by 8 h prothrombin (72 kDa protein band) was cleaved into fragments with an apparent molecular mass of 50, 38, 36, and 32 kDa. Incubation of prothrombin with HVSMC for either 12, 24, or 48 h (lanes 6, 7, and 8, respectively) did not result in any further cleavage. No cleavage of prothrombin was observed after its incubation with HVSMC for a shorter period (1 or 4 h; lanes 3 and 4, respectively). In the absence of HVSMC (control serum-free DMEM), prothrombin remained intact after 24 h of incubation at 37°C (lane 2). The concentration of prothrombin used in these experiments (6 μM) was chosen to enable clear visualisation of bands without the need to concentrate samples prior to electrophoresis. Similar cleavage of prothrombin occurred when physiological concentrations of prothrombin (1.2 μM) were incubated with HVSMC (data not shown). Immunoblotting confirmed that the various bands observed correspond to prothrombin fragments rather than to cell-derived proteins. A similar pattern of bands was obtained with Coomassie blue stained SDS gels (not shown). These data indicate that the observed prothrombin cleavage requires the presence of a cell surface or a cell-derived factor(s) and that no further prothrombin proteolysis occurs with prolonged incubation.

Analysis of Prothrombin Cleavage by HVSMC Conditioned Medium

To determine whether the observed cleavage of prothrombin by cultured HVSMC could occur in the absence of a cell surface membrane, experiments were performed using serum-free HVSMC-conditioned medium. Confluent cultures of HVSMC were rinsed several times in serum-free DMEM and incubated in similar medium for 24 h to ensure complete removal of serum. Such cultures were further incubated in new serum-free medium for different time intervals after which conditioned medium was collected and ultracentrifuged (100,000*g*, 1 h, 4°C) to remove any cell membrane constituents. Prothrombin was incubated in such conditioned medium (12 μM in 100 μl) or control serum-free DMEM for 12 h at 37°C after which samples were analysed by SDS-PAGE and immunoblotting. As shown in Figure 3, prothrombin cleavage occurred only with HVSMC-conditioned medium but not with control DMEM. These data indicate that limited proteolysis of prothrombin can occur in the absence of a cell surface membrane and is mediated by an HVSMC-secreted factor(s). As reported previously [Mann, 1977; Benezra et al., 1993], incubation of prothrombin with control DMEM resulted in the formation of a 50 kDa fragment which corresponds to pre-

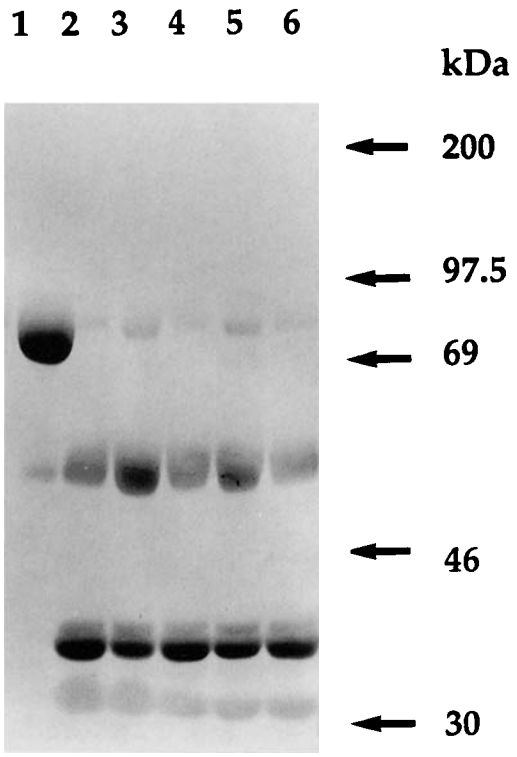

Fig. 3. Analysis of prothrombin cleavage by HVSMC conditioned medium. Prothrombin (12 μM) was incubated for 24 h at 37°C with either control DMEM (*lane 1*), or medium conditioned by HVSMC for 1 h (*lane 2*), 4 h (*lane 3*), 12 h (*lane 4*), 24 h (*lane 5*), and 48 h (*lane 6*). Aliquots were electrophoresed on SDS (10% acrylamide) gels followed by immunoblotting using a polyclonal anti-human prothrombin antibody which recognises prothrombin and its cleaved products. Immunodetection was performed by ECL.

thrombin 1. The formation of prethrombin 1 under these conditions without the addition of any exogenous protein, most likely results from minimal catalytic activity within the zymogen prothrombin molecule [Mann, 1977]. As seen in Figures 2 and 3, the majority of prothrombin was cleaved on the surface of HVSMC or in the presence of HVSMC-conditioned medium. The identity of the resulting fragments and whether prothrombin cleavage resulted in the generation of enzymatically active α-thrombin was investigated and is described in the following two sections.

N-Terminal Amino Acid Sequencing of Cleaved Prothrombin Fragments

The apparent molecular mass of the prothrombin cleavage products observed suggested that these might represent similar fragments to those generated during prothrombin activation by factor Xa which leads to active α-thrombin formation. This was further tested by sequencing the 5 N-terminal amino acids of each of the prothrombin fragments evident in Figure 2. Alignment of these sequences with the known amino acid sequence of the prothrombin molecule permitted identification of sites cleaved upon incubation of prothrombin with HVSMC. These results are presented in Table II and Figure 4. The N-terminal sequence of band 1 (72 kDa) confirmed that this represents uncleaved native prothrombin. Band 2 (50 kDa) corresponds to prethrombin 1 and resulted from cleavage at R^{155}-S^{156}, a known α-thrombin cleavage site. Band 3 (38 kDa), formed by cleavage at R^{271}-T^{272}, a factor Xa cleavage site, represents prethrombin 2. Since the N-terminal sequence of band 4 (36 kDa) is identical to the N-terminal sequence of the A-chain of mature human α-thrombin, this band was probably formed by α-thrombin autocatalytic cleavage at R^{284}-T^{285}. The molecular mass of this fragment (36 kDa) suggests that it encompasses the whole serine proteinase domain of prothrombin (prethrombin 2′). Therefore cleavage at R^{320}-I^{321}, which is required for the formation of active α-thrombin and known to be mediated by factor Xa during prothrombin activation, did not occur at a detectable level. Since the N-terminal sequence of band 5 (32 kDa) is identical to the N-terminal sequence of uncleaved prothrombin, this suggests that it corresponds to fragment 1.2 obtained via cleavage at R^{271}-T^{272}, which also generates prethrombin 2 (band 3; 38 kDa). Alternatively, this fragment could be derived from cleavage of prothrombin at R^{284}-T^{285}, which was also detected and resulted in the formation of fragment 4. In such a case, this band would represent fragment 1.2 with an additional 13 C-terminal amino acids.

The majority of prothrombin incubated with HVSMC was converted to both prethrombin 2 (band 3) and its N-terminally truncated form, prethrombin 2′ (band 4). During prothrombin activation, the prethrombin 2 generated is further cleaved by factor Xa at R^{320}-I^{321} and yields the two-chain disulphide-linked catalytically active α-thrombin. However, cleavage at R^{155}-S^{156} and R^{284}-T^{285}, both α-thrombin cleavage sites, were observed, suggesting that either α-thrombin was generated at low levels undetectable by SDS-PAGE and Western blotting, or that HVSMC produced a proteinase(s) which mediated these cleavages. Since R^{271}-T^{272} and R^{320}-I^{321} are both factor Xa cleavage sites but only

TABLE II. N-Terminal Amino Acid Analysis of Prothrombin Fragments Generated by Incubation With Cultured HVSMC*

Fragment/ band number	Apparent molecular mass (kDa)	N-terminal sequence	Cleavage site in prothrombin	Fragment identity
1	72	A-N-T-F-L	None	Prothrombin
2	50	S-E-G-S-S	R-155	Prethrombin 1
3	38	T-A-T-S-E	R-271	Prethrombin 2
4	36	T-F-G-S-G	R-284	Prethrombin 2'
5	32	A-N-T-F-L	R-271 or R-284	Fragment 1.2

*Prothrombin was incubated with HVSMC in DMEM for either 4 or 12 h and aliquots were electrophoresed under reducing conditions on an SDS (10% acrylamide) gel. Proteins were electrotransferred onto a PVDF membrane, stained with Coomassie blue, and 5 amino acids from the N-terminus of each protein band illustrated in Figure 4 were sequenced. Comparison between the N-terminal amino acid sequence obtained and the known amino acid sequence of prothrombin, together with the apparent molecular mass of each band, permitted the identification of the cleaved prothrombin fragments.

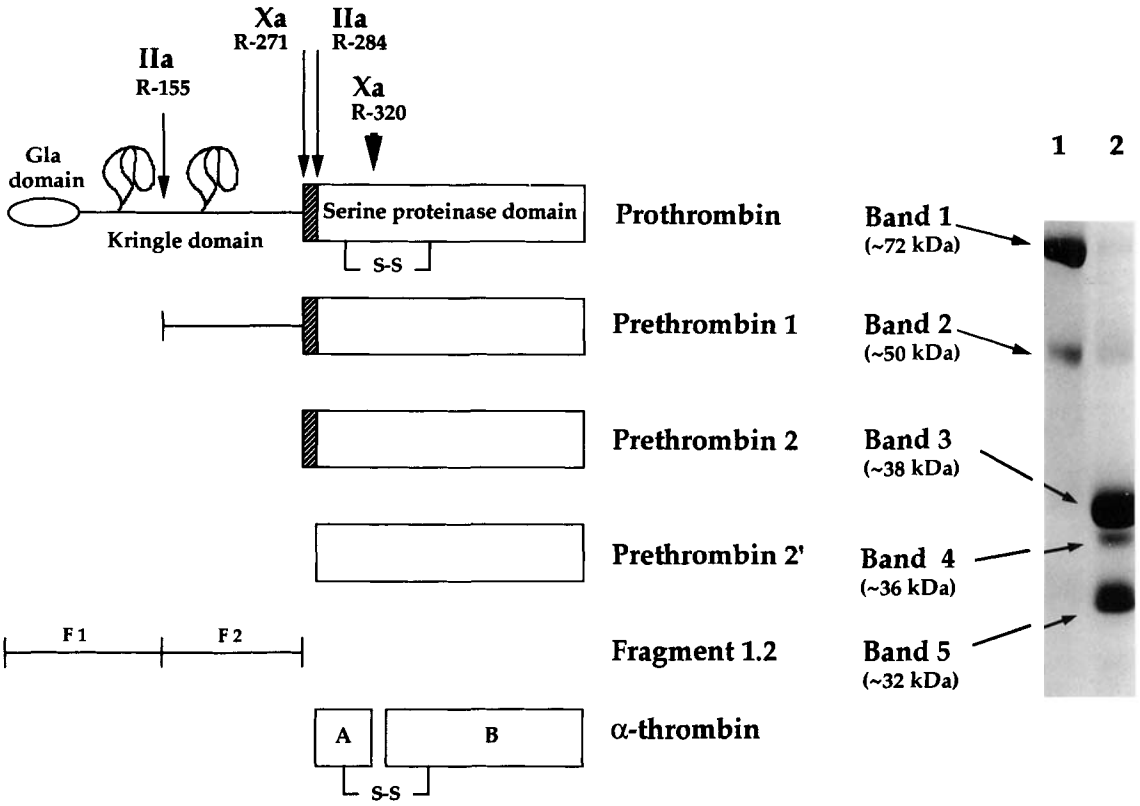

Fig. 4. Schematic representation of the domain structure of prothrombin and cleavage products obtained from prothrombin incubation with HVSMC monolayers. The N-terminal γ-carboxyglutamic acid (Gla) domain of prothrombin is followed by the kringle domain and the serine proteinase domain. Sites in prothrombin known to be cleaved by α-thrombin and factor Xa are indicated. The panel on the right illustrates the electrophoretic separation of prothrombin incubated with cultured HVSMC for 4 h (*lane 1*) or 12 h (*lane 2*) as described in Figure 2. Proteins were electrotransferred to PVDF membranes and 5 amino acids of the N-terminus of each band were sequenced. Based on the obtained N-terminal sequence and the apparent molecular mass of each band, specific prothrombin fragments were identified. α-Thrombin is also illustrated for comparison.

cleavage at R^{271}-T^{272} was observed, one may speculate that prothrombin cleavage by HVSMC was mediated, at least partially, via a proteinase(s) distinct from factor Xa. The analysis of prothrombin cleaved products reported here was carried out under reducing conditions. Examination of such prothrombin cleaved fragments under non-reducing conditions did not show any changes in the pattern or intensity of the bands obtained (data not shown). The structural as-

says used here (SDS-PAGE, Western blotting, and N-terminal amino acid analysis) failed to detect the formation of α-thrombin. Silver staining could not be used here as it would also reveal many interfering HVSMC-secreted proteins. The generation of α-thrombin activity was nevertheless suggested by the data derived from the mitogenic assays discussed above. The possibility that α-thrombin was formed at levels that remained undetectable by the above structural analyses could not be excluded. Hence with the aim of investigating this possibility, amidolytic assays were performed.

Analysis of Amidolytic Activity of Prothrombin Incubated With HVSMC-Conditioned Medium

Using the amidolytic assay with pure α-thrombin of known activity as a standard, the enzymatic activity of prothrombin cleavage products generated upon incubation of prothrombin with HVSMC-conditioned medium was determined. Incubation of prothrombin to a final concentration range of 1.2 to 12 μM with HVSMC-conditioned medium for 12 h at 37°C, resulted in the generation of a total of 1.5 to 4.5 U thrombin (equivalent to 0.5 to 1.4 μg α-thrombin) per 90 μg prothrombin. Complete activation of 90 μg prothrombin would theoretically be expected to result in the generation of approximately 45 μg (140 U) thrombin. Therefore, the yield of α-thrombin from prothrombin under the conditions above was approximately 1–3%. The amidolytic activity of prothrombin incubated with conditioned medium was abolished in the presence of 20 U/mL hirudin. No amidolytic activity was generated when prothrombin was incubated with control DMEM under identical conditions to those described above. Conditioned medium lacked any inherent amidolytic activity which is in agreement with data demonstrating that cultured HVSMC do not express the prothrombin gene as determined by both Northern blotting and reverse transcriptase polymerase chain reaction (RT-PCR) analysis (our unpublished observations). The amidolytic activity of α-thrombin incubated in conditioned medium (final concentration of 20 U/mL) at 37°C for a period of up to 24 h was identical to that of thrombin incubated in serum-free medium under the same conditions. Similarly, thrombin incubation at 37°C for 12 h in either serum-free or conditioned medium, resulted in only a 5% loss of amidolytic activity as compared to a standard thrombin preparation assayed without prior pre-incubation. Taken together, these data imply that the low yield of thrombin-like activity from prothrombin preparations incubated with conditioned medium is unlikely to be due to the inactivation of thrombin by factor(s) in the conditioned medium. This low yield most likely represents a true reflection of the amount of thrombin generated under the conditions described. The results of the amidolytic assays correlate well with the data shown in Table I where prothrombin incubation with HVSMC resulted in the generation of mitogenic activity to equivalent levels to those expected to be generated by 1–3% yield of enzymatically active α-thrombin. These data are also in agreement with the N-terminal sequencing analysis of prothrombin cleavage by HVSMC which confirm that HVSMC convert prothrombin to mainly prethrombin 2 and prethrombin 2′. Since enzymatic activity was generated after incubation of prothrombin with HVSMC conditioned medium but not DMEM, the possibility of autoactivation of prothrombin under the conditions used is excluded.

Characterisation of Prothrombin Cleaving Activity of HVSMC

The sensitivity of the activity of HVSMC-conditioned medium to various treatments was assessed. Aliquots of HVSMC-conditioned medium were treated with various agents prior to incubation with prothrombin (12 μM, final concentration) for 12 h followed by SDS-PAGE and immunoblotting analysis. The results are summarised in Table III. Heat treatment (95°C, 5 min), trypsin exposure (10 μg/mL, 15 min), exposure to reducing agent (DTT 10 mM, 1 h), or acidification (pH 2.0) completely abolished HVSMC-conditioned medium activity. Various classes of proteinase inhibitors affected the activity of HVSMC-conditioned medium differently. Pepstatin (inhibitor of aspartic proteinases), EDTA (inhibitor of metalloproteinases), amastatin (inhibitor of aminopeptidase), and E64 (inhibitor of cysteine proteinases), did not alter the activity of HVSMC-conditioned medium. By contrast, no prothrombin cleavage was observed with conditioned medium pre-incubated with either PMSF or leupeptin (inhibitors of serine proteinases). However, aprotinin, also a serine proteinase inhibitor with a restricted activity (inactive towards thrombin, factor Xa, or tPA) failed to alter the prothrombin cleavage activity of HVSMC-conditioned medium. These data

TABLE III. Investigation of the Nature of the Prothrombin Cleavage Activity of HVSMC-Conditioned Medium*

Treatment of HVSMC- conditioned medium	Effect on prothrombin cleaving activity of HVSMC-conditioned medium
Heat (95°C for 5 min)	Abolished
Trypsin (10 μg/mL for 5 min followed by 100 μg/mL soya bean trypsin inhibitor for 5 min)	Abolished
DTT (10 mM for 1 h followed by DTT removal on G25 column)	Abolished
Acidification (pH 2.0 with 1N HCl for 20 min followed by neutralisation with 1N NaOH)	Abolished
Proteinase inhibitors	
Pepstatin (1 μM)	No effect
Amastatin (10 μM)	No effect
EDTA (10 mM)	No effect
E64 (10 μM)	No effect
Aprotinin (2 μg/mL)	No effect
PMSF (1 mM)	Abolished
Antibodies	
Anti-factor Xa monoclonal (20 μg/mL every 2 h)	No effect
Anti-tPA monoclonal (100 μg/mL every 2 h)	No effect
Anti-uPA rabbit polyclonal (20 μg/mL every 2 h)	No effect
Hirudin (2–20 U/mL)	Formation of prethrombin 1 and prethrombin 2′ inhibited but formation of prethrombin 2 observed.

*HVSMC-conditioned media were treated with various agents and then incubated with 12 μM prothrombin for a period of 12 h at 37°C. Aliquots were subjected to SDS-PAGE (10% acrylamide) followed by either Coomassie blue staining or immunoblotting using a polyclonal anti-prothrombin antibody which recognises human prothrombin and its cleaved fragments. The effect of the various treatments on the prothrombin cleavage activity of HVSMC-conditioned medium responsible for the generation of fragments 2–5 described in Figure 4 is indicated.

strongly suggest that the prothrombin cleaving activity in HVSMC-conditioned medium is due to a serine proteinase-like enzyme.

Pre-incubation of HVSMC-conditioned medium with increasing concentrations (2–20 U/mL) of the specific thrombin inhibitor, hirudin, resulted in a dose-dependent alteration of the prothrombin cleavage pattern (data not shown). The generation of fragments 2, 4, and 5 (depicted in Fig. 4) was reduced, whereas generation of fragment 3 remained unaffected. The cleavage sites in prothrombin that resulted in the generation of fragments 2 and 4, were demonstrated by N-terminal amino acid sequencing to be identical to the known specific cleavage sites of α-thrombin (Fig. 4). Taken together, these results suggest that fragments 2 and 4 result from cleavage by catalytically active α-thrombin formed during the assay. In addition, the inhibition of the generation of band 5 in the presence of hirudin, suggests that this band is likely to represent an extended fragment 1.2 discussed previously or a combination of this fragment and fragment 1.2. Although α-thrombin was not detected as a product of HVSMC-mediated prothrombin cleavage by SDS-PAGE, immunoblotting, or N-terminal amino acid sequencing, its generation at low levels, undetectable by these techniques, is suggested. Therefore, prothrombin cleavage observed with HVSMC-conditioned medium most likely results from the combined action of α-thrombin generated at low levels, together with another, as yet unidentified, proteinase(s) which cleaves R^{271}-T^{272} to yield prethrombin 2. It is of interest to mention here that in the mitogenic, amidolytic, and prothrombin cleavage assays, the use of the thrombin inhibitor antithrombin-III yielded similar data to those obtained with hirudin.

VSMC are known to express and secrete several proteinases of the fibrinolytic cascade which include tPA and uPA [Wojta et al., 1993; Clowes et al., 1990; Booyse et al., 1981]. Prothrombin cleavage, mediated by HVSMC-conditioned medium, was compared to that obtained with the serine proteinases tPA, uPA, and factor Xa. In addition, the effect of antibodies, directed against the above serine proteinases, on the activity of HVSMC-conditioned medium, was studied. Both

uPA (up to 5 U/mL) and tPA (up to 400 U/mL), incubated with prothrombin (6 μM) for 12 h in DMEM at 37°C, failed to generate any significant cleavage of prothrombin (data not shown). As seen in Figure 5, under similar conditions, factor Xa (20 μg/mL), yielded a pattern of fragments distinctly different from that obtained with HVSMC-conditioned medium. The absence of the prothrombinase complex drastically reduces the efficiency of the activation of prothrombin by factor Xa which occurs mainly via the prethrombin 2 pathway. Fragment 1.2 is formed first and prethrombin 2 is further cleaved to generate enzymatically active α-thrombin [Mann, 1994] (Fig. 1). As seen in Figure 5, under the conditions of our assay (absence of phospholipid surface and factor Va) prothrombin cleavage by factor Xa yielded a prominent band migrating identically to the B-chain of α-thrombin and two other bands migrating identically with prethrombin 2′ and fragment 1.2, thus indicating that a certain proportion of prethrombin 2′ remained uncleaved. Pre-incubation of HVSMC-conditioned medium with anti-uPA (20 μg/mL), anti-tPA (100 μg/mL), or anti-factor Xa (20 μg/mL) antibodies and their repeated addition at 2 h intervals throughout the assay period, failed to alter the pattern or extent of prothrombin cleavage; neither did a combination of all three antibodies (data not shown). These antibodies were effective at neutralising the activities of the above serine proteinases in solution and were used at concentrations in excess of those required to inhibit amounts of uPA and tPA likely to be secreted by VSMC and amounts of factor Xa present in blood plasma [Wojita et al., 1993; Clowes et al., 1990; Booyse et al., 1981]. Previous studies have indicated that tPA and uPA associated with the endothelial cell extracellular matrix mediate the cleavage of prothrombin to enzymatically active α-thrombin [Benezra et al., 1993]. The contribution of uPA and tPA to the prothrombin cleavage activity of HVSMC-conditioned medium cannot be excluded on the basis of data presented here as this may imply that other as yet unidentified factor(s) may account for the non-neutralised HVSMC-conditioned medium activity by antibodies against uPA and tPA. However, uPA, tPA, or Xa alone are unlikely to be responsible for the cleavage of prothrombin by HVSMC as their addition in solution failed to induce a similar pattern of cleavage of prothrombin in solution to that observed by the HVSMC-conditioned medium.

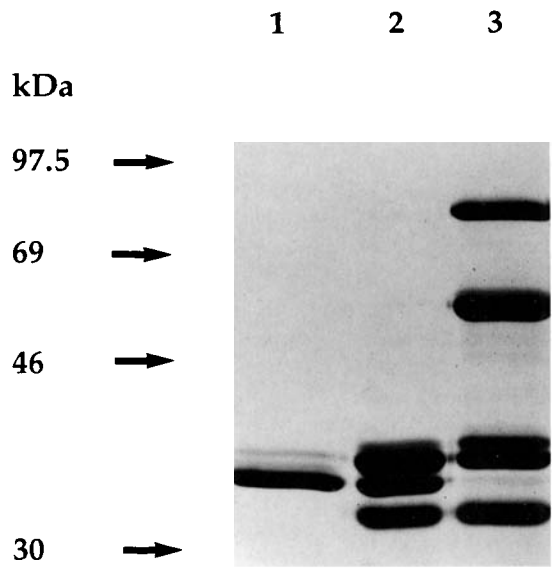

Fig. 5. Comparison of prothrombin cleavage by HVSMC-conditioned medium and factor Xa. *Lane 1* represents purified human α-thrombin (10 μg). Prothrombin (12 μM) was incubated with either 2 μg factor Xa in DMEM (100 μl) (*lane 2*), or HVSMC-conditioned medium (*lane 3*) for 12 h at 37°C. Aliquots were then electrophoresed on SDS 10% acrylamide gels followed by immunoblotting using a polyclonal anti-human prothrombin antibody which recognises prothrombin and its cleaved products. Immunodetection was performed by ECL. In lane 2, factor Xa mediated prothrombin cleavage resulted in the generation of three fragments: prethrombin 2′, B-chain of thrombin, and fragment 1.2.

HVSMC-conditioned medium obtained after a 24 h incubation of HVSMC in SFM was concentrated 20-fold and subjected to gel filtration chromatography on a P60 column and the prothrombin cleavage activity of each fraction assessed. The activity was eluted and concentrated as a single peak with an apparent molecular weight of 30–40 kDa, and totally converted prothrombin to prethrombin 2, prethrombin 2′, and fragment 1.2 (data not shown). It is of interest to note here that the activity of HVSMC-conditioned medium was dose-dependent. Its concentration by 20-fold greatly enhanced prothrombin cleavage whereas its dilution by 1:50 or 1:250 resulted in a substantial reduction and total loss of prothrombin-cleaving activity, respectively (data not shown).

DISCUSSION

Using purified factor Xa, factor Va, Ca^{2+}, and phospholipid vesicles, the activation of prothrombin has been extensively studied in several in

vitro systems [Mann, 1994; Mann et al., 1990; Doyle and Haley, 1993]. Two pathways of prothrombin activation involving the generation of either prethrombin 2 or meizothrombin as intermediate products have been described [Mann et al., 1990; Doyle and Haley, 1993] (Fig. 1). In the absence of factor Va and a phospholipid surface, activation of prothrombin by factor Xa occurs mainly via the prethrombin 2 pathway [Mann, 1994]. In this pathway, the activation peptide fragment 1.2 is released and prethrombin 2 is further cleaved to generate enzymatically active α-thrombin. Cleavage of prothrombin at R^{320}-I^{321} is required for the formation of an active catalytic site in thrombin. The intermediate product meizothrombin is generated by this cleavage and is enzymatically active against small synthetic substrates but not fibrinogen. The final product of the activation of prothrombin, α-thrombin, is generated via two cleavages at R^{320}-I^{321} and R^{271}-T^{272} sites and possesses full enzymatic activity. Neither the prethrombin 2 intermediate nor fragment 1.2 of prothrombin is known to possess any enzymatic activity.

Numerous laboratories have studied the mechanisms and pathways involved in prothrombin activation by various cell types [Esmon, 1993]. Platelets, macrophages, and endothelial cells have all been shown to support prothrombinase complex assembly by providing the phospholipid surface [Tracy et al., 1992; Lindahl et al., 1989; Tijburg et al., 1991]. Cultured endothelial cells express factor V and support prothrombin activation in the presence of exogenous factor Xa [Tijburg et al., 1991]. Macrophages express both factor X and V and therefore support prothrombin activation in the absence of an exogenous supply of both these factors [Lindahl et al., 1989]. However, the study of prothrombin interaction with cultured SMC or other cell system of the vascular wall in the absence of any exogenously added prothrombin activating factor (factor Xa) has not yet been reported despite its obvious importance for physiological and pathological processes [Nelken et al., 1992; Sarembock et al., 1991; Walters et al., 1994; Fuster et al., 1992; Wilcox et al., 1994]. The aim of the present study was to investigate the interactions of prothrombin with cultured HVSMC in the absence of serum or any other exogenous factors.

The biological activity of prothrombin incubated with HVSMC or conditioned medium was evaluated by both mitogenic and amidolytic assays. In addition, the interference of hirudin, a specific thrombin inhibitor, with the biological activity of prothrombin was assessed in both assays. Incubation of prothrombin with conditioned medium, generated amidolytic activity which was abolished in the presence of hirudin. Both meizothrombin and α-thrombin are known to be inhibited by hirudin [Stocker, 1991] and to possess amidolytic activity [Doyle and Haley, 1993]. It is therefore concluded that a molecule(s) inhibited by hirudin and possessing amidolytic activity is generated upon incubation of prothrombin with HVSMC. Hirudin could also be inhibiting the generation of an amidolytically active molecule(s). Prothrombin incubated with growth-arrested cultured HVSMC under serum-free conditions stimulated DNA synthesis and this effect was inhibited by hirudin. Several studies have demonstrated the requirement of an intact catalytic site for α-thrombin-induced mitogenicity in a variety of cultured cells including VSMC [Kanthou et al., 1992; McNamara et al., 1993] and the abolition of thrombin's mitogenic effect by hirudin [McNamara et al., 1993; Bar-Shavit et al., 1990]. The mitogenic activity of meizothrombin has not yet been studied. Using both the amidolytic and mitogenic assays, comparison of the activity of purified α-thrombin and that generated upon incubation of prothrombin with HVSMC suggests that a catalytically active form of thrombin was produced. Prethrombin 2, prethrombin 2′, and fragment 1.2 are not known to possess enzymatic activity which is a prerequisite for the activation of the thrombin receptor leading to the generation of mitogenic signals [McNamara et al., 1993]. However, their contribution towards proliferative signals, although unlikely, cannot be categorically excluded. Nevertheless, the purification of the various prothrombin cleavage fragments obtained upon incubation of prothrombin with HVSMC-conditioned media and the assessment of their possible mitogenic properties remains to be investigated. Such an approach, is complicated by the fact that cultured VSMC secrete a variety of autocrine mitogenic factors. Once prothrombin cleaving activity is purified from HVSMC-conditioned media, the assay could be performed in a more defined system comprising prothrombin and the purified factor(s). Such an assay will greatly facilitate the purification of the various prothrombin cleavage products and the assessment of their biological activity. These

approaches constitute the future aims of this study.

A combination of SDS-PAGE, immunoblotting, and N-terminal amino acid sequencing revealed that HVSMC mediated cleavage of prothrombin to several fragments. From the identification of the cleavage sites it was concluded that prothrombin cleavage by HVSMC occurs according to a pathway involving prethrombin 2 (Fig. 1). Prothrombin cleavage by HVSMC generated prethrombin 1, fragment 1.2, prethrombin 2, and prethrombin 2′. Prothrombin cleavage by HVSMC was affected by hirudin which inhibited the formation of fragments 2, 4, and 5 but not that of fragment 3. Thus the generation of a thrombin-like activity subsequent to prothrombin incubation with HVSMC was strongly suggested and was in agreement with the data on the mitogenic effects of prothrombin. Although R^{271}-T^{272} and R^{320}-I^{321} are both cleavage sites for factor Xa, only cleavage at R^{271}-T^{272} site was observed. The analysis of prothrombin cleavage by HVSMC could not be performed by a more sensitive technique such as silver staining, since in this case cell-secreted proteins would have also been visualised.

Proteolytic enzymes are known to be either localised in specialised intracellular granules, or are cell surface-associated or secreted to the cell exterior [Bond and Butler, 1987; Leytus et al., 1988]. An HVSMC-secreted factor(s) supported prothrombin cleavage with a similar pattern to that observed with cultured HVSMC. HVSMC are not known to express factor X and factor Xa-neutralising antibodies failed to inhibit the activity of HVSMC-conditioned medium. In addition, cleavage of R^{320}-I^{321}, a factor Xa cleavage site, did not occur at a detectable level. Taken together, these data indicate that factor Xa was not involved in the observed cleavage of prothrombin by HVSMC.

Benezra and colleagues [Benezra et al., 1993] analysed prothrombin cleavage by endothelial extracellular matrix using SDS-PAGE and immunoblotting. These authors described a pattern of prothrombin cleavage similar to that observed in the present study. However, since amino acid sequencing was not used to identify the various cleavage sites in the prothrombin molecule, no comparison can be made between the prothrombin cleavage pattern exhibited by the subendothelial matrix and that exhibited by HVSMC. HVSMC express both uPA and tPA [Clowes et al., 1990; Booyse et al., 1981] and previous studies [Benezra et al., 1993] have demonstrated that both these serine proteinases, bound to subendothelial extracellular matrix, can convert prothrombin to active α-thrombin. Neutralising antibodies against tPA and uPA did not alter the activity of HVSMC-conditioned medium. These data demonstrate that the activity of HVSMC responsible for prothrombin cleavage is due to an as yet unidentified factor(s) but do not exclude the contribution of both tPA and uPA to this activity. Using several classes of proteinase inhibitors, it was deduced that a serine proteinase is responsible for the observed prothrombin cleaving activity of HVSMC-conditioned medium. While this manuscript was in preparation, the activation of prothrombin by a novel membrane-associated proteinase has been reported [Sekiya et al., 1994]. This activity was mainly found associated with a feline kidney fibroblast cell line and its partial characterisation indicates that it is a different enzyme from the one described in the present study.

In vitro studies showed that in the presence of factor Xa and factor Va, cell-mediated prothrombin activation leads to thrombin generation within minutes [Mann et al., 1990; Doyle and Haley, 1993]. In this report, prothrombin activation by HVSMC was studied in the absence of serum and any other exogenous factors. Under these conditions, prothrombin cleavage by cultured HVSMC was not detected before 4 h of incubation and the pattern and extent of this cleavage did not vary between 8 and 48 h. In vitro experiments using cultured cells do not necessarily reflect the in vivo situation. This is because many other parameters may be involved in vivo, namely, infiltration of prothrombin through the endothelial barrier of the vessel wall, its clearance mechanism(s), the presence of other factors and cofactors, and the ability of HVSMC to synthesise and secrete serine proteinase like enzymes. Infiltration of circulating proteins through the endothelial barrier is a phenomenon postulated to occur during vessel wall injury [Ross, 1993]. Our immunocytochemical studies have revealed the presence of prothrombin and/or its cleavage products in the media of normal human arteries. A significant increase of this antigen is evident in human atherosclerotic plaques (our unpublished data). Several studies have shown that in both healthy and atherosclerotic human arteries, smooth muscle cells of the media of both normal and atherosclerotic ves-

sels express the LDL receptor-related protein/α2-macroglobulin receptor [Lupu et al., 1994]. This complex is a multifunctional cell surface receptor responsible for the clearance of lipoproteins, proteinases, cytokines, and growth factors in complex with α2-macroglobulin as well as plasminogen activator complexes with their inhibitors. The involvement of this receptor in the clearance of prothrombin or its cleavage products has not yet been reported.

Peritoneal macrophages have been shown to express a surface-bound serine proteinase activity that proteolytically cleaves thrombin and leads to its inactivation [Pejler and Seljelid, 1992]. Whether the observed prothrombin cleavage by HVSMC implies the generation of an activity which stimulates dormant SMC mitogenically and also leads to fibrin generation or, by contrast, represents a defence mechanism is unclear. Peritoneal macrophages proteolytically degraded and completely abolished any thrombin-like activity within 20 h [Pejler and Seljelid, 1992], whereas incubation of prothrombin with cultured HVSMC for up to 48 h failed to alter the prothrombin cleavage pattern or the yield (1–3%) of thrombin-like activity generated. If a similar reaction were to occur in vivo, a yield of 1–3% from circulating prothrombin (1.2 μM) would represent a level of activity (12 to 36 nM) likely to be sufficient for fibrin formation and stimulation of VSMC proliferation. However, the biological significance of such a yield will depend on the concentration of circulating thrombin inhibitors within the vessel wall. The assembly of prothrombin into the prothrombinase complex occurs via the binding of the Gla and kringle domains of the molecule which form the activation peptide (fragment 1.2) [Mann, 1994]. Cleavage of prothrombin by HVSMC which removes this activation peptide may imply that this molecule can no longer participate in prothrombinase complex formation. Therefore this could be regarded as a mechanism of prothrombin inactivation.

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