Evidence for a protein S receptor(s) on human vascular smooth muscle cells

Analysis of the binding characteristics and mitogenic properties of protein S on human vascular smooth muscle cells

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The presence of specific binding sites for the coagulation factor protein S (PS) on the surface of human vascular smooth muscle cells (HVSMC) is described. The binding characteristics of ^{125}I -PS to HVSMC were studied and found to be saturable, reversible and, as described by the Hill equation, co-operative (h 1.74; K_d 0.33 nM). Autoradiographic analysis of detergent extracts of HVSMC chemically cross-linked with ^{125}I -PS and separated by

SDS/PAGE revealed radioactivity associated with two proteins with apparent molecular masses of 220 and 230 kDa respectively. The mitogenic activity of PS on HVSMC was also investigated. Protein S was shown to stimulate DNA synthesis of growth-arrested HVSMC and to support their proliferation under low-serum conditions in a sustained and dose-dependent manner.

INTRODUCTION

The accumulation of neointimal smooth muscle cells (SMC) resulting from medial cell migration and proliferation in response to vascular injury is believed to play a key role in atheromatous plaque development and restenosis [1]. Using cultured SMC, numerous studies have identified various factors that are able to initiate and sustain the replication of SMC, i.e. platelet-derived growth factor, basic fibroblast growth factor and a member of the epidermal growth factor (EGF) family, heparin-binding EGF-like factor [2,3]. Although SMC derived from animal tissues provide reasonable models for the study of proliferative events associated with atherosclerosis and restenosis, they may exhibit quantitative or qualitative differences as compared with human vascular SMC (HVSMC) in terms of their responses to different growth modulators [4,5].

According to the 'response to injury' hypothesis for the development of atherosclerosis, the initial SMC proliferative event occurs because the damaged endothelium ceases to provide a non-thrombogenic barrier [1]. The cellular components of the vascular wall are therefore exposed to factors secreted by infiltrating cells, circulating plasma proteins and products resulting from the activation of the coagulation cascade. Indeed the contribution of thrombosis to atherosclerotic plaque formation is well documented [6,6a,7], and many enzymes and cofactors of the coagulation and fibrinolytic cascade, such as α -thrombin [8], tissue plasminogen activator [9] protein S (PS), factor X and factor Xa [10], may contribute to the mitogenic stimulation of the quiescent SMC in the arterial wall. A thrombin receptor has been characterized, and shown to be involved in thrombin-induced SMC mitogenicity [11,12]. However, no receptors for other coagulation-related factors involved in SMC mitogenesis have been described until now. Recently, a novel cell

surface receptor for factor Xa was cloned from a human T-cell cDNA library [13].

PS is a vitamin K-dependent plasma protein which functions as a cofactor to activated protein C in the inactivation of factors Va and VIIIa [14–16]. Gasic *et al.* [10] have shown that PS is mitogenic for rat SMC. However, the binding of PS to SMC and the subsequent transduction of its mitogenic signals have not yet been reported. In the present study, we demonstrate that PS is mitogenic for HVSMC and supports their proliferation under low-serum conditions. Additionally, by a combination of ^{125}I -PS binding assays and chemical cross-linking of ^{125}I -PS to HVSMC, we provide evidence for the presence of a PS receptor(s) on the surface of HVSMC.

EXPERIMENTAL

Materials

Human PS, purified from plasma, was obtained from Calbiochem (Nottingham, U.K.) and was described by the manufacturer as 99% pure. Silver-stained SDS/PAGE of pure human PS under reducing conditions showed the presence of a doublet migrating at 86 kDa and 76 kDa as described previously [17–19]. Thrombin treatment for 15 min at 37 °C converted all of the PS to a single 76 kDa protein band. Anti-(human smooth muscle cell α -actin) monoclonal antibody and anti-(human PS) monoclonal antibody HS-3 (MAb HS-3) were purchased from Sigma (Poole, Dorset, U.K.). MAb HS-3 recognizes an epitope on both free PS and thrombin-cleaved PS, and was used in all experiments at a 1:250 dilution. [^3H]Thymidine ([^3H]TdR; specific radioactivity 5 mCi/mmol) and carrier-free Na^{125}I (specific radioactivity 15.3 mCi/ μg of iodine) were obtained from Amersham

Abbreviations used: SMC, smooth muscle cells; HVSMC, human vascular smooth muscle cells; PS, protein S; MAb, monoclonal antibody; TdR, thymidine; BS³, bis(sulphosuccinimidyl)suberate; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle's medium.

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(Amersham, Bucks., U.K.). Bis(sulphosuccinimidyl)suberate (BS³) was from Pierce Chemicals (Chester, U.K.). Enzymobead radioiodination reagents were from Bio-Rad laboratories (Hemel Hempstead, U.K.).

Cells and cell culture

HVSMC were isolated from sections of normal abdominal aorta obtained from kidney transplant donors (10–45 years of age) as described previously [8]. Briefly, the aortic tissue was transferred into Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin sulphate, 100 µg/ml neomycin and 2.5 µg/ml Fungizone (Gibco, Paisley, U.K.). Dissected aortic media was rinsed in DMEM with antibiotics, finely cut and digested under constant agitation (18–20 h; 37 °C) in DMEM, 15 mM Hepes, pH 7.4 (ICN Flow, Thame, U.K.), 0.1 % (w/v) collagenase type II (Lorne Labs, Reading, U.K.), 0.05 % (w/v) elastase type I (Sigma) and 10 % (v/v) foetal calf serum (FCS). Digested cells were cultured in L-glutamine/antibiotics-supplemented DMEM/Hepes, pH 7.4, and 20 % (v/v) FCS, which was decreased to 10 % after the second passage. Cells were subcultured at a split ratio of 1:4. 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 as described previously [20]. HVSMC were used between passages 2 and 8.

Measurement of DNA synthesis

HVSMC were plated (96-well plates; 1×10^4 cells per well) in DMEM/15 mM Hepes, pH 7.4, supplemented with 10 % (v/v) FCS and antibiotics. At confluence, cells were growth-arrested for 72 h in 0.5 % (v/v) FCS and then treated with the indicated factor for 16 h. DNA synthesis was measured by labelling with 1 µCi/ml [³H]TdR for a further 24 h (16–40 h post-stimulation). Cells were harvested on to Whatman GF/B filters using an automatic cell harvester, and [³H]TdR incorporated into the DNA was quantified by scintillation counting as described previously [8].

Radioiodination of PS

PS was radiolabelled with ¹²⁵I using Enzymobead radioiodination reagents according to the manufacturer's protocol. Briefly, 100 µg of PS was incubated with 1 mCi of carrier-free Na¹²⁵I in 10 mM sodium phosphate, pH 7.3, and 0.2 % (w/v) glucose in a final volume of 150 µl and with an appropriate quantity of Enzymobeads. Labelled PS was separated from free iodine by gel filtration on a Sephadex G25 column (10 cm $\times$ 1 cm). The specific radioactivity of the protein was determined by trichloroacetic acid precipitation and was found to be 0.6 mCi/mg of protein. Autoradiographic analysis of radiolabelled PS subjected to SDS/PAGE under reducing conditions showed a doublet migrating at 86 kDa and 76 kDa, which was converted to a single 76 kDa form by thrombin treatment described previously [17–19].

¹²⁵I-PS binding studies

HVSMC were plated in 24-well culture plates (Costar, High Wycombe, U.K.) in 10 % (v/v) FCS/DMEM at a density of 5×10^4 cells/well. After 24 h, the cells were washed extensively with binding buffer (DMEM containing 25 mM Hepes, pH 7.4, and 1 mg/ml BSA) and incubated in binding buffer for 2 h to allow dissociation of any bound PS derived from the serum. After a further wash in binding buffer, increasing amounts

(1–150 nM) of radiolabelled PS diluted in binding buffer were added to the cells in a total volume of 200 µl. The plates were then incubated at 20 °C for 3 h (equilibrium). Aliquots of each supernatant were then removed and radioactivity was counted to obtain the level of free radiolabelled ligand. The cells were then washed four times with pre-chilled binding buffer, dried and lysed in 0.5 ml of 1 M NaOH. Radioactivity in cell lysates was then counted using a γ -radiation counter to obtain the level of bound ligand. Non-specific binding was determined either by incubating ¹²⁵I-PS with a 100-fold excess of unlabelled ligand or by adding 10 mM EDTA 10 min prior to the end of the incubation period. Both the addition of 10 mM EDTA and incubation in the presence of a 100-fold excess of unlabelled ligand resulted in similar values for non-specific binding. PS-specific binding was calculated as the difference between total and non-specific binding.

The association kinetics of PS were determined essentially as described above, except that a single concentration of ¹²⁵I-PS (5 nM) was used and the amount of bound ¹²⁵I-PS was determined at timed intervals. For the dissociation and reversibility studies, pre-bound ¹²⁵I-PS was displaced by dilution with binding buffer or addition of a 100-fold excess of unlabelled ligand respectively. The association and dissociation studies were used to determine the rate constants for the forward and reverse reactions and to derive an empirical value for K_d as described previously [21]. Briefly, determination of the slope of the curve of the association kinetic, expressed as $-\ln [1 - (RL^*/RL^*_{eq})]$ versus time, where RL^* and RL^*_{eq} represent bound ligand at a specific time point and bound ligand at equilibrium respectively, allowed the calculation of the 'on-rate constant' $K_{forward}$ according to the equation $[\text{slope} - K_{reverse} = K_{forward} \times L]$, where L represents the total ligand. The value of $K_{reverse}$ was given by the slope of the dissociation kinetics plot expressed as $\ln (RL^*)$ versus time.

Cross-linking studies

HVSMC were plated in 10 % (v/v) FCS/DMEM in 3 cm-diameter dishes at 2×10^5 cells per dish. After 48 h, the cells were washed in PBS (three times) followed by a 2 h incubation with binding buffer. ¹²⁵I-PS (15 nM) was then added and cells were incubated for a further 4 h at room temperature. Cells were washed a further three times with binding buffer without BSA and then incubated with 2 ml of DMEM containing 1 nM BS³ for 15 min at 4 °C. The cells were then washed with DMEM and extracted by scraping in 0.5 ml of a solution containing 25 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA and 0.3 mM PMSF. The cell lysate was centrifuged at 12000 g at 4 °C and the resulting pellet was solubilized in 25 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 % (v/v) Triton X-100 and 10 mM PMSF. The supernatant was analysed by electrophoresis on an SDS/7.5 %-PAGE gel under reducing conditions according to the method of Laemmli [22], followed by autoradiography.

RESULTS AND DISCUSSION

PS has been shown to act as a mitogen for rat SMC [10]. In the present study, the effects of this protein on the stimulation of DNA synthesis in growth-arrested HVSMC, as well as its effect on their proliferation under low-serum conditions, were investigated. Since α -thrombin had been shown previously to stimulate DNA synthesis in HVSMC [8], it was used here for comparison. Growth-arrested HVSMC responded mitogenically to PS in a dose-dependent manner (Table 1). A concentration of 10 nM PS gave a clear stimulation, and the stimulation observed with 200 nM PS was equivalent to that induced by 100 nM α -thrombin. The mitogenic response induced by PS on HVSMC

Table 1 Effect of PS on [³H]TdR incorporation in HVSMC

HVSMC were plated (96-well plates; 1×10^4 cells per well) in DMEM/15 mM Hepes, pH 7.4, containing 10% (v/v) FCS and antibiotics. At confluency, cells were growth-arrested [72 h; 0.5% (v/v) FCS]. Cells were either untreated (control) or treated for 16 h with different concentrations of PS, 200 nM PS pre-incubated with MAb HS-3 for 20 min, or 100 nM α -thrombin. The cells were then radiolabelled with 1 μ Ci/ml [³H]TdR for 24 h and after this period they were harvested (Brandelhaesle/Walma GF/B filter) and the radioactivity associated with the filter determined by scintillation counting. Results are means $\pm$ S.E.M. of four independent determinations.

Treatment	[³ H]TdR incorporation (c.p.m.)
Control	2900 $\pm$ 136
10 nM PS	3600 $\pm$ 1079
20 nM PS	5600 $\pm$ 1316
50 nM PS	6000 $\pm$ 210
100 nM PS	7700 $\pm$ 62
200 nM PS	10400 $\pm$ 171
100 nM α -thrombin	9100 $\pm$ 1972
200 nM PS + MAb HS-3	3100 $\pm$ 40

Table 2 Effect of various concentrations of PS on HVSMC proliferation

Cells were plated (3000 cells per dish) in 10% (v/v) FCS. After 24 h, the cells were growth-arrested in DMEM containing 0.5% (v/v) FCS for 48 h. The numbers of live cells were determined in two different dishes (starting number) and found to be 25000 $\pm$ 270 cells. HVSMC were either untreated (control) or treated with different concentrations of PS. After 3 and 6 days, the numbers of cells were determined by counting them under the microscope using a haemocytometer. Each value represents the mean $\pm$ S.E.M. of two independent determinations.

Treatment	Cell count 3 days after start of treatment	Cell count 6 days after start of treatment
Control	2400 $\pm$ 208	2100 $\pm$ 175
1 nM PS	2480 $\pm$ 154	2590 $\pm$ 129
10 nM PS	4020 $\pm$ 301	9670 $\pm$ 508
20 nM PS	5100 $\pm$ 465	19600 $\pm$ 297
100 nM PS	9060 $\pm$ 759	14200 $\pm$ 509
200 nM PS	9000 $\pm$ 295	17000 $\pm$ 490

was drastically reduced by pre-incubating it with an anti-(human PS) MAb for 20 min prior to its addition to the cells. This confirms that the mitogenic effect was due to PS and not to any putative contaminant(s) in the PS preparation. Cell counts performed 3 and 6 days after the addition of PS to growth-arrested HVSMC (maintained during the assay in 0.5% serum) confirmed that the [³H]TdR incorporation assay was a fair index of cell division (Table 2). During the assay period, control HVSMC maintained in 0.5% (v/v) serum did not divide and decreased in number, probably owing to loss of cell adhesion and cell death. After 6 days in the presence of 200 nM PS, however, HVSMC had undergone four cycles of division, which clearly demonstrates that PS is able to support HVSMC growth and division in a sustained manner. Some variability was, however, observed between different HVSMC primary cultures in terms of the extent of their mitogenic response to PS, but all HVSMC cultures examined responded mitogenically to PS (results not shown).

Having established that PS is a potent mitogen for HVSMC, we next studied the characteristics of its interaction with HVSMC monolayers. Figure 1 represents cell-associated radioactivity determined at various times in experiments in which HVSMC were incubated with ¹²⁵I-PS at 20 °C in the absence (total binding)

Figure 1 Time course of ¹²⁵I-PS binding to HVSMC

Tissue culture wells (1.5 cm in diameter) each containing 5×10^4 cells were incubated at 20 °C with 200 μ l of binding buffer containing 2 nM ¹²⁵I-PS for selected intervals of time. Cells were then washed and cell-bound radioactivity quantified as described in the Experimental section. Values given represent the means $\pm$ S.E.M. of two determinations. The points for which the error bars are not visible had an S.E.M. of less than 5%. Specific (■) and non-specific (□) binding are presented. The inset represents the association kinetics, expressed as $-\ln[1 - (RL^*/RL_{eq}^*)]$ versus time, where RL^* and RL_{eq}^* represent, respectively, the bound ligand at a specific time point and bound ligand at equilibrium.

or presence (non-specific binding) of a 100-fold excess of unlabelled PS. This binding was slow, with 50% of maximum binding occurring at 1 h and equilibrium being attained after 3 h. Accordingly, subsequent equilibrium binding experiments were performed at 20 °C with an incubation time of 3 h. The inset of Figure 1 depicts a semi-logarithmic analysis of the association kinetics from which an empirical value of the on rate constant $K_{forward}$ ($6 \times 10^6 \text{ M} \cdot \text{s}^{-1}$) was deduced. The kinetics of dissociation of bound ¹²⁵I-PS were studied by dilution of unbound ligand at equilibrium and were found to be relatively rapid: after 20 min, bound ¹²⁵I-PS was completely dissociated (results not shown). From these experiments, an empirical value of $K_{reverse}$ (off-rate constant) was determined (10^{-2} s^{-1}) and used to calculate the value of the equilibrium dissociation constant K_d ($K_{forward}/K_{reverse} = 1.6 \text{ nM}$). However, these determinations of the K_d remain largely speculative, since the non-linearity of the representation $-\ln[1 - (RL^*/RL_{eq}^*)]$ versus time indicates that the association of PS with HVSMC is not a simple bimolecular interaction; its biphasic shape suggests that first-order-like kinetics occur only after a lag phase of approx. 80 min. The biphasic shape of the representation $-\ln[1 - (RL^*/RL_{eq}^*)]$ versus time was observed in more than four independent experiments. Pre-incubation of ¹²⁵I-PS with an anti-(human PS) MAb (HS-3) for 20 min completely abolished its binding to HVSMC (results not shown). These data provide evidence that the observed binding was due to PS and not to any contaminant(s) in the PS preparation. PS binding to HVSMC was reversible, since the addition of 100-fold excess of unlabelled ligand at equilibrium completely displaced bound ¹²⁵I-PS (results not shown).

To determine the nature of PS binding to HVSMC and the receptor level, saturation binding of ¹²⁵I-PS to HVSMC was performed (Figure 2). The sigmoidal binding isotherm obtained suggested that the association of PS with its putative receptor(s) constitutes a co-operative phenomenon. Indeed, the analysis of equilibrium binding could best be described by application of the Hill equation [23], from which a K_d of 0.33 nM and a Hill

Figure 2 Saturation binding of ^{125}I -PS to HVSMC

Tissue culture wells (1.5 cm in diameter) each containing 5×10^4 cells were incubated for 3 h at 20 °C with various amounts of ^{125}I -PS. Total (○), specific (▲) and non-specific (■) binding were determined as described in the Experimental section. Values given represent the means $\pm$ S.E.M. of two independent determinations. The inset depicts the Hill plot of the specific binding, where F and $1 - F$ represent the fraction of occupied and unoccupied sites respectively.

coefficient of 1.74 were obtained. Since the Hill coefficient is a minimum estimate of the number of binding sites per receptor, it is likely that a minimum of two molecules of PS associate with each receptor molecule. From the value of the saturation levels of bound PS, an estimate of the number of receptors per cell was obtained, i.e. 3×10^6 . This number may, however, constitute an overestimate of receptor number per cell by a factor of two (or more), because the Hill coefficient indicated at least two binding sites per receptor. It is of interest to note here that there is a discrepancy between the deduced K_d (1.6 nM) and the concentration required to observe a half-maximal biological effect (~ 50 nM). This type of discrepancy is a common phenomenon encountered in many receptor binding studies [24] and may be accounted for by the co-operativity observed in the binding of PS to its putative receptor(s). Moreover, the conditions of the receptor binding assay (2 h, 20 °C) imply that increases in cell and receptor numbers are negligible. These parameters are, however, likely to change under the conditions of the mitogenic assay (40 h, 37 °C).

In order to provide further evidence for the existence of a PS receptor(s) on HVSMC, chemical cross-linking of ^{125}I -PS to monolayers of HVSMC was performed (Figure 3). Autoradiographic analysis of detergent extracts of cross-linked HVSMC separated on an SDS/7.5%-PAGE gel under reducing conditions showed that the radioactivity was associated with two bands migrating at 220 and 230 kDa. These bands represent complexes of ^{125}I -PS linked to its putative receptor(s). The specificity of the interaction of PS with these two proteins was demonstrated by the absence of these bands in lanes containing samples prepared in the presence of either a 100-fold excess of unlabelled ligand or 10 mM EDTA. The presence of two bands of different sizes suggests that the binding of PS to HVSMC may occur either via a multimeric complex or to two distinct receptors. This observation is consistent with the deduction from the Hill equation of the presence of at least two different binding sites for PS on HVSMC. The bands present at the bottom of the gel and at the 76 kDa position most likely correspond to free ^{125}I and degraded ^{125}I -PS and non-cross-linked ^{125}I -PS respectively. The

Figure 3 Chemical cross-linking of ^{125}I -PS to HVSMC

Tissue culture dishes (3 cm in diameter) each containing 2×10^5 cells were incubated with 1 ml of binding containing 15 nM ^{125}I -PS for 3 h at 20 °C. Cells were then washed with binding buffer, and cell-associated ^{125}I -PS was cross-linked to HVSMC by treatment with 1 nM BS³. Detergent extracts from the cells were analysed by SDS/PAGE under reducing conditions followed by autoradiography. In lanes 1 and 2 the binding was performed in the presence of an excess (1500 nM) of unlabelled PS and 10 mM EDTA respectively. The arrow indicates the position of ^{125}I -PS-associated proteins in lane 3.

Table 3 Summary of binding parameters

Parameter	Value	Remarks
K_{forward}	$6 \times 10^6 \text{ M} \cdot \text{s}^{-1}$	On-rate constant determined from association kinetics
K_{reverse}	10^{-2} s^{-1}	Off-rate constant determined from dissociation kinetics
K_d	1.6 nM	Affinity constant determined from the ratio $K_{\text{forward}}/K_{\text{reverse}}$
Hill coefficient	1.74	Minimum estimate of the number of protein S molecules binding per receptor
K_d	0.33 nM	Affinity constant calculated from the Hill plot
Minimum binding sites per cell	3×10^6	Deduced from the value of maximum ligand bound and calculated by normalization to the total number of cells
Chemical cross-linking	Two bands at 220 and 230 kDa	Radioactivity associated with ^{125}I -PS cross-linked to HVSMC, analysed by SDS/PAGE and autoradiography

different physical parameters of the binding of PS to HVSMC determined in this study are summarized in Table 3.

The proliferative effects of PS on HVSMC were obtained with concentrations of PS far below physiological human plasma levels (270 nM). This suggests that PS-induced SMC proliferation could occur *in vivo* provided that SMC are exposed to plasma components, a process which is believed to occur during vascular injury and perturbation of the endothelium [1]. Since human endothelial cells synthesize PS [25], one may speculate as to the existence of a paracrine mechanism by which PS might induce SMC proliferation. Recently, the protein encoded by a growth-arrest-specific gene, *gas6*, was described and shown to be a secreted factor with significant amino acid similarity to human

PS [26]. The function of the *gas6* protein, its effect on cell growth and its possible association with the putative PS receptor(s) described here remain to be investigated.

The hypothesis that this putative receptor(s) transduces the PS-dependent mitogenic signals in HVSMC remains purely speculative, and its confirmation requires further investigation. Potential approaches would include the inhibition of PS-induced mitogenesis on HVSMC by antibodies generated against the purified receptor(s) or by the cloning and *in vitro* expression of the gene(s) encoding these putative receptor(s). Finally, the present work highlights the potential role of PS in SMC proliferation and provides the basis for further studies aiming to elucidate its signal transduction pathways.

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