Protein S attenuates the invasive potential of THP-1 cells by interfering with plasminogen binding on cell surface via a protein C-independent mechanism

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Abstract Protein S, a cofactor for activated protein C (aPC) to inactivate coagulation factors, also plays a pivotal role in inflammation. Based on our recent findings that aPC and protein S modify tissue plasminogen activator (tPA)-catalyzed activation of Glu-plasminogen (Glu-plg), we analyzed possible role of protein S in cell-associated plasminogen activation and invasive potential of inflammatory cells. Monocyte-like THP-1 cells, to which both plasminogen and tPA bind, enhanced tPA-catalyzed plasminogen activation, which was partially abolished by protein S but not by aPC. Protein S attenuated both the plasminogen binding to THP-1 cells and associated their invasive potential through Matrigel.

Keywords: Protein S; Plasminogen; Tissue plasminogen activator; Cell invasion

1. Introduction

Protein S is a vitamin K-dependent anticoagulant protein that functions as a cofactor for activated protein C (aPC) to inactivate factors Va and VIIa by limited proteolysis [1]. Its physiological relevance in regulation of the coagulation cascade is revealed by the development of life-threatening thrombotic complications in infants homozygous for protein S deficiency [2,3]. In addition, recent findings suggest that protein S plays an important role in inflammation [4]. This depends mainly on the fact that, in plasma, a fraction of protein S exists as a high molecular mass complex with C4b-binding protein (C4BP), a soluble inhibitor of the classic complement pathway. Monocyte-like THP-1 cells, which both plasminogen and tPA bind, enhanced tPA-catalyzed plasminogen activation, which was partially abolished by protein S but not by aPC. Protein S attenuated both the plasminogen binding to THP-1 cells and associated their invasive potential through Matrigel.

Binding of Glu-plasminogen (Glu-plg), the native form of plasminogen, onto cell surfaces is also involved in inflammatory events. Glu-plg, bound to either fibrin or the cell surface through its lysine-binding site in kringle domains, acquires a looser conformation allowing it to be activated more readily by plasminogen activators (PAs) [7]. This enables plasmin, a broad-range enzyme, to be generated and located precisely at the site where proteolytic activity is needed. Recently, we reported that tissue plasminogen activator (tPA)-catalyzed Glu-plg activation in the presence of poly-lysine, which mimics C-terminal lysine bearing either fibrin or plasminogen-specific binding protein on the cell surface, was modified by aPC [8]. To assess the physiological relevance of this phenomenon, we analyzed the effect of aPC and protein S on cell-associated plasminogen activation. It was found that protein S, but not aPC, suppressed the extent of Glu-plg binding to the cell surface, as well as tPA-catalyzed Glu-plg activation on THP-1 cells.

2. Methods

2.1. Reagents

Human protein S was purchased from Enzyme Research Laboratories (South Bend, IN, USA) and monoclonal chemotactic protein I was from Sigma (St. Louis, MO, USA). Human recombinant aPC was kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Tranexamic acid and human recombinant single chain tPA were kindly provided by Daiichi Sankyo (Tokyo, Japan). Human Glu-plg was purified from freshly frozen human plasma by affinity chromatography on lysine-Sepharose. Glu-plg was labeled with biotin, using ECL protein biotinylation module (Amersham Life Sciences, Little Chalfont, UK).

2.2. Cells

THP-1 cells were obtained from Riken Cell Bank (Tokyo, Japan) and cultured in RPMI-1640 (Sigma) supplemented with 2 mM L-glutamine and 10% fetal calf serum (Biological Industries, Beth Haemek, Israel) in a 5% CO2 and 95% air atmosphere. Cell density was maintained at 0.1–0.6 × 106 cells/ml.

2.3. Plasminogen activation assay

Cell-dependent plasminogen activation was monitored by a continuous chromogenic assay using a plasmin-specific substrate of S-2251. Cells were washed three times in Hank’s balanced salt solution (HBSS) before assay and resuspended at 2 × 106 cells/ml. When required, the cells were preincubated with protein S (100 nM) for 30 min. Glu-plg (0.5 µM) and S-2251 (0.4 nM) were then added to cells and plasminogen activation was initiated by the addition of tPA (1.9 nM). tPA-catalyzed Glu-plg activation was monitored by the increase in the absorbance at 405 nm.
2.4. Plasminogen binding assay
After washing three times in HBSS, cells were resuspended at 2 x 10^6 cells/ml and were preincubated with different concentrations of protein S (0–300 nM) for 2 h. Biotin-labeled Glu-plg was then added (25 nM final concentration) and incubated for another 90 min. After washing three times in HBSS, a 100-μl aliquot of plasminogen elution buffer (2 mM tranexamic acid in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl) was added to the cells and incubated for 20 min. Cells were then pelleted and the supernatant was frozen at −80 °C until assayed. The amount of plasminogen eluted into the supernatant was assayed by an enzyme-linked immuno-sorbent assay (ELISA).

2.5. ELISA for plasminogen
The amount of plasminogen was assayed by an ELISA method, using anti-plasminogen polyclonal IgG as a catching antibody (5.3 μg/ml) and its conjugate with biotin as a tagging antibody. The buffer was phosphate-buffered saline, containing 0.1% BSA and 0.1% Tween 80.

2.6. Invasion assay
Invasive potential of THP-1 cells was evaluated using invasion chambers (BD BioCoat Matrigel invasion chambers; BD Biosciences Discovery Labware, Bedford, MA, USA), as described previously [9]. Inserts, coated with Matrigel, were rehydrated for 2 h with RPMI-1640 containing 2 mM l-glutamine. THP-1 cells, resuspended in RPMI-1640 (4 x 10^6/ml) with Glu-plg (106 nM) and protein S (100 nM) when required, were placed in the upper chambers and monocyte chemotactic protein-1 was placed in the lower chambers containing 100 mM NaCl) was added to the cells and incubated for 30 min. Plasminogen activation was monitored as in Fig. 1. Data obtained in the absence of supplemented tPA, was subtracted, though the value was negligible. Protein S at 100 nM attenuated Glu-plg activation by tPA in the presence of THP-1 cells (Fig. 2), whereas it did not evoke any significant change in their absence. Since protein S is a cofactor for aPC and, as reported previously [8,11], aPC modifies tPA-catalyzed plasminogen activation, possible effects of aPC, both in the presence and absence of protein S, were analyzed. aPC did not modify tPA-catalyzed Glu-plg activation further in the presence of both THP-1 cells and protein S. Thus, the inhibitory effect of protein S on tPA-catalyzed Glu-plg activation appears to be cell surface-dependent, but aPC-independent.

3. Results

3.1. Protein S attenuates Glu-plg activation by tPA
TPA-catalyzed Glu-plg activation was assayed by a continuous chromogenic assay. The presence of the THP-1 cell suspension (2 x 10^6 cells/ml) did not interfere with the assay. Activation was enhanced by THP-1 cells (2 x 10^6 cells/ml), as reported previously [10] (Fig. 1). To avoid the possible effect of inborn PAs produced in THP-1 cells, the increase in absorbance at 405 nm, obtained in the absence of supplemented tPA, was subtracted, though the value was negligible. Protein S at 100 nM augmented Glu-plg activation by tPA in the presence of THP-1 cells (Fig. 2), whereas it did not evoke any significant change in their absence. Since protein S is a cofactor for aPC and, as reported previously [8,11], aPC modifies tPA-catalyzed plasminogen activation, possible effects of aPC, both in the presence and absence of protein S, were analyzed. aPC did not modify tPA-catalyzed Glu-plg activation further in the presence of both THP-1 cells and protein S. Thus, the inhibitory effect of protein S on tPA-catalyzed Glu-plg activation appears to be cell surface-dependent, but aPC-independent.

3.2. Effect of protein S on plasminogen binding to THP-1 cells
Since the capacity of a cell surface to bind plasminogen is a factor in controlling the efficacy of cell surface-dependent plasminogen activation, a possible effect of protein S on plasminogen binding to THP-1 cells was analyzed. As shown in Fig. 3, protein S attenuated the binding of biotin-labeled Glu-plg to THP-1 cells in a dose-dependent manner. The concentration of protein S needed to inhibit plasminogen binding by 50% (ID50) was approximately 200 nM. When THP-1 cells were incubated with protein S for different time periods (1.5–3.5 h), the inhibition of plasminogen-binding was not significantly changed (data not shown).

3.3. Invasive potential of THP-1 cells was suppressed by protein S
Since the enhancement of invasion potential is a representative phenomenon induced by plasmin bound on the surface of inflammatory cells [12], the effect of protein S on the potential of THP-1 cells to invade through the matrix protein was analyzed. The number of THP-1 cells invading through Matrigel was markedly suppressed by protein S at 100 nM (Fig. 4).
4. Discussion

In the present study, it was shown that protein S suppressed plasminogen-binding on the surface of THP-1 cells, as well as their invasive potential. We propose that this mechanism explains, at least in part, the anti-inflammatory function of protein S.

As has been reported, THP-1 cells potentiated tPA-catalyzed Glu-plg activation, most probably by providing a specific binding protein for both tPA and plasminogen on the cell surface. Among several plasminogen-binding molecules on the cell surface, proteins possessing carboxy-terminal lysine are principally responsible for the enhancement of plasminogen activation in cells [7]. The enhancement is believed to be the result of either conformational or proteolytic alteration of Glu-plg, after binding to the cell surface, to a form that is more susceptible to activation by Pas, as well as a template mechanism to accumulate both plasminogen and tPA on the cell surface [7]. Several different molecules are reported as candidates, including α-enolase [13], the S-100A10-annexin II complex [14], cytokeratin-8 [15] and TIP49a [16]. The responsible molecule seems to vary depending on cell type, but the number of plasminogen-binding sites on the cell surface essentially determines the ability of the cells to enhance plasminogen activation.

Protein S suppressed plasminogen activation only in the presence of THP-1 cells, suggesting that protein S possibly diminished the binding capacity of either tPA or plasminogen on the cell surface. This was confirmed by the finding that protein S suppressed the lysine binding site-dependent binding of biotin-labeled Glu-plg on THP-1 cells. Two different mechanisms are suggested to modify the plasminogen-binding capacity of the cell surface. One is modification by proteases either to upregulate by exposing newly developed carboxy-terminal lysine after cleavage of cell-surface protein by trypsin-like proteases, including plasmin, or to downregulate by removal of the carboxy-terminal lysine by carboxyl peptidase, including the thrombin activatable fibrinolysis inhibitor (TAFI) [17]. Another is a non-protease-dependent modification of exposure of the plasminogen-binding molecule to the cell surface, which is often induced by various stimuli to alter the architecture of the cell membrane and/or cytoskeleton. The mechanism for protein S in suppressing plasminogen binding to THP-1 cells is not known, but the facts that protein S does not possess protease activity and that the downregulation was aPC-indepen-dent suggest that the mechanism is not protease-dependent. Since protein S is known to bind to negatively charged phospholipids on the cell surface through its Gla-domain [1], membrane structure might be modified by its binding to the cell surface, thus affecting the exposure of plasminogen-binding protein on the cell surface. Annexin II, a plasminogen-binding protein, as a complexed form with S-100A10, is a candidate to be influenced by protein S to be exposed to the cell surface, since it also binds to phosphatidyl serine [18].

Cell-associated fibrinolysis plays an important role in a variety of physiological and pathological events. It facilitates migration and invasion of leukocytes, cancer cells and bacterial cells, while participating in effective clot dissolution and/or angiogenesis in the case of vascular endothelial cells. On the surface of macrophages, plasminogen activation enhances their migration to sites of injury and inflammation [19,20], the physiological relevance of which has been demonstrated clearly by annexin II gene knockout animals [12] and by blocking antibody against α-enolase [21]. In our experiments, suppression of plasminogen binding to THP-1 cells by protein S resulted in the attenuation of the potential of THP-1 cells to invade through Matrigel toward a chemoattractant of monocyte chemotactic protein-1. Hyper-inflammatory responses in either congenital [2,3] or acquired [22] protein S deficiency, be-
sides procoagulant states, may signify the physiological importance of this phenomenon. Our findings in the present study support the idea that lower concentrations of protein S favor an exacerbated host response by increasing the accumulation of inflammatory cells in tissues.

In summary, protein S attenuated the potential of THP-1 cells to migrate through the extracellular matrix by suppressing the binding of plasminogen on the cell surface. This newly identified property of protein S may be involved in the pathogenesis of hyper-inflammatory host response and increased accumulation of cells in tissues when plasma levels of free protein S are decreased.

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References