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Further Characterization of the Cellular Plasminogen Binding Site: Evidence That Plasminogen 2 and Lipoprotein *a* Compete for the Same Site†

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ABSTRACT: Specific cell surface receptors for plasminogen (Pg) are expressed by a wide variety of cell types and serve to promote fibrinolysis and local Pg proteolysis. Pg types 1 and 2, separated by chromatography on concanavalin A–Sepharose, were utilized to determine their binding to the monocytoid U937 cell line. Both forms bind in a dose-dependent manner. However, Pg 2 binds to the cellular receptor considerably better than Pg 1 and at equilibrium demonstrates approximately 10-fold greater binding. Lipoprotein *a* [Lp(*a*)], which possesses a subunit showing considerable homology to Pg, competes with Pg 2 for the Pg receptor in U937 cells. Moreover, Pg 1 is not able to displace Pg 2 from the receptor. These studies suggest that high levels of Lp(*a*) may alter the profibrinolytic activity at the cell surface and increase the risks of atherosclerosis and thrombosis. This hypothesis is in accord with the 2–5-fold increased risk of atherosclerosis in patients having high levels of Lp(*a*).

Plasminogen (Pg), the plasma zymogen of the fibrinolytic enzyme plasmin, is a single-chain glycoprotein. There are two major isozymes of Pg in human plasma, Pg 1 and 2 (Brockway & Castellino, 1972). Pg 1 has a greater molecular weight (93 000) with two carbohydrate chains linked to Asn-280 and Thr-345, while Pg 2 has a lower molecular weight (89 000) with one carbohydrate chain linked to Thr-345 (Hayes & Castellino, 1979a–c). Both forms seem to have identical amino acid composition (Powell & Castellino, 1983), and the amino acid sequence is also identical (Sottrup-Jensen et al., 1978a,b). However, many physicochemical properties of the two forms are different (Takada et al., 1985). An interesting feature of the Pg molecule is the presence of five tandem repeats, called kringles. These structures, containing about 80 amino acids, are present in the amino-terminal region of the protein (Sottrup-Jensen et al., 1978a). They are homologous to two kringles present in the amino-terminal region of tissue plasminogen activator (Pennica et al., 1983), two kringles present

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in the amino-terminal region of prothrombin (Magnusson et al., 1975), and a single kringle present in the amino-terminal region of urokinase (Gunzler et al., 1982).

Recent studies have also shown that the apoprotein *a* [apo(*a*)] subunit of lipoprotein *a* [Lp(*a*)] is a homologue of Pg. Apo(*a*) contains a hydrophobic signal sequence for secretion, followed by approximately 37 copies of kringle 4 of Pg, followed by kringle 5 and the proteinase domain, all highly conserved with respect to Pg (McLean et al., 1987; Eaton et al., 1987), and has immunoreactivity with antibodies against Pg (Karadi et al., 1988). The 36th copy of kringle 4 is altered to contain an extra unpaired cysteine, the likely site of disulfide linkage with apo B-100, the other constituent of Lp(*a*) (Fless et al., 1984).

Pg receptors originally identified on platelets (Miles & Plow, 1985) have now been identified on a variety of peripheral blood cells (Miles & Plow, 1987) and on cultured cells including adherent cells such as fibroblasts (Plow et al., 1986) and endothelial cells (Hajjar et al., 1986) and nonadherent cells such as monocytoid U937 cells (Plow et al., 1986). Pg binds to its receptor in a kringle-dependent reaction (Hajjar et al., 1986; Miles et al., 1988). There are, however, no reports in the literature concerning the binding of separated Pg 1 and 2 to these cells. All the studies have been done with total Pg isolated from plasma (Miles & Plow, 1988). In the present paper we have concluded the question of whether the Pg receptor in the U937 cell line discriminates between Pg 1 and 2. In addition, we have assessed whether Lp(*a*) is recognized and binds effectively to the Pg receptor on these cells.

Materials and Methods

Proteins. Pg was purified from human plasma by affinity chromatography on L-lysine–Sepharose (Deutsch & Mertz, 1970). Pg 1 and 2 were separated by affinity chromatography on concanavalin A–Sepharose as previously described (Gonzalez-Gronow & Robbins, 1984). Heavy chains from both Pg forms were prepared as described by Gonzalez-Gronow et al. (1977).

Lp(*a*) was isolated from ten subjects with normal levels of Lp(*a*) by using a two-step chromatographic procedure. The pooled plasma was applied to a heparin–agarose column (3 × 45 cm) and the protein eluted with a NaCl gradient from 200 to 500 mM in 50 mM Tris-HCl, pH 7.4. The peak between 300 and 350 mM NaCl was concentrated by lyophilization and then gel filtered by employing a Sephacryl S-200 column (3 × 120 cm) equilibrated with 50 mM ammonium bicarbonate. The protein in the void volume was collected and lyophilized, and the Lp(*a*) identity was confirmed by immunodiffusion analysis and Western blot techniques (Towbin et al., 1979) with a polyclonal IgG raised against human Pg (Gonzalez-Gronow & Robbins, 1984).

The concentration of the human Pg 1 and 2 forms was determined spectrophotometrically at 280 nm by using the extinction coefficient of 16.8 for Glu–Pg (Wallen & Wiman, 1972) and 15.4 for the heavy chains 1 and 2 (Nilsson et al., 1982). The concentration of the isolated Lp(*a*) was determined by a modified Lowry method (Peterson, 1979) and by using a protein molecular weight of 1×10^6 as determined by SDS–PAGE.

Plasminogen Antibodies. Goat antihuman Pg antibodies were prepared and purified as previously described (Gonzalez-Gronow & Robbins, 1984). Monoclonal antibodies, 10-F-1, specific for an epitope on the kringle 4 domain of human Pg were a kind gift from Dr. F. J. Castellino (Ploplis et al., 1982).

Radioiodination of Proteins. Radioiodination was carried out by the method of Markwell (1982). Radioactivity was measured in a LKB 1272 γ counter. Incorporation of ^{125}I was approximately 8×10^6 cpm/nmol of protein.

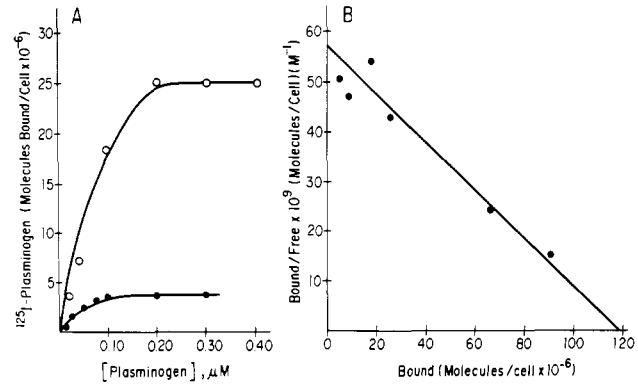

FIGURE 1: Binding of Pg 1 and 2 to U937 cells. Each assay tube contained 1×10^5 cells. (A) ^{125}I-Pg 1 (●) or 2 (○) was added at increasing concentrations in the presence or absence of 10 mM EACA to determine specific binding, as described under Materials and Methods. (B) Scatchard plot of the data in (A) for Pg 2 (●).

Cells and Ligand Binding Analyses. U937 cells were grown in RPMI 1640 culture medium (Gibco Ltd.) containing 10% fetal calf serum. Prior to use in binding assays, the cells were washed three times in Hanks' balanced salt solution and resuspended in Tyrode's buffer, pH 7.2, containing 20 mg/mL bovine serum albumin (Marguerie et al., 1980). For binding assays the U937 cells were incubated at a final concentration of 1×10^6/mL with radiolabeled ligands in the presence of unlabeled competitor or buffer. After incubation for 30 min at 22 °C, bound ligand was separated from free by layering triplicate 100-μL aliquots over 500 μL of 20% sucrose in Tyrode's buffer in 1.5-mL conical polypropylene tubes. The tips containing the cells were amputated and counted in a LKB 1272 γ counter. Molecules of ligands bound were calculated after subtraction of nonspecific binding measured in the presence of 10 mM EACA (Hajjar et al., 1986) with a molecular weight of 93 000 for Pg 1 and 89 000 for Pg 2. Scatchard plots and inhibition curves were drawn by linear regression analysis using a Hewlet-Packard HP-32E calculator.

Results

Pg 1 and 2 purified after chromatography on concanavalin A–Sepharose were utilized to determine their binding to the monocytoid U937 cell line. Both forms bind in a dose-dependent and saturable manner (Figure 1A). However, Pg 2 binds to the cellular receptor considerably better than Pg 1. The binding of Pg 2 to U937 cells was time dependent and rapidly saturable. Specific binding was essentially complete by 20–30 min (data not shown). When the data were analyzed by a Scatchard plot (Figure 1B), a K_d of 2 μM and 119×10^4 sites/cell were obtained. Not only does Pg 1 show little total or specific binding but also it is completely unable to displacd PG 2 from the receptor (Figure 2). As shown in Table I, Pg 2 and its heavy chain are able to inhibit the binding of ^{125}I-Pg 2 to U937 cells. Of particular note is the inhibitory activity observed with the heavy chain derived from Pg 1, suggesting a receptor recognition site not exposed in the parent native Pg 1 molecule.

Experiments were also performed to assess whether the binding of Pg 2 to the receptor was inhibited by Lp(*a*). The results are shown in Figure 3. As can be seen, Lp(*a*) was almost as effective as Pg 2 in blocking the binding of ^{125}I-Pg 2 to U937 cells. Moreover, as also shown in the figure, preincubation of Lp(*a*) with a monoclonal antibody to kringle

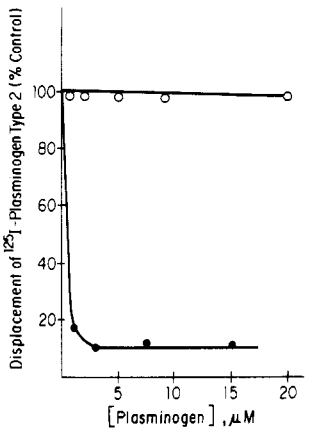

FIGURE 2: Inhibition of ^{125}I-Pg 2 binding to U937 cells by Pg 1 and 2. ^{125}I-Pg 2 (0.2 μM) and unlabeled Pg 1 (○) and 2 (●) were incubated with 1×10^5 cells at 22 °C for 30 min.

Table I: Inhibition of ^{125}I-Plasminogen 2 Binding to U937 Cells by Plasminogen and Its Derivatives[a]

plasminogen derivative added	concn added (μM)	^{125}I-Pg 2 bound (molecules/cell × 10^{-6})	inhibition (%)
none	0	92	0
Pg 2	0.5	30	68
	1.0	22	75
Pg 1	0.5	92	0
	1.0	92	0
heavy-chain 2	0.5	45	51
	1.0	36	60
heavy-chain 1	0.5	65	29
	1.0	51	44

[a] U937 cells (1×10^6 cells/mL) were incubated with 0.2 μM ^{125}I-Pg 2 and the indicated competitors for 30 min at 22 °C.

4 abolished the inhibitory effect in ^{125}I-Pg 2 binding. A similar, but less extensive, effect was observed in Pg 2.

Discussion

Many types of cells bind Pg in a kringle-dependent reaction (Hajjar et al., 1986; Plow et al., 1986; Miles et al., 1988; Miles & Plow, 1988; Hajjar & Nachman, 1988). It is important to note that none of these investigators studied Pg 1 and 2 separately, but rather pooled protein was employed in the binding experiments. Pg 1 and 2 not only differ in their carbohydrate contents (Hayes & Castellino, 1979a–c) but also differ in many of their physicochemical properties. The activation of Pg 1 is enhanced more than that of Pg 2 in the presence of fibrin by either urokinase or streptokinase (Takada et al., 1985). Pg 2 is degraded by elastase faster than Pg 1 (Takada et al., 1988). Pg 1 appears in plasma at half the rate of Pg 2 (Siefring & Castellino, 1974), and secondary glycosylation is essential for the secretion of Pg 1 (Powell et al., 1981). The concentration of Pg 2 in the circulation is twice that of Pg 1, but the latter seems to be a better fibrinolytic enzyme (Takada et al., 1985).

Clearance experiments in mice, moreover, with ^{125}I-labeled human Pg 1 and 2 and a nonglycosylated recombinant Pg showed that the rate of clearance is augmented as their content of carbohydrate decreases (Pizzo et al., 1988). We speculated then that binding of Pg to its receptor though dependent on the lysine binding sites of the kringles (Miles & Plow, 1988) is also somewhat affected by the presence of carbohydrate. We tested this hypothesis employing the monocytoid U937 cell line. These cells, a prototypic nucleated cell with respect to expression of plasminogen receptor, exhibit a recognition

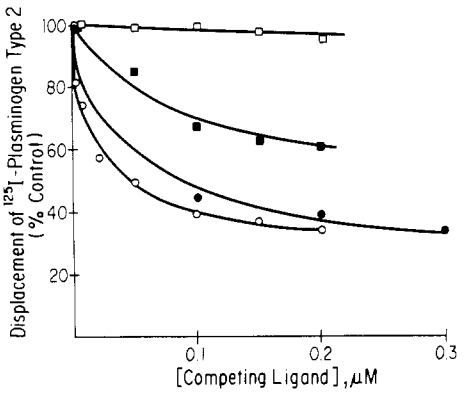

FIGURE 3: Inhibition of ^{125}I-Pg 2 binding to U937 cells by Lp(*a*) and Pg 2. ^{125}I-Pg 2 (0.2 μM) and unlabeled Lp(*a*) (○) and Pg 2 (●) were incubated with 1×10^5 cells at 22 °C for 30 min. Equimolar amounts of antikringle 4 IgG were added to Lp(*a*) (□) and Pg 2 (■) and incubated for 2 h prior to the competition experiment.

specificity similar to that of platelets (Plow et al., 1986).

Binding data for Pg 1 and 2 demonstrate that Pg 2 binds to the cellular receptor considerably better than Pg 1. The latter is completely unable to displace Pg 2 from the receptor. The K_d observed for binding of Pg 2 to U937 cells is 2 μM, in agreement with previous calculations derived after binding of total Pg to either endothelial or U937 cells (Miles & Plow, 1988). Analysis of the data in Table I reveals that although native Pg 1 does not compete with Pg 2 for the receptor, heavy-chain 1 can successfully compete for the receptor. This observation suggests that a receptor-recognition site is masked in native Pg 1 but is revealed as a consequence of conversion of Pg 1 to plasmin. It is well-known that the environment of the kringles influences the affinity of the binding sites in Pg for the ligand. Conversion of native Pg to plasmin leads to a much more open conformation and large changes in the relative position of entire domains (Vuk-Pavlovic & Gafni, 1979). A high-affinity lysine binding site, present in kringle 1, shows a loss of affinity, and a weak site present in kringle 4 increases in affinity about 20-fold (Marcus et al., 1978a,b, 1979; Vali & Pathy, 1982). Bauer et al. (1984) have shown that subphysiologic concentrations (10 nM) of human plasmin, but not Pg, bind to cultured mini-pig aortic endothelial cells. This observation is consistent with an increase in affinity of plasmin for the Pg receptor and suggests that the site in kringle 4 is an important determinant of this interaction.

The apo(*a*) subunit of Lp(*a*) contains approximately 37 copies of kringle 4. The capacity of Lp(*a*) to compete with Pg 2 for the Pg receptor in U937 cells is clearly demonstrated by our data. This ability is abolished when either Lp(*a*) or Pg 2 is previously incubated with a monoclonal antibody to kringle 4. Blocking kringle 4 in Pg 2 with the antibody does not prevent completely the binding since the region of kringles 1–3 in the Pg molecule is also recognized by the receptor (Miles et al., 1988).

From these observations we can conclude that kringle 4 plays an important receptor-recognition role in the Pg molecule. We suggest that Pg 2 binds better since its only carbohydrate chain does not prevent it from being recognized by the receptor. Since Pg 1 contains two closely located carbohydrate chains (Asn-280 and Thr-345), the receptor recognition site is hindered. This hindrance disappears when Pg 1 is converted to plasmin, with a drastic change in conformation, making it possible to bind to the receptor. The physiological relevance of this observation must be further explored. Known functions of the Pg receptors include enhancement of Pg activation (Miles & Plow, 1985, 1988; Plow

et al., 1986), localization of active plasmin on cell surfaces (Miles & Plow, 1985; Hajjar et al., 1986), and protection of cell-associated plasmin from inactivation by the primary plasmin inhibitor α_2-antiplasmin (Plow et al., 1986; Miles & Plow, 1988). The evidence presented in this paper that Pg 2 is the preferred ligand for the cellular receptor and that Lp(*a*) may compete with Pg for this receptor needs to be considered in evaluating the regulation of fibrinolysis on cell surfaces. The delicate balance between profibrinolytic and antifibrinolytic activities in maintaining hemostasis while preventing thrombosis is regulated by specific cell-mediated mechanisms, as discussed above. High levels of Lp(*a*) may alter this balance, increasing the rate of atherosclerosis and thrombotic disease. Moreover, a recent report (Silverstein et al., 1988) suggests that the entire fibrinolytic system is assembled on the cell surface. The results of the present study suggest that cell surface plasminogen activation will predominantly produce type 2 plasmin.

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