Defensin Modulates Tissue-type Plasminogen Activator and Plasminogen Binding to Fibrin and Endothelial Cells*

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Defensins are naturally occurring antimicrobial peptides that may participate in host defense against microorganisms. We previously reported that the amino acid sequence of leukocyte defensins resembles the lysine-binding site in the kringle of plasminogen and that defensin inhibits fibrinolysis mediated by tissue-type plasminogen activator (tPA) and plasminogen. In the present paper we analyze the mechanisms of this inhibition. Defensin binds specifically to cultured human umbilical vein endothelial cells (HUVEC) (half-maximal binding = 3 μM) as well as to fibrin. At saturating concentrations (5-10 μM), defensin stimulates the maximum binding of plasminogen to HUVEC and to fibrin approximately 10-fold. However, defensin inhibits plasminogen binding to both surfaces at concentrations > 10 μM. Defensin also inhibits tPA and plasminogen-mediated fibrinolysis in a dose-dependent manner at all concentrations tested. Fibrinolysis is almost totally inhibited by 5 μM defensin, a concentration that stimulates the binding of plasminogen to fibrin. Discordance between the enhancement of plasminogen binding and its activation cannot be explained by an inhibitory effect of defensin on tPA binding nor by inhibition of plasmin activity, each of which occur only at higher concentrations. Rather, these results suggest that plasminogen bound to fibrin in the presence of defensin is less susceptible to activation by tPA.

Human defensins comprise a family of closely related peptides, each of which is 29–35 amino acids in length and contains 3 intramolecular disulfide bonds (1–5). Three defensins, human neutrophil peptides HNP-(1–3),1 constitute more than 5% of the total protein in human neutrophils (PMN) (6). Other members of the human defensin peptide family are also found in the Paneth cells of the intestine, and structurally-related β-defensins have been detected in the urogenital tract (7–9). Human defensins comprise a family of closely related peptides, each of which is 29–35 amino acids in length and contains 3 intramolecular disulfide bonds (1–5). Three defensins, human neutrophil peptides HNP-(1–3),1 constitute more than 5% of the total protein in human neutrophils (PMN) (6). Other members of the human defensin peptide family are also found in the Paneth cells of the intestine, and structurally-related β-defensins have been detected in the urogenital tract (7–9). The concentration of defensins HNP-(1–3) in plasma is normally less than 15 nM, but levels approaching 50 nM have been measured in patients with sepsis or bacterial meningitis (10).

The physiologic effects of human defensins are not completely understood. Defensins clearly express potent antimicrobial activity in vitro, a function that is consistent with their cellular origins (1, 6–9). Defensins are also cytotoxic to many types of normal and malignant cells, including PMNs themselves (11–14). The cytolytic effects of defensin are abolished by serum (11–14), presumably because the peptide binds to various plasma proteins such as specific complement components, serpins, and activated αvβ3-macroglobulin (15–17). The biological activity of some plasma proteins may, in turn, be modulated by interactions with defensins (17).

In addition to their role in host defense, defensins may also contribute to the pathophysiologic consequences of inflammation. We previously reported that defensins inhibit fibrinolysis (18) and could thereby promote the development of thrombotic microvascular occlusion typical of delayed hypersensitivity reactions, some forms of vasculitis, and in disseminated intravascular coagulation (19–21). We also noted that defensins share certain similarities in their amino acid sequence with the lysine binding sites found in the kringle of plasminogen (18), and that, like plasminogen and lipoprotein(a) (Lp(a)), defensins can be purified by their affinity for lysine-Sepharose (22–24). Further, we reported preliminary studies which suggest that defensins inhibit fibrinolysis by interacting with tPA and plasminogen.

Plasminogen activation occurs not only on the surface of fibrin clots, but on the surface of vascular endothelial cells. Endothelial cells express specific binding sites for tPA and for plasminogen (25–29), and plasmin formation is accelerated when both reactants bind to their respective receptors (25, 30). Conversely, interruption of plasmin generation at these sites could contribute to fibrin formation and microvascular occlusion in situations where thrombin formation is accelerated, such as after infection and tissue injury. In the present paper, we report that leukocyte defensins bind to endothelial cells as well as to fibrin and indeed inhibit plasmin formation. These effects are seen at concentrations that are well below those reported to cause cytotoxicity and, in contrast to its cytotoxic effects, occur in the presence of serum albumin.

EXPERIMENTAL PROCEDURES

Materials—Glu-plasminogen, Lys-plasminogen, and single-chain tPA were provided by American Diagnostica (Greenwich, CT); fibrinogen (plasminogen-free), bovine serum albumin, human thrombin, and 6-aminohexanoic acid (6-AHA) were obtained from Sigma. Defensin (HNP-2) was prepared and characterized as described previously (1, 31).

Iodination of Proteins—Proteins were radiolabeled with 125I using lactoperoxidase (Sigma). Labeled proteins were separated from free iodine using gel filtration (PD-10; Pharmacia Biotech Inc.), and the protein concentration was determined using the Bio-Rad microprotein enzyme-linked immunosorbent assay. In each experiment, the specific activities of each protein ranged between 1000 and 4000 cpm/μg. To test whether iodination altered the binding properties of defensin, aliquots

1 The abbreviations used are: HNP, human neutrophil peptides (defensins); tPA, tissue-type plasminogen activator; HUVECs, cultures of human umbilical vein endothelial cells; 6-AHA, 6-aminohexanoic acid; PMN, polymorphonuclear leukocytes; Lp(a), lipoprotein(a).

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of defensin were iodinated with nonradioactive potassium iodide for 1 h using the same method. There was no difference in capacity of unlabeled and nonradioactive iodinated defensin to compete for the binding of 125I-labeled defensin to cultured human umbilical vein endothelial cells (see below), consistent with preservation of other functional activities described previously (34).

Ligand Binding Assay— Cultures of human umbilical vein endothelial cells (HUVECs) were prepared and characterized as described previously (32). Cells were grown to confluence in 48-well Falcon multilayers (Becton Dickinson, Lincoln Park, NJ) to a final density of approximately 5 x 10^4 cells/well. To impede cytotoxicity by defensin and to prevent internalization of ligands, the cells were pre-chilled to 4°C for 30 min and washed twice with a prechilled binding buffer composed of phosphate-buffered saline and 1% albumin. 125I-Labeled ligands were added in the same buffer for 90 min at 4°C in the absence of unlabeled competitor to determine total binding. The same experiments were performed in the presence of 10-50-fold molar excess unlabeled ligand to determine nonspecific binding. In each case, unbound ligand was removed, the cells were washed four times with binding buffer, and the cell surface associated radioactivity released with 50 mM glycine HCl, pH 2.8, was counted. Specific binding was defined as the difference between total and nonspecific binding. In the absence of the indicated concentrations of defensin, and the binding activity was measured. The mean ± S.D. of three experiments is shown.

RESULTS

Human neutrophil extracts containing defensin inhibit tPA-mediated fibrinolysis by inhibiting the binding of Glu-plasminogen to fibrin (18). Since endothelial cells express binding sites for plasminogen, we examined whether defensins have a similar effect on the binding of plasminogen to its cellular receptors. Defensin had a biphasic effect on the binding of 125I-Glu-plasminogen to HUVECs (Fig. 1). Binding of plasminogen was augmented approximately 8-fold at low concentrations of defensin (6 μM), but was inhibited to almost basal levels at higher concentrations (34 μM).

Additional experiments were performed to elucidate the mechanism by which defensin augmented the binding of plasminogen. We first asked whether the increase in binding of Glu-plasminogen seen at low concentrations of defensin is attributable solely to a conformational change in the ligand that makes it similar to Lys-plasminogen which is known to bind to HUVECs with higher affinity and capacity (B_max) (34). Defensin showed a similar biphasic effect on the binding of 125I-Lys-plasminogen to HUVECs, making this explanation unlikely (Fig. 1).

Alternatively, the stimulatory effect of defensin on the binding of Glu-plasminogen may be explicable by one of the following mechanisms. 1) Defensin may bind to endothelial cells providing novel sites to which plasminogen can bind; 2) defensin may bind to Glu- and to Lys-plasminogen directly, and the resultant complex may express novel cell binding epitopes; or 3) both mechanisms may be operative.

To explore these possibilities, we began by asking whether defensin binds to HUVECs. The results shown in Fig. 2 indicate that defensin indeed binds to HUVECs in a dose-dependent, specific, and saturable manner, with half-maximal binding attained at a concentration of 3 μM. The same results were seen when total binding was studied at 37°C. Binding of defensin to HUVECs was not stimulated by physiologic concentrations of plasminogen (Fig. 3). Indeed, binding of defensin was inhibited at high plasminogen concentrations (Fig. 3). This finding can be explained by the capacity of plasminogen to bind defensin through the same epitopes that participate in its binding to the cell. Consistent with this possibility, plasminogen bound directly to defensin in solution (Fig. 4).

These results led us to re-explore the interaction between defensin and plasminogen on fibrin in greater detail, since only an inhibitory effect of defensin was observed in our previous study (18). However, these experiments were performed at a
single, high concentration of defensin (approximately 17 μM). When we repeated these experiments over a wider range of defensin concentrations, a biphasic effect on the binding of plasminogen to fibrin was seen, similar to the effect on HUVECs (Fig. 5). We next examined the mechanism by which defensin stimulated the binding of Glu-plasminogen to fibrin and to HUVECs. Increasing concentrations of [125I-Glu]plasminogen were incubated with fibrin in the presence of a fixed concentration of defensin (8 μM). Defensin increased the binding of plasminogen at each concentration tested with little change in its binding affinity (half-maximal concentration) (Fig. 6). Defensin also increased the binding of plasminogen to HUVECs (~10-fold increase in the B_max) with a ~5.5-fold decrease in the affinity of binding (not shown). These results suggest that additional, low affinity plasminogen binding sites are exposed or are generated on fibrin and on HUVECs in the presence of defensin. To determine whether the defensin-induced binding is through the lysine binding sites in plasminogen, the effect of 6-AHA was examined. 6-AHA (20 mM) inhibited binding of Glu-plasminogen to HUVECs in the presence and in the absence of defensin 78% (not shown). To determine whether defensin and plasminogen share binding sites on fi-
brane and on HUVECs, we examined the capacity of Lys-plasminogen to compete with defensin for binding. In accord with this possibility, Lys-plasminogen inhibited the binding of defensin to fibrin in a dose-dependent manner (Fig. 7). Lys-plasminogen also inhibited the binding of defensin to HUVECs (not shown).

Based on this, we next examined whether low concentrations of defensin, which stimulated the binding of plasminogen to fibrin, stimulated fibrinolysis to the same extent. To our surprise, defensin inhibited plasminogen activator activity at concentrations that promoted binding of Glu-plasminogen to fibrin (Fig. 8). This result cannot be attributed to inhibition of plasmin since we have previously reported that defensin is a weak inhibitor of plasmin-mediated fibrinolysis (18). Therefore, we explored two other possible mechanisms to explain the discordance between plasminogen binding and activation. One possibility is that defensin inhibits the binding of tPA to fibrin. An alternative possibility is that defensin renders plasminogen less susceptible to cleavage by tPA. Indeed, defensin inhibited the binding of tPA to fibrin at all concentrations tested, including those that stimulated the binding of plasminogen to fibrin (Fig. 9). Further, tPA inhibited the binding of defensin to fibrin and to HUVECs (Fig. 10), suggesting a competitive mechanism of inhibition. However, the concentrations required to inhibit tPA binding were well above those required to inhibit fibrinolysis, suggesting that plasminogen bound to the surface of fibrin in the presence of defensin is less susceptible to cleavage by tPA.

**DISCUSSION**

The observation that leukocyte defensin binds to human endothelial cells in a dose-dependent, saturable, and specific manner without causing cytotoxicity has not been reported previously to our knowledge. The cellular localization of such sites suggests this family of peptides may express important physiological or pathophysiologic effects on hemostasis in addition to their capacity to lyse prokaryotic and eukaryotic cells. Binding of defensin to HUVECs was half-maximal at 3 μM which is approximately 15-fold less than has been reported to cause cytotoxicity upon prolonged incubation (12). Binding did not follow a typical hyperbolic pattern. This suggests, among other possibilities, participation of more than one class of binding sites.

Our results also indicate that defensin binds plasminogen in solution. The likely explanation for this finding rests in the fact that defensin is an arginine-rich peptide. Kringle-kringle interactions can be mediated by binding of arginine in one kringle to the lysine binding sites in other kringles (35, 36). It is likely that the arginine residues on the surface of the defensin behave in a similar manner. This direct interaction also probably accounts for the observation that low concentrations of defensin that bind to fibrin as well as to cells increase the binding of Glu-plasminogen to these surfaces. The possibility that defensin induces a conformational change from the strained Glu-plasminogen to the relaxed conformation assumed by Lys-plasminogen, which is known to bind with higher affinity and capacity to HUVEC, does not provide a sufficient explanation since defensin stimulated the binding of Lys-plasminogen to a comparable extent.

It appears likely that the enhanced binding of plasminogen to HUVECs is mediated primarily by a direct interaction of defensin with the cell surface on the one hand and plasminogen on the other, rather than binding of soluble complexes through sites on both molecules. In support of this contention, plasmin-
inhibits the binding of tPA to fibrin which can be explained by one of several possible explanations. First, defensin directly (36). In the case of defensin, such steric considerations are only and that Lp(a) impairs plasmin-mediated fibrinolysis directly (18). There may be a similar explanation for the dissociation between binding of plasminogen to fibrin and its activation in the presence of Lp(a).

All of the binding experiments described in this study were performed in protein-containing buffers at concentrations of defensin at or below 34 μM. Plasma concentrations of defensin have been reported to approach 50 μM in some patients during sepsis and bacterial meningitis (10). Thus, it is likely that defensin binds to endothelial cells and to fibrin in vivo under these conditions. The capacity of leukocyte defensins to inhibit fibrinolysis may have a beneficial effect by helping to contain microbial invasion (21). However, it is also clear that defensin may impair fibrinolysis or plasmin generation required for other physiologic process such as matrix remodeling, activation of transforming growth factor β, etc. (39). Whether interrupting the interaction of defensins with fibrin or the endothelium may exert favorable effects on fibrinolysis requires further study.

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FIG. 10. tPA inhibits the binding of defensin to fibrin and to HUVECs. 1-Defensin (3 μM) was incubated with fibrin or with HUVECs in the presence of the indicated concentrations of tPA or 20-fold molar excess unlabeled defensin, and bound radioactivity was measured. The mean ± S.D. of three experiments is shown.

The biphasic effect of defensin on the binding of plasminogen is reminiscent of the effect of Lp(a) (36). Binding of plasminogen to fibrin is inhibited at low concentrations of Lp(a), but stimulated at high concentrations (36). The stimulatory effect has been attributed to a direct binding of several molecules of plasminogen to each molecule of fibrin-bound Lp(a) (36). This stimulatory effect is similar to our purported explanation for the increase in plasminogen binding to cells and fibrin by defensin.

Inhibition of plasminogen- and tPA-mediated fibrinolysis by defensin at the same concentrations that stimulate plasminogen binding is also reminiscent of the effect of Lp(a) (36). It has been reported that the plasminogen molecules that bind to Lp(a) with low affinity are activated by tPA with less efficiency and that Lp(a) impairs plasmin-mediated fibrinolysis directly (36). In the case of defensin, such steric considerations are only one of several possible explanations. First, defensin directly inhibits the binding of tPA to fibrin which can be explained by its ability to interact with the lysine binding site in kringle 2 of tPA (38) However, tPA-mediated fibrinolysis was inhibited at lower concentrations of defensin than were required to inhibit the binding of tPA. This result suggests that plasminogen becomes less susceptible to activation by tPA in the presence of defensin. Thus, even at low concentrations of defensin where plasminogen binding is enhanced, the bound plasminogen molecules have lost their susceptibility to be cleaved, either because they have undergone a conformational change or because defensin causes a redistribution of plasminogen on fibrin which physically separates it from bound tPA. The fact defensin inhibits plasminogen activation by tPA even in the absence of fibrin provides some support for the former explanation, especially in view of the modest effects of defensin on plasmin activity (18). There may be a similar explanation for the dissociation between binding of plasminogen to fibrin and its activation in the presence of Lp(a).

The capacity of plasminogen to bind defensin in solution also helps to explain why high concentrations of the peptide inhibit plasminogen binding to cells or fibrin. When defensin is present at concentrations that exceed its binding capacity, the equilibrium between binding sites for plasminogen on surfaces and those in solution are disturbed. The same mechanism may explain why high concentrations of plasminogen inhibit binding of defensin to cells or fibrin. However, the possibility that both molecules compete for a single binding site at these concentrations must also be considered (18).

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