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Heparin Oligosaccharides Enhance Tissue-Type Plasminogen Activator: A Correlation between Oligosaccharide Length and Stimulation of Plasminogen Activation†

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ABSTRACT: The rate of plasminogen (Pg) activation by tissue-type Pg activator (t-PA) is enhanced by heparin-drived oligosaccharides. Kinetic analysis of the effects of heparin oligosaccharides, ranging in size from di- to dodecasaccharides, on Pg activation demonstrates that stimulation of the reaction is dependent on the size of the heparin oligosaccharides. Di- and tetrasaccharides enhance the activation through 2-fold increases in k_{cat} and 4-fold decreases in K_m. Hexasaccharide and larger oligosaccharides stimulate the reaction by increasing the k_{cat} by as much as 4-fold, but do not affect the K_m. Previous experiments have shown that lipoprotein(a) [Lp(a)] inhibits Pg activation by t-PA, but only in the presence of a template which enhances t-PA activity such as fibrinogen fragments or intact heparin. Similiarly, Lp(a) inhibits the enhancement of t-PA activity by the larger heparin oligosaccharides but has no effect on t-PA activity in the presence of di- and tetrasaccharides. The results of this study when considered with our previous observations (Edelberg & Pizzo, 1990) suggest that the enhancement in Pg activation by the smaller oligosaccharides is mediated exclusively via binding to t-PA while the larger oligosaccharides may interact with both t-PA and Pg. Furthermore, studies of Pg activation in the presence of both heparin oligosaccharides and fibrinogen fragments demonstrate that t-PA is stimulated preferentially by fibrinogen fragments.

Plasminogen (Pg) is a zymogen which is activated by conversion to plasmin, the enzyme which degrades fibrin clots. In the vasculature, this activation is mediated primarily by tissue-type Pg activator (t-PA) (Bachmann & Kruithof, 1984). The rate of endogenous t-PA activation of Pg is extremely slow (Hoyalerts et al., 1982) but increases in the presence of fibrinogen, CNBr fibrinogen fragments, or partially degraded fibrin (Hoyalerts et al., 1982; Rijken et al., 1982; Nieuwenhuizen et al., 1983; De Serrano et al., 1989; Harpel et al., 1985). Recent studies demonstrate that t-PA-mediated Pg activation is enhanced 5–25-fold by the glycosaminoglycan heparin (Andrade-Gordon & Strickland, 1986; Paques et al., 1986; Fears, 1988; Edelberg & Pizzo, 1990).

Heparin interacts specifically with the amino-terminal finger domain of t-PA, and thereby stimulates the rate of Pg activation (Stein et al., 1989; Edelberg & Pizzo, 1990). The finger domain is also involved in the fibrinogen fragment enhance-

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ment of t-PA-mediated Pg activation (Van Zonneveld et al., 1986a,b; De Munk et al., 1989). These fragments also stimulate plasmin generation by binding to the t-PA kringle 2 domain (Van Zonneveld et al., 1986a,b).

Heparin is well-known for its physiologic and pharmacologic capacity to decrease fibrin clot deposition in the vasculature where it is believed to function as both an anticoagulant and a pro-fibrinolytic agent. The anticoagulant properties of heparin result in a down-regulation of fibrin polymer formation by enhancing the rate of antithrombin III inhibition of thrombin and coagulation factors IXa, Xa, XIa, and XIIa (Rosenberg et al., 1986). Heparin stimulates this inhibition by acting as a template for both the inhibitor and its target proteinase (Rosenberg et al., 1986). Therefore, the anticoagulant activity of heparin is dependent on the size of the polymer. Studies with low molecular weight heparin demonstrate that only heparin decasaccharides and larger oligosaccharides promote the antithrombin-factor Xa interaction (Barrowcliffe & Thomas, 1989).

The mechanism of heparin pro-fibrinolytic activity is less well characterized. Recently, Andrade-Gordon and Strickland (1990) proposed that heparin may stimulate plasmin generation by serving as a template for binding both t-PA and Pg, in a manner analogous to that by which heparin binds to both antithrombin III and thrombin to inactivate thrombin. Thus, if binding of heparin to both enzyme and substrate is required, the stimulation of Pg activation may correlate with the size of the heparin oligosaccharide employed in the studies.

In addition to enhancing the rate of Pg activation, heparin serves as a surface for the inhibition of plasmin generation. Lipoprotein(a) [Lp(a)] is a low-density lipoprotein with an apoprotein (a) subunit which has a high degree of homology to Pg, but cannot be activated to form plasmin (Eaton et al., 1987; McLean et al., 1987). Lp(a) inhibits t-PA-mediated Pg activation, but only in the presence of templates, such as fibrinogen fragments and heparin, which can simultaneously bind both t-PA and Lp(a) (Edelberg & Pizzo, 1990; Edelberg et al., 1990; Loscalzo et al., 1990). These studies suggest that there may be a minimum size of the heparin polymer necessary for the inhibition by Lp(a).

The present study describes a further examination of the interaction between the heparin oligosaccharides and t-PA in which the effects of heparin oligosaccharides of defined molecular weight on the kinetics of t-PA-mediated Pg activation are measured in the presence and absence of fibinogen fragments and Lp(a).

Materials and Methods

Reagents. The plasmin substrate H-D-Val-L-Leu-L-Lys-*p*-nitroanilide dihydrochloride (VLK-pNA) was purchased from Helena Laboratories, Beaumont, TX. All other reagents were of the best grade commercially available.

Glycosaminoglycans. Heparin oligosaccharides were prepared by dissolving 10 g of hog mucusa heparin (170 IU/mg) and 345 g of $NaNO_2$ in 80 mL of H_2O at pH 6.0. The pH of this solution was adjusted to 1.5 with 6 N HCl and maintained at 1.5 by dropwise addition of 6 N HCl or 2 M Na_2CO_3 until the reaction reached completion (cessation of N_2 evolution, about 6 min). When the reaction was complete, the pH was adjusted to 8.5 with 2 M Na_2CO_3, and the mixture was centrifuged for 10 min at 8000 rpm in a Sorvall GSA rotor to removal a fine white precipitate which formed during the rise in pH. The supernatant was decanted, degassed under vacuum, and then concentrated by lyophilization or rotary evaporation to 30 mg of heparin/mL. The oligosaccharide mixture was chromatographed on a BioGel P10 chromatography system using two columns (5 cm × 128 cm) connected in tandem, and containing a total of 5 L of BioGel P10. The columns were packed and developed in 0.5 M NH_4HCO_3 at a flow rate of 0.7 mL/min. Fractions (18 mL) were analyzed for oligosaccharides by a carbazole procedure for quantification of uronic acid content (Bitter & Muri, 1962). The peak that emerged last from the column was a mixture of disaccharides. Each successive earlier peak was a mixture of tetra-, hexa-, octa-, deca-, dodeca-, and larger oligosaccharides. The mixture of disaccharides was further fractionated by gel filtration on a BioGel P2 column (1 cm × 60 cm) to obtain unsulfated, monosulfated, and disulfated disaccharides. With the exception of the disulfated disaccharide L-iduronosyl-2-sulfate-anhydromannose-6-sulfate that was used in the these experiments, all of the oligosaccharide preparations were purified only according to size. Within each size class, these fractions were mixtures of oligosaccharides which vary in their ratio of D-glucuronic acid to L-iduronic acid and in the degree of sulfation. However, all oligosaccharide fractions had similar compositions with respect to relative amounts of the individual disaccharide units. None of the fractions possessed anticoagulant activity as determined by effects of these preparations on the activated partial thromboplastin time.

Proteins. Human Pg glycoform 2 was isolated by affinity chromatography as previously described by Deutsch and Mertz (1970) and modified by Brockway and Castellino (1972) and Gonzalez-Gronow and Robbins (1984). t-PA was the generous gift of Dr. Henry Berger, Wellcome Research Laboratories, Research Park, NC, and the specific activity was determined as described previously (Edelberg et al., 1991). Lp(a) was purified as previously described (Edelberg et al., 1989). Human fibrinogen, purchased from Kabivitrum, Stockholm, Sweden, was rendered Pg-free and used to prepare CNBr fragments as previously described (Nieuwenhuizen et al., 1983).

Determination of Kinetic Activation Constants of the Heparin Oligosaccharides. Pg was activated with t-PA as previously described (Edelberg & Pizzo, 1990). Briefly, various concentrations of Pg were incubated in a buffer of 50 mM Tris-HCl, 0.05% gelatin, and 0.01% Tween 80, pH 7.4, with t-PA (35 IU/mL; 1 nM), in th presence of the plasmin substrate VLK-pNA (300 μM) and increasing concentrations of the various heparin oligosaccharides. The hydrolysis of VLK-pNA was monitored by continuous spectrophotometry (A_{405nm}). The initial rate of substrate hydrolysis was determined from a plot of the first-order derivative of hydrolyzed substrate versus time, and the initial slope (b) was calculated when greater than 95% of the initial concentrations of both substrate and Pg were present. From this, the initial rate of plasminogen activation was determined using the equation $V_i = b(1 + K_E/[S_0])/\epsilon k_e$ where K_E is the apparent Michaelis constant of VLK-pNA hydrolysis by plasmin (0.3 mM), k_e is the catalytic rate constant for hydrolysis of VLK-pNA by plasmin 2 [2.3×10^3 M (mol of plasmin)$^{-1}$ s^{-1}], and ϵ is the molar extinction coefficient of the hydrolyzed substrate at A_{405nm} (8.8×10^3 M^{-1} cm^{-1})(Erlanger et al., 1961). The kinetic constants for Pg activation were determined by a double-reciprocal plot of initial rates versus Pg concentrations in the absence of any heparin oligosaccharides. In the presence of heparin oligosaccharides, the kinetic activation constants were determined using the equation $V_i = \{V_m([S]/K_m + \beta\text{-}[A][S]/\alpha K_m K_a)\}/\{1 + [S]/K_m + [A]/\alpha K_m + [S][A]/\alpha K_m K_a\}$ (Dixon & Webb, 1979). α and β are the activation constants which modify K_m and k_{cat}, respectively, and K_a is the dissociation constant of the activator from the enzyme. From a

double-reciprocal plot of initial rates of plasmin formation versus Pg concentrations in the presence of increasing concentrations of heparin oligosaccharides, the change (Δ) from control in both abscissa intercepts and slopes was determined for each concentration of heparin oligosaccharide. The constants, α, β, and K_a, were calculated from a double-reciprocal plot of Δ intercept and slope values versus heparin oligosaccharide concentration. The rate of VLK·pNA hydrolysis by plasmin was unaffected by the presence of the various heparin oligosaccharides, similar to intact heparin as previously reported (Andrade-Gordon & Strickland, 1986; Edelberg & Pizzo, 1990). Lp(a) also has no effect on hydrolysis of this substrate by plasmin as previously reported (Edelberg et al., 1990).

Determination of Lipoprotein(a) Inhibition of Enhancement by Heparin Oligosaccharides. Pg was activated by t-PA in a solution containing the various heparin oliogosaccharides (50 μg/mL) in the presence and absence of Lp(a) (50 nM). The initial rate of plasmin formation was determined as described above. The Lp(a) inhibitory activity was calculated for reactions with the various oligosaccharides. The percent of activity for a given reaction was determined as follows. Fraction of activity for any given heparin oligosaccharide in the presence of Lp(a):

$$\phi_{oligo} = V_{i,oligo+Lp(a)}/V_{i,oligo} \quad (1)$$

Fraction of activity for intact heparin in the presence of Lp(a):

$$\phi_{heparin} = V_{i,heparin+Lp(a)}/V_{i,heparin} \quad (2)$$

The percent of maximal inhibitory activity (M) in the presence of a given heparin oligosaccharide relative to the Lp(a) inhibitory in the presence of intact heparin in the presence of Lp(a):

$$M_{Lp(a)} = 100\left(\frac{1-\phi_{oligo}}{1-\phi_{heparin}}\right) \quad (3)$$

The percent of activity for a given heparin oligosaccharide was then plotted versus the degree of polymerization of the oligosaccharide (DP).

Determination of Enhancement in the Presence of Heparin Oligosaccharides and Fibrinogen Fragments. Pg was activated by t-PA as described above in a solution containing 50 μg/mL fibrinogen fragments or 50 μg/mL various heparin oligosaccharides, or in the presence of both. The initial rate of plasmin formation was determined as described above.

Results

Determination of Kinetic Activation Constants of the Heparin Oligosaccharides. The heparin oligosaccharides increased t-PA activation of Pg by two kinetic mechanisms: increases in the catalytic rate constant (k_{cat}) and decreases in the Michaelis constant (K_m) of the reaction. The kinetic constants describing the stimulation of Pg activation by heparin oligosaccharides were calculated from the replots of the data and are listed in Table I. These results show that the disulfated disaccharide and the tetrasaccharide fractions behave in a similar manner; they both lower the K_m and raise the k_{cat}. By constrast, the hexa-, octa-, and dodecaoligosaccharide fractions all increase the k_{cat} but have no effect on the K_m. In similar experiments, it was found that the monosulfated disaccharide fraction had no effect on Pg activation. For all of the assays reported here, concentrations of these materials that gave maximal effects were used: that is, the oligosaccharides were saturating with respect to enhancement of the t-PA-mediated activation of Pg. Thus, the observations reported here on the effects of increasing oligosaccharide size on activation of t-PA appear to reflect the effects of size on these reactions. Determination of the effects of individual oligosaccharides within each size class would require the extremely difficult task of purification of the individual components of these mixtures.

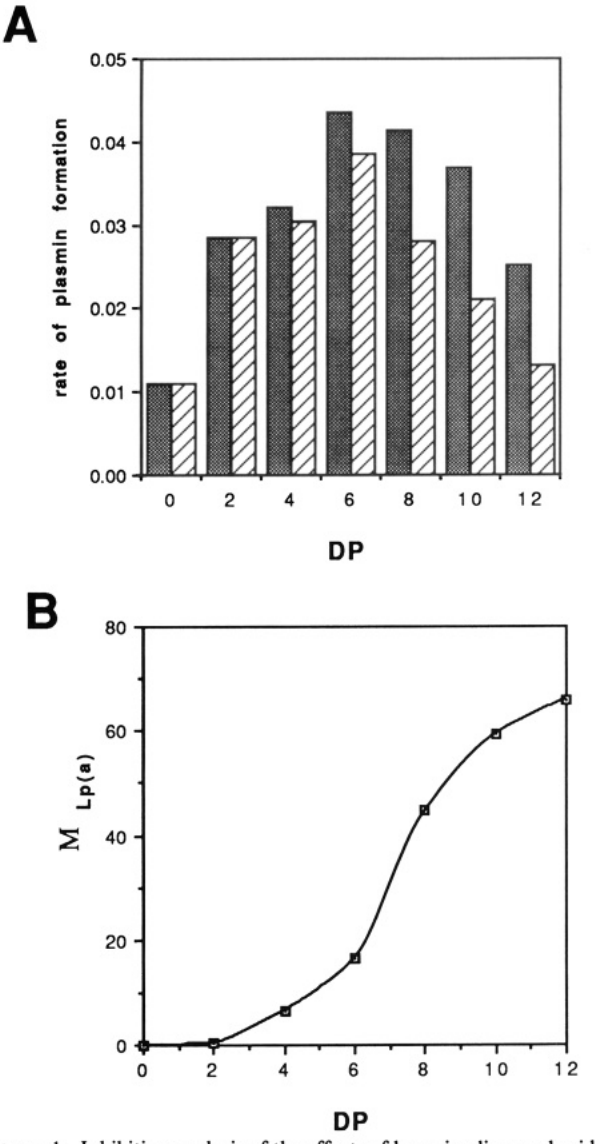

FIGURE 1: Inhibition analysis of the effects of heparin oligosaccharides and lipoprotein(a) on plasmin formation. (Panel A) Bar graph of plasmin formation (10^{11} mol/s) versus the degree of heparin polymerization (DP). Pg, 100 nM, was activated with (1 nM) t-PA in a buffer of 50 mM Tris-HCl, pH 7.4, 0.05% gelatin, 0.1% Tween 80, and 300 μM VLK·pNA with 50 μg/mL each heparin oligosaccharide alone (shaded) and also with 50 nM Lp(a) (striped). Plasmin formation was calculated as described previously under Materials and Methods. (Panel B) Replot of the maximal inhibitory activity of Lp(a) [$M_{Lp(a)}$] versus DP. The calculation of $M_{Lp(a)} = 100[(1-\phi_{oligo})/(1-\phi_{heparin})]$ was previously described under Materials and Methods.

Determination of Lipoprotein(a) Inhibition of Enhancement by Heparin Oligosaccharides. Lp(a) inhibited the heparin oligosaccharide-enhanced t-PA activation of Pg (Figure 1A). In the absence of oligosaccharides, or in the presence of the disaccharide fraction, Lp(a) had little effect on the t-PA-catalyzed reaction; however, the degree of inhibition by Lp(a) increased with increasing size of the heparin oligosaccharides. In the presence of the heparin octasaccharide fraction, Lp(a) demonstrated 50% of its inhibitory capacity (Figure 1B).

Determination of Enhancement in the Presence of Heparin Oligosaccharides and Fibriongen Fragments. Both heparin

Table I: Kinetic Constants of Plasminogen Activation for Various Size Heparin Oligosaccharides[a]

DP	α	β	K_a (μg/mL)
2	0.25	1.5	20
4	0.25	2.0	10
6	1.0	3.0	40
8	1.0	3.5	20
10	1.0	4.0	25
12	1.0	2.0	10

[a] Abbreviations: DP, degree of heparin polymerization; α, nonessential activator factor of K_M; β, nonessential activator factor of k_{cat}; K_a, dissociation constant for a nonessential activator.

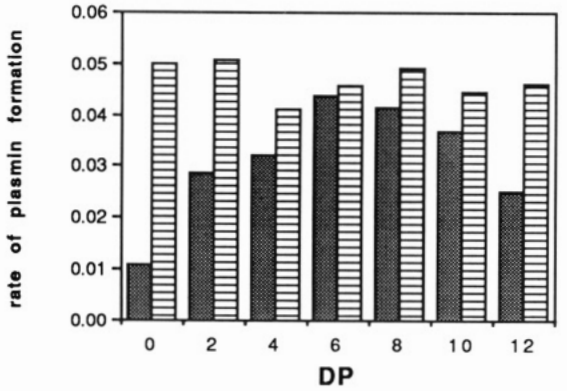

FIGURE 2: Comparative analysis of the effects of heparin oligosaccharides and fibrinogen fragments on plasmin formation. Bar graph of plasmin formation (10^{11} mol/s) versus the degree of heparin polymerization (DP). Pg, 100 nM, was activated with t-PA (1 nM) in a buffer of 50 mM Tris-HCl, pH 7.4, 0.5% gelatin, 0.1% Tween 80, and 300 μM VLK-pNA with 50 μg/mL each heparin oligosaccharide alone (shaded) and also with 50 μg/mL fibrinogen fragments (striped). Plasmin formation was calculated as described previously under Materials and Methods.

oligosaccharides and fibrinogen fragments increased the rate of t-PA-mediated Pg activation (Figure 2). However, the rate of Pg activation in the presence of fibrinogen fragments was not further increased by any of the heparin oligosaccharide fractions (Figure 2).

Discussion

Markwardt and Klocking (1977) proposed that heparin may promote fibrinolysis. Several studies have demonstrated that heparin enhances the rate of Pg activation by the physiologic Pg activator t-PA at a rate which is increased 5–25-fold (Andrade-Gordon & Strickland, 1986; Paques et al., 1986; Fears, 1988; Edelberg & Pizzo, 1990). Recent studies have identified the domain of t-PA involved in its interaction with heparin. Stein et al. (1989) and Edelberg and Pizzo (1990) demonstrated that heparin interacts with the amino-terminal finger domain of t-PA to stimulate the rate of plasmin generation. In addition, Andrade-Gordon and Strickland (1990) fractionated heparin into pools with high affinity and low affinity for t-PA and showed that only the high-affinity fraction enhanced Pg activation, although both fractions retained anticoagulant activity.

The rate of t-PA-mediated Pg activation is markedly stimulated by vascular components such as fibrin(ogen) and its derivatives such as CNBr-derived fibrinogen fragments and partially degraded fibrin (Hoyalerts et al., 1982; Rijken et al., 1982; Nieuwenhuizen et al., 1983; De Serrano et al., 1989) and heparin (Andrade-Gordon & Strickland, 1986; Paques et al., 1986; Fears, 1988; Edelberg & Pizzo, 1990). These components serve as templates for the interaction between enzyme and substrate, increasing their local concentrations and thereby stimulating the rate of the activation. In addition, these vascular components increased the catalytic rate of the reaction by interaction with the amino-terminal domains of t-PA to further increase the rate of the activation. In the presence of a template which cannot simultaneously bind both the enzyme and substrate, the stimulation from the increase in local concentrations of t-PA and Pg cannot take place, but the enhancement due to the interaction between the template and the t-PA amino-terminal regulatory domains can be detected.

These vascular surfaces also serve as templates for Lp(a) inhibition of t-PA-mediated Pg activation. Previous studies have demonstrated that Lp(a) inhibition of t-PA requires a template to which both t-PA and Lp(a) may bind, such as fibrinogen fragments or intact heparin (Edelberg & Pizzo, 1990; Edelberg et al., 1990; Loscalzo et al., 1990). In the presence of these templates, Lp(a) acts as a competitive inhibitor of Pg activation, binding to the template and sterically preventing Pg from interacting with the simultaneously template-bound t-PA. Furthermore, Lp(a) does not displace the enzyme from the stimulatory template, which kinetically would be detected as an uncompetitive inhibitor.

With this background, it is possible to consider the present results and examine what analogies can be draw with the prior literature. The data presented in Table I demonstrate that the large heparin oligosaccharides stimulate Pg activation via increases in k_{cat}. Furthermore, this stimulation by the large heparin oligosaccharides (octasaccharides and greater) is inhibited by Lp(a), indicating that the larger heparin oligosaccharides are also large enough to interact with both t-PA and Lp(a). The enhancement of Pg activation by heparin oligosaccharides may be dependent on the capacity of the oligosaccharides to interact with both t-PA and Pg. Our studies suggest that the effects of the larger oligosaccharides are mediated through binding of the oligosaccharides to both t-PA and Pg, as has been previously proposed by Andrade-Gordon and Strickland (1990). Previous studies demonstrate that the catalytic domain of Pg interacts with heparin (Soeda et al., 1989), although the affinity of heparin for Pg is 2 orders of magnitude lower than its affinity for t-PA (Andrade-Gordon & Strickland, 1986).

The kinetic effects of smaller oligosaccharides on Pg activation may be due to binding of these oligosaccharides to only t-PA. On the basis of the data in Table I, these small oligosaccharides (di- and tetrasaccharides) are clearly large enough to bind to either t-PA or Pg, and increase the t-PA affinity for and hydrolysis of Pg. However, these oligosaccharides are not likely to be large enough to interact simultaneously with Pg and t-PA. Lp(a) only minimally inhibits the stimulation of the activation by these smaller oligosaccharides, suggesting these oligosaccharides do not simultaneously bind both Lp(a) and t-PA. Furthermore, the lower degree of Lp(a) inhibition suggests that the smaller oligosaccharide's stimulatory interaction is with t-PA and not Pg. If the disaccharide exerted its effect by binding Pg rather than t-PA, Lp(a) should reverse this effect (Edelberg & Pizzo, 1990). However, since the disaccharide effect is not reversed by Lp(a), the disaccharide does not bind to Lp(a) and, by analogy, does not bind to Pg. We conclude that the disaccharide binds only to the t-PA and the lowering of the K_m and the raising of k_{cat} must occur as a result of this interaction.

The size of the template is important in the inhibition of t-PA by Lp(a), with the heparin octasaccharide representing the minimal size as a template for Lp(a) inhibition. In an analogous manner, the anticoagulant activity of heparin also depends on its ability to act as a template for binding of both

antithrombin III and the target proteinase. Pharmacokinetic studies with low molecular weight heparin demonstrated that the heparin decasaccharide is the minimal oligosaccharide size with anticoagulant activity (Palm et al., 1990). Heparin octasaccharides are too small to stimulate the interaction between antithrombin III and thrombin [for a review, see Barrowcliffe and Thomas (1989)].

The interaction between the heparin oligosaccharides and t-PA is dependent on sulfate groups. The experiments employing disaccharides with varying sulfate content demonstrated that only the heparin-disulfated disaccharide enhanced the rate of t-PA-mediated Pg activation, suggesting that heparin oligosaccharides may bind to one or more Arg or Lys residues of t-PA. These results are consistent with previous studies which fould that intact heparin enhanced Pg activation to a greater extent than did the related but less sulfated glycosaminoglycan heparan sulfate (Edelberg & Pizzo, 1990).

The heparin oligosaccharides and intact heparin appear to interact with t-PA in a similar manner. The kinetics of enhanced plasmin generation suggest a common interaction of the heparin oligosaccharides with the t-PA finger domain. Furthermore, the binding constants of both the various fragments and the intact molecule to the activator are similar ($\sim$20 mg/L). Heparin oligosaccharides which bind to only the finger domain may leave the t-PA kringle 2 domain exposed. The free kringle 2 could then bind the Lys residues of fibrinogen fragments. The initial interaction between the kringle 2 domain and fibrinogen fragments promotes a further interaction between the finger domain and the fibrinogen fragments (Van Zonneveld et al., 1986a,b; De Munk et al., 1989). The binding of fibrinogen fragments to the t-PA finger domain will then likely displace the bound heparin oligosaccharides. This may explain why only the effects of fibrinogen fragments are detected in Pg activation studies in which both heparin oligosaccharides and fibrinogen fragments are present, and suggest that in vivo t-PA may be transferred from vascular heparin to developing fibrin clots.

In conclusion, the results described here demonstrate that a range of heparin oligosaccharides, from di- to dodecasaccharides, stimulate t-PA-mediated Pg activation. These experiments suggest that in enhancing Pg activation the smaller heparin oligosaccharides may bind only to t-PA. Kinetic analysis demonstrates that the smaller oligosaccharides increase t-PA affinity for and turnover of Pg but do not act as a template for Lp(a) inhibition of Pg activation. The larger oligosaccharides may interact with both t-PA and Pg to increase plasmin generation. The larger oligosaccharides only increase the rate of Pg turnover but dot not affect the overall affinity of t-PA for Pg. These larger oligosaccharides also serve as templates for Lp(a) inhibition of plasmin generation. Furthermore, the heparin oligosaccharides interact with the finger domain of t-PA and are competed away by fibrinogen fragments.

The clinial implications of these observations are difficult to determine. Grailhe and Anglis-Cano (1991) recently suggested that heparin does not enhance t-PA-mediated plasminogen activation in the presence of fibrin. By contrast, Marsh and Born (1991) have concluded that low molecular weight heparin sulfate stimulates the fibrinolytic system both in vitro and in vivo. Clearly, additional studies are required to address the issue of the biological relevance of heparin as a stimulator of fibrinolysis.

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