Neonatal Plasminogen Displays Altered Cell Surface Binding and Activation Kinetics

Correlation with Increased Glycosylation of the Protein

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Abstract

Plasminogen isolated from 60 full-term newborns differs from adult plasminogen in carbohydrate composition, kinetic activation constants, and cell binding. Amino acid composition and amino-terminal sequence analysis data indicate that the plasminogens of neonates and adults have the same amino acid sequence. Like the adult, the neonate has two glycoforms, but both have significantly more mannose and sialic acid than the adult forms. The difference in the neonatal glycosylation is probably responsible for the altered migration observed by isoelectric focusing. Moreover, the difference in carbohydrate composition appears to be the basis of the decreased functional activity of the neonatal plasminogen. The k_{cat}/K_m ratios indicate that the overall activation rates of the two neonatal plasminogen glycoforms are lower compared with the adult glycoforms. In addition, neonatal plasminogen does not bind as well to cellular receptors compared with adult plasminogen. These studies suggest a basis for the decreased fibrinolytic activity observed in neonates. (*J. Clin. Invest.* 1990. 86:107–112.) Key words: plasminogen • fibrinolysis • neonatal coagulation • neonatal fibrinolysis • altered glycosylation

Introduction

Fibrinolytic activity in the neonatal human is markedly lower than that of the adult, rendering the full-term newborn more prone to thrombotic disease than at any other time in development (see reference 1 for review). This reduced ability to lyse fibrin clots correlates with an increased risk of developing renal vein thrombosis (2, 3), pulmonary embolism (4), severe systemic arterial embolism (5), and hyaline membrane disease (6, 7). Several clinical investigations have correlated this decreased fibrinolytic activity with lower levels of functional plasminogen in the full-term newborn. Phillips and Skrodelis (8) measured plasminogen activation by substrate hydrolysis, and found that levels in the full-term newborns are 25% of maternal levels. Several other studies (9–11) also observed lower functional plasminogen levels in the neonate as compared with the adult. Based on amino acid composition, Estelles and co-workers (12) suggested that this decreased function is due to structural differences between the adult and neonatal plasminogen forms.

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Plasminogen is a fibrinolytic zymogen which, when activated by streptokinase, urokinase, or tissue-type plasminogen activator, forms plasmin, the proteinase that digests fibrin (13). As initially synthesized in the liver, the protein has an amino-terminal Glu residue, but plasmin cleavage of this molecule can occur, removing an amino-terminal, 76-residue peptide (13). The resulting form of plasminogen has an amino-terminal Lys. In adult plasma, plasminogen has two major glycoforms that have identical amino acid sequence (13). Hayes and Castellino (14–16) determined the carbohydrate composition and structure of the two adult plasminogen variants. Adult plasminogen 1 has both an *N*- and an *O*-linked carbohydrate chain, while adult plasminogen 2 contains only an *O*-linked carbohydrate chain.

The carbohydrates of the adult plasminogen variants affect the functional properties of the molecules. Adult plasminogen 2 has a higher affinity for cell surface binding sites than plasminogen 1 (17). These cell binding sites are important as sites for plasminogen activation (17–20). The carbohydrate-dependent differences in binding affinity may result in different rates of activation by tissue-type plasminogen activator and urokinase for the two adult glycoforms.

This paper presents studies of the two plasminogen glycoforms isolated from the plasma of 60 full-term newborns. Our data suggest that the amino acid sequences of adult and neonatal plasminogen are identical. However, significant differences in carbohydrate composition were observed between the neonatal and adult plasminogen glycoforms. These changes may be the basis for the decreased fibrinolytic activity found in clinical studies of neonates.

Methods

Reagents. The plasmin substrate, H-D-Val-L-Leu-L-Lys-*p*-nitroanilide-dihydrochloride (S-2251), was purchased from Helena Laboratories, Beaumont, TX. All other reagents were of the best grade commercially available.

Proteins. Plasma samples were collected from umbilical venous blood of 60 nondiabetic full-term newborns. Plasminogen was purified from pooled plasma by affinity chromatography as previously described by Deutsch and Mertz (21), and modified by Brockway and Castellino (22). The glycoform 1 was separated from glycoform 2 by affinity chromatography on concanavalin A–Sepharose in a buffer of 50 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$, pH 7.4, as previously described (23). The less glycosylated glycoform 2 does not bind to the column, while the mannose-rich glycoform 1 binds to the column and can be eluted with 200 mM α-methylmannopyanoside. The concentrations of both fetal and adult plasminogens 1 and 2 forms were determined spectrophotometrically at 280 nm using an extinction coefficient of 16.8 1%/1 cm for Glu-plasminogen (24). Two-chain recombinant tissue-type plasminogen activator, 500,000 IU/mg, was the generous gift of Dr. Henry Burger, Wellcome Research Laboratories, Research Triangle Park, NC. Urokinase, 2025 IU/ml, was purchased from Calbiochem-Behring Corp., San Diego, CA.

Human fibrinogen was purchased from Helena Laboratories and used to prepare CNBr fragments as previously described (25).

Electrophoresis. SDS-PAGE was performed on 8% polyacrylamide slab gels using 0.1% SDS and 0.1 M Tris-glycine buffer, pH 8.6 (26).

Isoelectric focusing. Isoelectric focusing was performed in an FMC resolute HPM chamber system (Marine Colloids Division, Rockland, ME) with Isogel agarose plates with pH gradients of 5.5–8.5. The electrode solutions were 0.1 M L-histidine for the cathode and 0.1 M Hepes for the anode. Electrophoresis was performed at constant voltage (500 V) for 90 min with constant cooling at 10°C. After focusing, the pH gradient was determined using a surface electrode (Radiometer America Inc., Westlake, OH) with measurements at 3-mm intervals. The gels were fixed in a solution containing 3.5% sulfosalicylic acid/10% trichloroacetic acid for 30 min. The gels were stained with 0.25% Coomassie blue R-250 in 45% methanol/10% acetic acid for 1 h and destained at room temperature with 25% methanol/10% acetic acid.

Amino acid composition and protein sequence analysis. 50-μg protein samples were hydrolyzed in gas phase for 24 h at 110°C as previously described (27). The samples were then dried in a Speed Vac concentrator (Savant Instruments, Inc., Hicksville, NY), and the amino acid composition of the samples was determined in a high-performance amino acid analyzer (model 6300; Beckman Instruments, Inc., Palo Alto, CA) with sodium citrate buffers provided by the manufacturer. The proteins, 100 pmol, were sequenced by automated Edman degradation in a gas/liquid phase sequencer (model 477A; Applied Biosystems Inc., Foster City, CA) with on-line PTH analysis using HPLC (model 120A; Applied Biosystems Inc.). The instruments were operated as recommended in the user bulletins and manuals distributed by the manufacturer.

Carbohydrate analysis. Composition analysis of the uncharged carbohydrate was performed by Dr. Robert Haltiwanger at Johns Hopkins University (Baltimore, MD) by anion exchange on a pellicular exchange resin (HPIC-AS6; Dionex Corp., Sunnyvale, CA). The instrument was operated as recommended in user bulletins and manuals distributed by the manufacturer. Sialic acid content was determined by fluorometric assay as previously described (28). Endoglycosidase F treatment of neonatal plasminogen 1 was performed as previously described (29).

Kinetics. Steady-state kinetics determined by triplicate initial rate measurements were performed as previously described (30). Briefly, plasminogen at various concentrations was activated with 70 IU/ml tissue-type plasminogen activator in the presence of 50 μg/ml fibrinogen fragments in 1 ml of 50 mM Tris-HCl, pH 7.4, 0.05% gelatin, and 0.1% Tween 80 containing 300 μM S-2251. The incubations were conducted in a cuvette at 22°C and the plasmin hydrolysis of S-2251 was monitored continuously at $A_{405\ nm}$. The plasminogen activation progress curve was transformed by plotting instantaneous plasmin hydrolysis of S-2251 ($dA_{405\ nm}/dt$) versus time. The slope (b) of this plot gives the initial velocity of substrate hydrolysis by plasmin. The plasminogen activation velocity was determined by the equation $Vi = b(1 + K_E/S_0)/\epsilon k_c$, where K_E is the apparent Michaelis constant of S-2251 hydrolysis by plasmin (0.3 mM); k_c is the catalytic rate constant for plasmin hydrolysis of S-2251 determined for fully activated individual plasminogen isoforms; and ϵ is the molar extinction coefficient of the hydrolyzed substrate at $A_{405\ nm}$ ($10^4\ M^{-1} \cdot cm^{-1}$). The inverse Vi was plotted versus inverse plasminogen concentrations to determine k_{cat} and K_m.

Cell binding analyses. Cell binding studies were conducted as previously described (17) using U937 cells grown in RPMI 1640 culture medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum. Adult plasminogen 2 was radiolabeled as described by Markwell (31) and the radioactivity was measured in a γ counter (model 1272; LKB Instruments, Inc., Gaithersburg, MD). The specific activity of the adult plasminogen 2 was 8×10^6 cpm/nmol. The radiolabeled ligand was incubated with 1×10^6 U937 cells/ml in the presence of unlabeled competitor or buffer. The samples were incubated for 30 min at 22°C. Bound ligand was separated from free and the cell-bound ligand counted as previously reported (14).

Results

Full-term newborn plasminogen glycoforms. The two adult plasminogen glycoforms can be separated on the basis of mannose content (23). Plasminogen 1 binds concanavalin A through its *N*-linked oligosaccharide. Similarly, neonatal plasminogen also has two major glycoforms that are separable on concanavalin A–Sepharose. The total amount of plasminogen recovered from the full-term newborn plasma was 11 mg/dl, compared with 20 mg/dl for adult plasma. The ratios of the glycoforms 1 and 2 were 2:3 and 3:7 for adult and neonate, respectively. PAGE under denaturing conditions (Fig. 1) demonstrates that the neonatal plasminogens 1 and 2 are similar in size to their adult counterparts. Moreover, Fig. 1 shows that the plasmin derived from both the neonatal forms is similar in size to adult plasmin.

Amino acid composition and protein sequence analysis. The amino acid compositions of neonatal plasminogens 1 and 2 are similar both to each other and to those of the adult plasminogens 1 and 2 (Table I) and essentially identical within the limits of this technique. Amino-terminal analysis of both neonatal glycoforms revealed that these preparations consisted of Glu-plasminogen and contained < 1% Lys-plasminogen. Furthermore, amino-terminal sequence analysis of the first 20 residues from the adult and neonatal Glu-plasminogen glycoforms are identical. Plasmin treatment of both adult and neonatal plasminogen removes the first 76 residues, yielding Lys-77-plasminogen (13). An amino-terminal sequence analysis of the first 20 residues of the adult and neonatal Lys-plasminogen again demonstrates complete identity.

Carbohydrate composition. The notable structural difference between the neonatal plasminogen and the adult plasminogen is in the carbohydrate content. Altered elution profile from concanavalin A–Sepharose indicated that the two neonatal plasminogen isoforms would have different carbohydrate structures. The carbohydrate content of neonatal plasminogens 1 and 2 (Table II) differs markedly from that of adult plasminogens 1 and 2. Neonatal plasminogen 1 contains almost ten times more mannose and over three times more sialic acid than adult plasminogen 1. Neonatal plasminogen 2 contains more than four times more mannose and nearly twenty times more sialic acid residues than adult plasminogen 2.

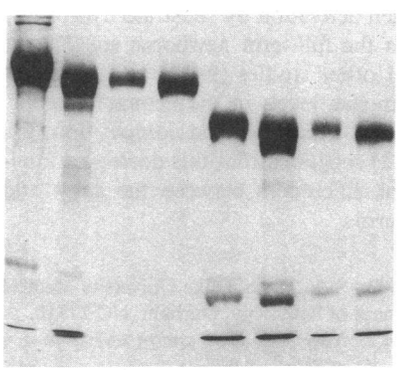

Figure 1. SDS-PAGE of plasminogen and plasmin glycoforms. Lane *a*, adult plasminogen 1; lane *b*, adult plasminogen 2; lane *c*, neonatal plasminogen 1; lane *d*, neonatal plasminogen 2; lane *e*, adult plasmin 1; lane *f*, adult plasmin 2; lane *g*, neonatal plasmin 1; lane *h*, neonatal plasmin 2. Plasmin was obtained by urokinase activation of plasminogen. Each lane contained 25 μg of protein to correct for poor staining of the neonatal plasminogen and plasmin glycoforms. Each preparation contained < 1% Lys-plasminogen as determined by amino-terminal sequence.

Table I. Amino Acid Composition of Plasminogen Forms

Amino acid (mol/mol protein)	Plasminogen form	
	Adult	Neonate
Asx	77	79
Thr	61	62.5
Ser	55	57
Glx	81	80
Pro	69	69
Gly	60	54
Ala	37	39
Cys	48	46
Val	46	50
Met	10	10
Ile	21	22
Leu	42	41
Tyr	30	28
Phe	20	23
His	23	24
Lys	47	48
Arg	53	51

Isoelectric focusing. The isoelectric focusing of the adult plasminogen isoforms is similar in distribution to previously published reports by Summaria and co-workers (32) and Robbins and Summaria (33). Isoelectric focusing (Fig. 2) demonstrates that the neonatal plasminogen 2 isoforms migrate at more acidic positions than the adult plasminogen 2 isoforms, consistent with the difference in the sialic acid content, as described above. There is also a different distribution of the adult and neonatal plasminogen 1 isoforms, and there are fewer neonatal variants. Additionally, the isoelectric focusing demonstrates that there was no cross-contamination of the neonatal plasminogens 1 and 2 preparations.

Plasminogen activation kinetics. The kinetics of activation of the neonatal plasminogens are markedly different compared with those of adult plasminogens. The plasminogen isoforms were activated by tissue-type plasminogen activator in the presence of CNBr fibrinogen fragments, which increases the rate of the plasminogen activation (25). Kinetic activation plots of both neonatal and adult plasminogens 1 and 2 are shown in Fig. 3, *A* and *B*, respectively. The kinetic constants are shown in Table III. While the activity of the adult and neonatal plasmin molecules was similar, the activation of the plasminogens was sharply different. Neonatal plasminogen 1 had a k_{cat} less than one-third that of adult plasminogen 1 and a

Table II. Carbohydrate Content of Plasminogen Glycoforms

Sugar (mol/mol protein)	Plasminogen glycoforms			
	Adult 1	Adult 2	Neonate 1	Neonate 2
Mannose	3.0	0.6	27.4	2.7
Galactose	3.0	1.0	0.41	0.69
N-Acetyl glucosamine	3.9	0.6	4.8	6.7
Sialic acid	2.5	1.1	8.0	18.5

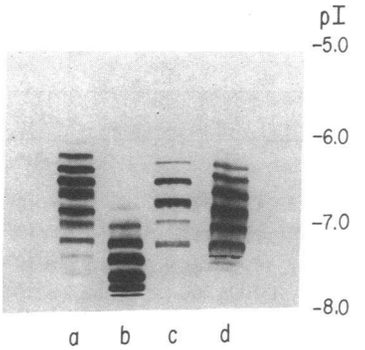

Figure 2. Isoelectric focusing gel of plasminogen glycoforms. Lane *a*, adult plasminogen 1; lane *b*, adult plasminogen 2; lane *c*, neonatal plasminogen 1; lane *d*, neonatal plasminogen 2.

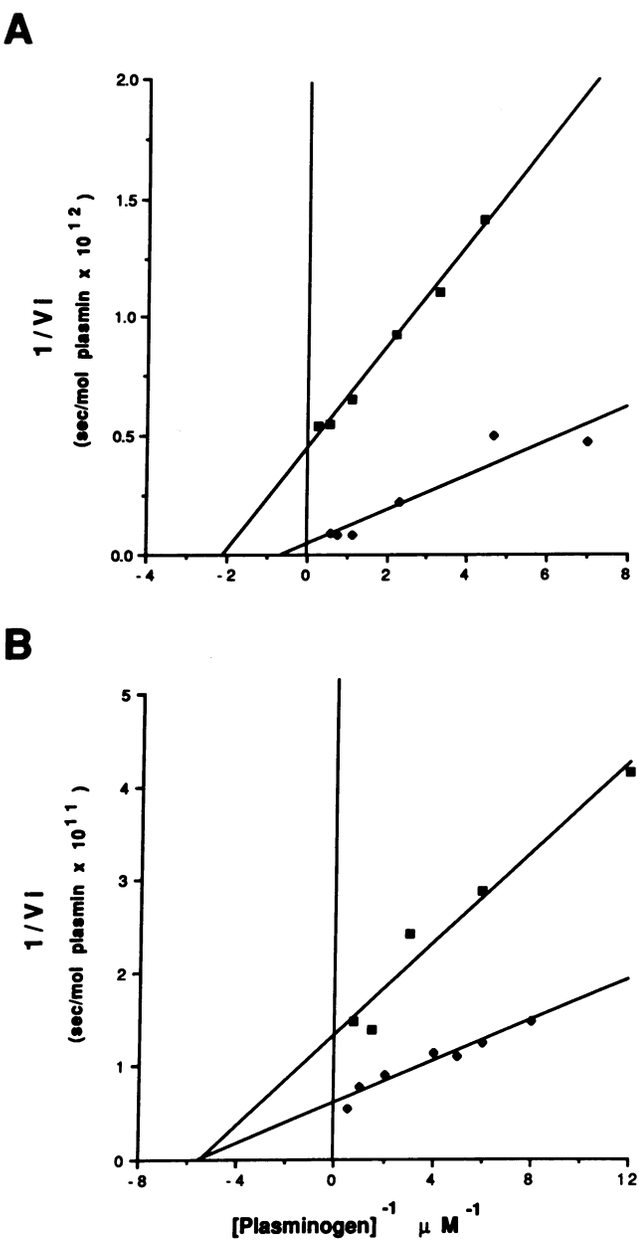

Figure 3. Double reciprocal kinetic plots of the activation of plasminogen glycoforms. *A* is derived from kinetic plots of neonatal plasminogen 1 (*open squares*) and neonatal plasminogen 2 (*solid diamonds*); *B*, kinetic plots of adult plasminogen 1 (*open squares*) and adult plasminogen 2 (*solid diamonds*).

Table III. Kinetic Constants of Activation of Plasminogen Glycoforms

Plasminogen	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	nM	$s^{-1} \cdot M^{-1}$
Adult 1	1.5±0.6	180±7.0	8.3×10^6
Adult 2	3.3±0.2	180±11.0	1.8×10^7
Neonate 1	0.44±0.004	490±2.0	9.0×10^5
Neonate 2	4.6±0.55	1,700±200	2.7×10^6

K_m more than double that of adult plasminogen 1. The neonatal plasminogen 1 k_{cat}/K_m ratio is one-ninth that of adult plasminogen 1. Neonatal plasminogen 2 has a k_{cat} almost one and a half times that of adult plasminogen 2, but it has a K_m value close to 10 times that of adult plasminogen 2. Endoglycosidase F treatment of neonatal plasminogen 1, which removed the *N*-linked carbohydrate, increased the k_{cat} of activation sixfold, while the K_m remained the same. The k_e, the catalytic rate constant, of the plasmin isoforms for hydrolysis of the S-2251 were comparable at 2×10^3 M/s per mol adult plasmin 1 and 2, and 2.3×10^3 M/s per mol neonate plasmin 2, but was 75% lower for neonate plasmin 1 at 620 M/s per mol plasmin.

Cell binding. Adult plasminogen 2 binds through its kringle domains to sites on U937 monocytoid cells to a considerably greater extent than adult plasminogen 1 (17). Neonatal plasminogen 2 also binds to the U937 cells, but more poorly than adult plasminogen 2. Analysis of the binding data of neonatal plasminogen 2 by the method of Cheng and Prusoff (34) demonstrates that the affinity of the neonatal plasminogen 2 was only one-third that of the adult plasminogen 2 (Fig. 4). Neonatal plasminogen 1 did not bind to U937 cells, as also demonstrated by these radiolabeled competition studies.

Discussion

Multiple clinical studies have found that neonatal fibrinolytic capacity is far lower than observed in adults. Phillips and Skrodelis (8) found that functional plasminogen levels in the full-term and premature neonates are only 25 and 12% of maternal levels, respectively. Several other studies (9–11) also found that neonatal plasminogen levels were only a fraction of adult values. Ambrus et al. (6) noted the decreased fibrinolytic capacity and proposed a link between the abnormal plasminogen and the neonatal disease hyaline membrane disease. Beller et al. (35) observed increased clot lysis time in studies with neonatal plasma, and Corrigan et al. (36) reported failure of urokinase to induce thrombolysis in a neonate. Estelles and co-workers (12) examined the plasminogen of the neonate and found that its amino acid composition differed from that of adult plasminogen.

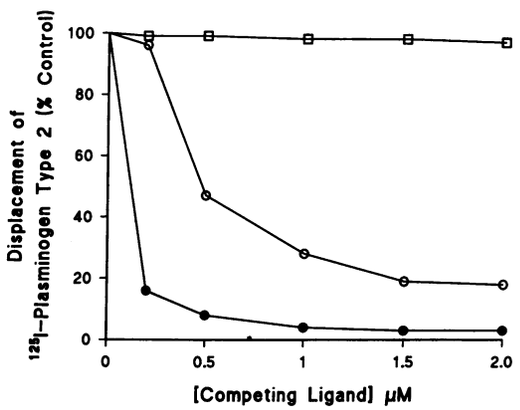

Figure 4. Inhibition of ^{125}I-adult plasminogen 2 binding to U937 cells by adult plasminogen 2 and neonatal plasminogen 2. ^{125}I–Adult plasminogen 2 (0.2 μM) and unlabeled adult plasminogen 2 (*solid circles*), unlabeled neonatal plasminogen 1 (*open squares*), and unlabeled neonatal plasminogen 2 (*open circles*) were incubated with 1×10^5 cells at 22°C for 30 min.

We have studied the two glycoforms of neonatal plasminogen and found that they differ from the adult forms in their extent in glycosylation, not primary structure. Adult and neonatal plasminogen and plasmin exhibited similar migration on SDS-PAGE, respectively, providing evidence of the similarity between the adult and neonatal molecules. In addition, amino acid composition of the plasminogen glycoforms is virtually identical, as has been noted in a previous study by Summaria (37). Moreover, the amino acid sequences of neonatal and adult plasminogen glycoforms appear to be identical. Amino-terminal sequences of 20 residues from the amino-terminal Glu residue as well as 20 residues following Lys-77 reveal identical sequences.

Though the primary structures of the adult and neonatal plasminogen glycoforms are identical, their isoelectric focusing distribution was markedly different. The adult plasminogen isoform pattern was similar to those found by Summaria and co-workers (32) and Robbins and Summaria (33). The isoform distribution of the adult plasminogen results from differences in carbohydrate composition (32, 33). Sialic acid content has been demonstrated to be responsible for differences in isoelectric focusing migration (38). The neonatal plasminogen 1 has fewer isoforms, and the neonatal plasminogen 2 isoforms have more acid migration compared with the adult glycoforms, which may be due to their differences in carbohydrate composition.

The primary difference between the glycoforms, both adult and neonatal, is in the carbohydrate composition. The carbohydrate composition and structure of the two adult glycoforms has been well characterized by Hayes and Castellino (14–16). In our studies, neonatal plasminogen 1 and 2, isolated from 60 full-term newborns, contained more mannose than the adult forms. Treatment of neonatal plasminogen 1 with endoglycosidase F revealed that > 90% of the mannose content was on the *N*-linked oligosaccharide.

N-Linked oligosaccharide is initially synthesized as a high mannose carbohydrate with as many as 12 mannose residues linked to each other or an *N*-acetylglucosamine, and is then processed to remove a majority of the mannose content (39, 40). The excess mannose content of the neonatal glycoforms could be due to incomplete processing. In addition, the difference in galactose, *N*-acetylglucosamine, and sialic acid composition may indicate that there are other glycosylation sites on the neonatal protein. The increased sialic acid content is consistent with our observations of a much more acidic migration of the neonatal plasminogen by isoelectric focusing gels. An earlier study using plasminogen isolated from two full-term newborns showed little difference in migration between the neonatal and adult plasminogens (37). However, in

these studies the two glycoforms were not separated, nor was carbohydrate analysis performed. It should be noted that in our study of plasminogen from 60 neonates, no heterogeneity was seen by isoelectric focusing. In particular, no adult forms of less acidic migration were noted.

The variation in the carbohydrate composition and structure may account for the differences in kinetic constants for activation of the adult and neonatal plasminogen glycoforms by tissue-type plasminogen activator. The retarded fibrinolysis in the neonate may be due to the slower activation kinetics of the neonatal plasminogen isoforms compared with the adult plasminogen. Comparison of the k_{cat}/K_m ratios obtained in the kinetic studies demonstrates that the activation of both neonatal glycoforms is slower than the adult plasminogen glycoforms. Neonatal plasminogen 1 is overall a poor substrate for activation compared with the adult glycoforms. Neonatal plasminogen 1 has a lower affinity for the tissue-type plasminogen activator–fibrin fragment complex, and is activated more slowly when it does bind. Moreover, endoglycosidase F treatment of neonatal plasminogen 1, which removes the mannose rich *N*-linked oligosaccharide, increases the activation rate sixfold, but has no effect on the affinity of the plasminogen for the activation complex. Neonatal plasminogen 2 activation differs markedly compared with the other isoforms. Neonatal plasminogen 2 has a much lower affinity for the activation complex than the other species, but it has the highest rate of turnover. These high values for both K_m and k_{cat} may indicate that the transition state of neonatal plasminogen 2 is unstable: the high K_m reflects the decreased probability of the molecule reaching the transition state, and the k_{cat} reflects the instability of the transition state as the plasminogen is converted to plasmin. Our data differ from those of Summaria (37), who showed similar activation for neonatal and adult plasminogen. However, because these studies used plasminogen from only two neonates, relative to the 60 in this study, it is likely that our data more closely reflect the neonatal plasminogen population. The differences in kinetic constants between the adult and neonatal plasminogen activation suggest a biochemical basis for the decreased fibrinolytic activity in the full-term newborn.

The decreased fibrinolytic activity in neonates may also be due to poor cell surface activation of plasminogen. We have recently demonstrated that adult plasminogen 2 preferentially binds to cell surface binding sites as compared with plasminogen 1 (17). This difference appears to result from the lower carbohydrate content of plasminogen 2. We have further probed this question with recombinant plasminogen expressed in *Escherichia coli* (41), and our data suggest that completely nonglycosylated plasminogen has an even greater binding affinity. The decreased affinity of neonatal plasminogen 2 that is hyperglycosylated is consistent with these observations. In the adult fibrinolytic system, activation of plasminogen bound to cellular binding sites results in a surface-regulated fibrinolytic system (18–20). Since neonatal plasminogen 2 binds poorly to cell surface binding sites, the amount of surface-bound plasminogen available for activation should be present at a much lower level in the neonate compared with the adult. This does not rule out the possibility of other binding sites in the neonatal vasculature to which the neonatal plasminogen may bind. However, this hypothesis appears unlikely since the human umbilical endothelial vein cell receptor for plasminogen appears to be similar to the receptor on U937 cells (18).

The differences in activation and binding properties of neonatal plasminogen relative to the adult molecule probably result from differences in carbohydrate composition. Similarly, neonatal fibrinogen contains an increased sialic acid content, which is associated with a decreased rate of fibrin polymerization (42, 43). Interestingly, a similar fibrinogen appears to be the prominent form in patients with acquired dysfibrinogenemia (44). Levels of glycosylation have been shown to play important roles in the activity of other proteinases. Studies of tissue-type plasminogen activator by both Wittwer et al. (45), and Parekh et al. (46) demonstrate that glycosylation influences the kinetics of fibrin-stimulated plasminogen activation. The level of glycosylation also influences the binding of antithrombin III to thrombin (47), and fibronectin to gelatin (48). The decreased overall activation of the neonatal plasminogen compared with the adult plasminogen may be important in understanding the lower level of fibrinolytic activity in the neonate.

Acknowledgments

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