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Inhibition of Blood Coagulation Factor $XIII_a$-Mediated Cross-Linking Between Fibronectin and Collagen by Polyamines

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Soluble fibronectin is found in body fluids and media of cultured adherent cells. Insoluble fibronectin is found in tissue stroma and in extracellular matrices of cultured cells. Fibronectin is a substrate for factor $XIII_a$ (plasma transglutaminase) and can be cross-linked to collagen and to the α chain of fibrin. We have used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to investigate the possibility that factor $XIII_a$ -mediated cross-linking is influenced by polyamines. Spermidine inhibited cross-linking between fibronectin and type I collagen, isolated α1 (I) collagen chains, or iodinated cyanogen bromide fragment 7 of α1 (I) chains (^{125}I-α1 (I)-CB7). Half-maximal inhibition of cross-linking between ^{125}I-α1 (I)-CB7 and fibronectin was observed when 0.1 mM spermine or spermidine was present. Spermidine, 0.7 mM, partially inhibited cross-linking between fibronectin and the α chain of fibrin but failed to inhibit cross-linking between the fibrin monomers of a fibrin clot. Spermidine also failed to inhibit cross-linking between fibronectin molecules when aggregation of fibronectin was induced with dithiothreitol. In contrast, 0.7 mM monodansylcadaverine inhibited fibronectin-collagen, fibronectin-fibrin, fibronectin-fibronectin, and fibrin-fibrin cross-linking. Spermidine or spermine, 0.7 mM, enhanced the cross-linking between molecules of partially amidinated fibronectin, suggesting that $N^{1,8}$-(di-γ-glutamyl)-polyamine cross-linkages were formed. Spermidine and spermine failed to enhance cross-linking between monomers of amidinated fibrin. These results indicate that physiologic concentrations of polyamines specifically disturb transglutaminase-catalyzed cross-linking between fibronectin and collagen.

Key words: fibronectin, factor XIII, transglutaminase, collagen, polyamine, ε(γ-glutamyl)-lysine

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Fibronectin is a glycopretein found in extracellular fluids, and connective tissues and associated with basement membranes (reviewed in Vaheri and Mosher [1] and Yamada and Olden [2]) and is present in the extracellular fibrillar matrix of substrate-attached cells in culture [3–6]. Collagen binds to fibronectin [7–10] and is a second component of the matrix [11, 12]. A specific region of the type I collagen α1 (I) chain, which is generated by cyanogen bromide cleavage and designated α1 (I)-CB7, comprises residues 552–822 and contains the principal binding site for fibronectin [9, 10].

Transglutaminases are a class of calcium ion-dependent enzymes that catalyze an acyl transfer reaction in which γ-carboxamide groups of peptide-bound glutaminyl residues are acyl donors and a variety of primary amines, including the -ε-amino groups of peptide-bound lysyl residues, are acyl acceptors. By this reaction, transglutaminases catalyze the formation of ε-(γ-glutamyl)-lysine linkages between proteins. As recently reviewed by Folk and Finlayson, transglutaminases are widely distributed and are throught to catalyse biologically important cross-linking of fibrin, hair proteins, keratin, and proteins of seminal fluid [13]. Fibronectin is a substrate for transglutaminases of plasma [14, 15] and liver [16] and can be cross-linked to itself [14, 16], fibrin [14, 17, 18], and collagen [19]. Fibronectin in the extracellular fibrillar matrix of cultured fibroblasts can be extensively cross-linked by exogenous plasma transglutaminase [20].

Polyamines are widely distributed and are thought to play important roles in cell proliferation and other biologic processes (reviewed in Tabor and Tabor [21] and Jänne et al [22]). These compounds can serve as acyl acceptors for liver [23, 24] and plasma [24] transglutaminase. Polyamines also dissociated fibronectin from gelatin [25]. In the present paper, we report that polyamines specifically inhibit transglutaminase-catalyzed cross-linking between collagen and fibronectin.

METHODS

Materials

Bolton Hunter reagent (1,500 Ci/mmole) was from New England Nuclear, Coomassie brilliant blue R-250 was from BioRad Laboratories, spermine and spermidine were from Sigma Chemicals, and human thrombin was a generous gift from Dr. John Fenton II, New York State Department of Health.

Purification of Proteins and Protein Fragments

Human fibrinogen, human factor XIII, human plasma fibronectin, lathyritic rat type I collagen, calf skin type III collagen, and cyanogen bromide peptides of α1 (I) chains of calf skin type I collagen were prepared as described elsewhere [19].

Radiolabeling of α1 (I)-CB7

Cyanogen bromide fragment 7 of bovine α1 (I) chains [α1 (I)-CB7] was reacted with Bolton Hunter reagent as described elsewhere [19]. Approximately 10^{-2} moles of ^{125}I-reagent was incorporated per mole of α1 (I)-CB7.

Amidination of Proteins

Fibronectin and fibrinogen in 20 mM sodium borate, 0.15 M sodium chloride, pH 8.3, were incubated for 2 h at 0° with 0.33 M and 0.28 M ethyl acetimidate, respectively. Unreacted reagent was removed by extensive dialysis. Approximately 84% of the lysyl residues in fibronectin and 99% of the lysyl residues in fibrinogen were modified, as assessed by spectroscopy after reaction of the modified proteins with trinitrobenzene sulfonate [26].

Cross-Linking Reactions

Factor $XIII_a$-mediated cross-linking was performed in 10 mM Tris, 140 mM sodium chloride, pH 7.4 (Tris-buffered saline), containing the indicated amount of calcium chloride. Concentrated stock solutions of the polyamines were made in Tris-buffered saline and titrated to pH 7.4 prior to addition. Proteins to be added were in Tris-buffered saline, with the exception of type I and type III collagen. Prior to being added to the incubations, the collagens were either in 5 mM acetic acid or in 10 mM Tris, 400 mM sodium chloride, pH 7.4. Solutions of Tris and sodium chloride were added along with the collagens in order to make the final ionic strengths and hydrogen ion concentrations constant. The incubations were terminated by addition of an equal volume of 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, followed by heating for 3 min at 95°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed in cylindrical gels or discontinuous slab gels as described previously [19]. After staining with Coomassie brilliant blue R-250, collagen bands were metachromatic [27] and could be identified by their red color. Slabs containing ^{125}I-proteins were dried and analyzed by autoradiography. Inhibition of cross-linking by polyamines was quantitated by analysis of densitometry tracings of autoradiograms:

$$\% \text{ Inhibition} = 100 - 100X$$

$$X = \frac{\text{Peak heights of 2.2 and } > 4 \times 10^5 \text{ molecular weight bands in presence of inhibitor}}{\text{Peak heights of all bands in presence of inhibitor}} \Big/ \frac{\text{Peak heights of 2.2 and } > 4 \times 10^5 \text{ molecular weight bands in absence of inhibitor}}{\text{Peak heights of all bands in absence of inhibitor}}$$

RESULTS

Fibronectin could be cross-linked to isolated α1 (I) collagen chains at 22° or 37° and to native type I collagen at 37° (Fig. 1). Complexes with apparent molecular weights of 3.3, 5.0, and $> 10 \times 10^5$ were formed. These complexes were not formed if factor XIII, thrombin, calcium ion, fibronectin, or collagen was omitted from the reaction mixture [19]. Inclusion of 1 mM spermidine in the reaction mixture completely inhibited formation of the complexes (Fig. 1).

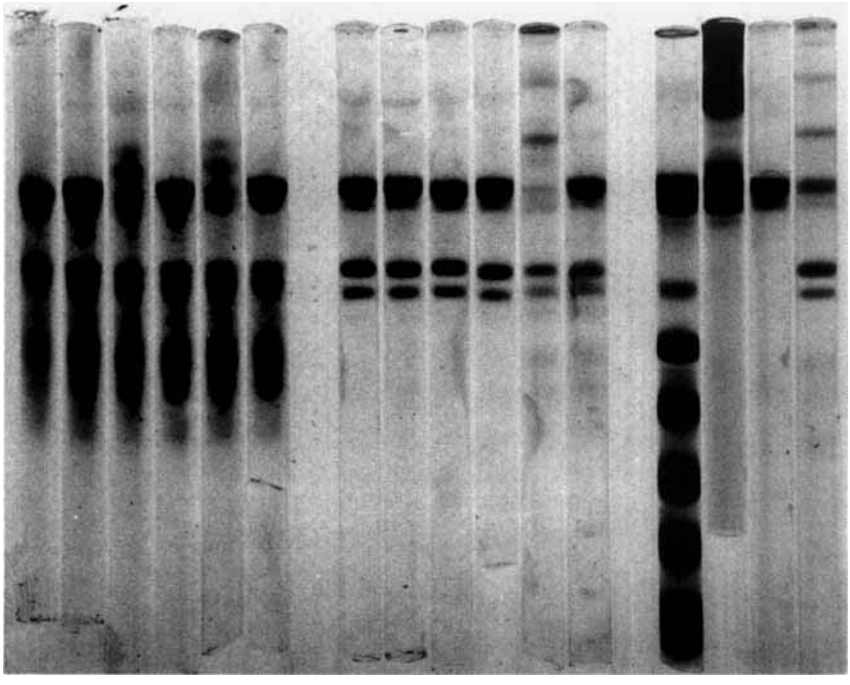

Fig. 1. Inhibition by spermidine of factor $XIII_a$-mediated cross-linking between fibronectin and α1 (I) collagen chains or fibronectin and type I collagen. The reduced products of 13 different incubations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 4% cylindrical gels. All incubations contained fibronectin, 270 μg/ml; factor XIII, 25 μg/ml; thrombin 1 unit/ml; and 10 mM calcium ion. Incubations 1–6 contained α1 (I) collagen chains, 1 mg/ml; incubations 7–12 contained type I collagen, 400 μg/ml. Even-numbered incubations contained 1 mM spermidine. Incubations were for 2 h at the following temperatures: incubations 1, 2, 7, and 8 at 0°; incubations 3, 4, 9, and 10 at 22°; and incubations 5, 6, 11, and 12 at 37°. Gel 13 contains the following reduced molecular-weight markers: fibronectin, 2.0×10^5; phosphorylase, 9.3×10^4; albumin, 6.8×10^4; ovalbumin, 4.3×10^4; chymotrypsinogen, 2.45×10^4; and hemoglobin, 1.65×10^4. Gel 14 contains nonreduced fibronectin; the major band has a nominal molecular weight of 4.0×10^5. Gel 15 contains the products of an incubation in which fibronectin and cross-linking reagents but no collagen were present. Gel 16 contains products of the same incubation as gel 11; the incubation was sampled at 5 min rather than 2 h.

Fibronectin also could be cross-linked to ^{125}I-α1(I)-CB7, which is known to contain a binding site for fibronectin [9, 10] (Fig. 2). Cross-linking was not observed if factor XIII, thrombin, calcium ion, or fibronectin was omitted from the reaction mixture. Cross-linking was partially inhibited by 1–100 μM spermine or spermidine (Fig. 2) and completely inhibited by 1 mM spermine or spermidine (data not shown). Half-inhibition was observed when these polyamines were present at concentrations of approximately 100 μM.

Spermidine, 0.7 mM, inhibitied fibronectin-collagen and fibronectin-fibrin but not fibronectin-fibronectin and fibrin-fibrin cross-linking (Fig. 3). As shown in gels 1–4, fibronectin can be cross-linked to itself in the presence of dithiothreitol [14] to form dimers of 4.0×10^5 MW and higher-molecular-weight oligomers. Formation of oligomers and loss of staining from the 2.0×10^5 MW monomer band were inhibited by monodansylcadaverine but not by spermidine. As shown in gels 5–8 of Figure 3, both spermidine and monodansylcadaverine inhibited the cross-linking of fibronectin and α1 (I) collagen chains. Gels 9–12

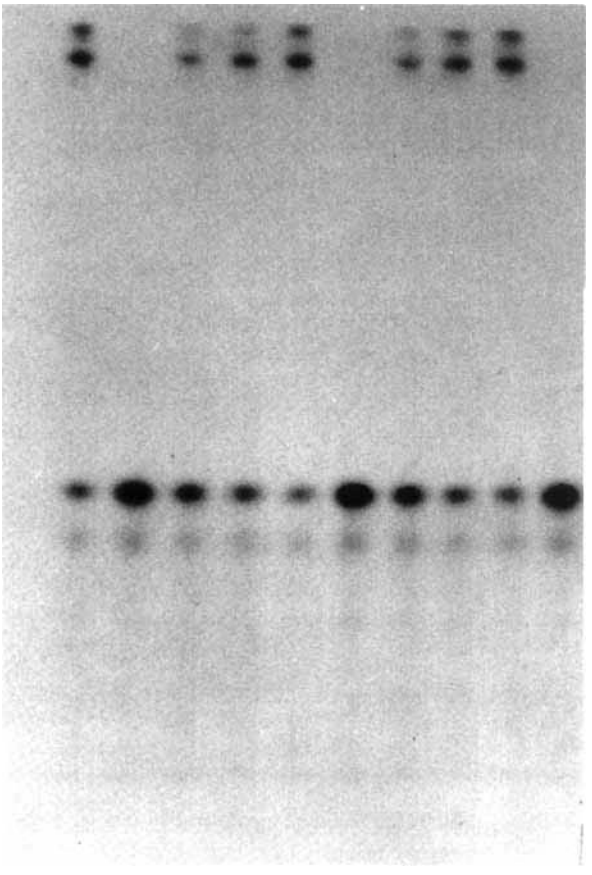

Fig. 2. Inhibition of factor $XIII_a$-mediated cross-linking between fibronectin and ^{125}I-α1 (I)-CB7 of α1 (I) collagen chains by spermine and spermidine. The reduced products of ten different incubations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through a 3% /8% discontinuous slab gel. The slab was analyzed by autoradiography. All incubations contained fibronectin, 270 μg/ml, and ^{125}I-α1 (I)-CB7, 3 μg/ml. Incubations 1, 3–5, and 7–9 contained 10 mM calcium ion; factor XIII, 25 μg/ml; and thrombin, 1 unit/ml. Incubations 3, 4, and 5 contained 100 μM, 10 μM, and 1 μM spermine, respectively; incubations 7, 8, and 9 contained 100 μM, 10 μM, and 1 μM spermidine, respectively. Incubations 2, 6, and 10 contained 10 mM ethylenediaminetetraacetic acid (EDTA) instead of calcium and no factor XIII or thrombin. Incubations were for 2 h at 37°C. Approximately 5×10^3 CPM were added to each slot of the gel. Apparent molecular weights of the non-cross-linked ^{125}I-α1 (I)-CB7 bands (narrow arrows) are 3.3×10^4 and 2.9×10^4; apparent molecular weights of cross-linked ^{125}I-α1(I)-CB7 (broad arrows) are 2.2 and $> 4.0 \times 10^5$.

of Figure 3 show that formation of 2.6 and 3.0×10^5 MW complexes, which consist of fibronectin and the α chains of fibrin [14, 18], and loss of staining from the band representing fibronectin monomer were partially inhibited by spermidine and more completely inhibited by monodansylcadaverine. Gels 9–16 show that formation of the cross-linked 9.3×10^4 MW dimer of the γ-chains of fibrin, loss of staining from the 4.7×10^4 MW γ-chain band, formation of the various cross-linked multimers of the α chains of fibrin, and loss of staining from the 7.2×10^4 MW α-chain band were inhibited by monodansylcadaverine but not by spermidine.

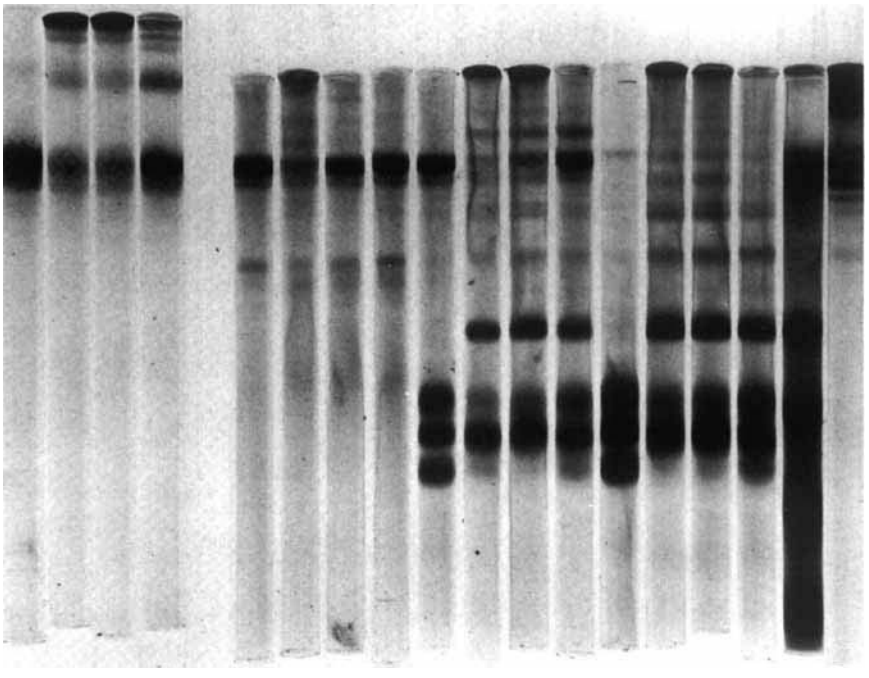

Fig. 3. Inhibition of factor $XIII_a$-mediated fibronectin-collagen and fibronectin-fibrin but not fibronectin-fibronectin or fibrin-fibrin cross-linking by spermidine. The reduced products of 16 different incubations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 4% (gel 1–4) or 5% (gels 5–18) cylindrical gels. Incubations 1–4 contained fibronectin, 710 μg/ml, and 10 mM dithiothreitol; incubations 5–8 contained fibronectin, 270 μg/ml, and isolated α1 (I) collagen chains, 250 μg/ml; incubations 9–12 contained fibronectin, 270 μg/ml, and fibrinogen, 730 μg/ml; and incubations 13–16 contained fibrinogen, 1.4 mg/ml. Incubations 2–4, 6–8, 10–12, and 14–16 contained factor XIII, 25 μg/ml; thrombin, 1 unit/ml; and 10 mM calcium ion. Incubations 1, 5, 9, and 13 contained 10 mM EDTA instead of calcium ion and no factor XIII or thrombin. Incubations 3, 7, 11, and 15 contained 0.67 mM spermidine; incubations 4, 8, 12, and 16 contained 0.63 mM monodansylcadaverine. Incubations 1–8 were for 2 h at 37°C; incubations 9–16 were for 2 h at 0°. Gels 17 and 18 contained reduced size markers and nonreduced fibronectin, respectively (see Fig. 1). Fibronectin-fibrin cross-linking was studied at 0° rather than 37° because fibrin α-chain polymer formation is less marked at the lower temperature [14].

Spermidine could enhance cross-linking between peptide chains by means of a transfer reaction between the carboxamide group of a glutaminyl residue in each chain and both primary amino groups of the polyamine [24]. To examine this possibility, we followed the lead of Schrode and Folk [24] and attempted to cross-link amidinated fibronectin and fibrin in the presence and absence of polyamines (Fig. 4). Modification of lysyl residues in fibrinogen was virtually complete, yielding a product that could not be cross-linked (Fig. 4, slot 7). Lysyl residues in fibronectin were not completely modified and the amidinated fibronectin could be partially cross-linked in the presence of dithiothreitol (Fig. 4, slot 2). Spermine or spermidine enhanced cross-linking of amidinated fibronectin (Fig. 4, slots 3 and 4) but not of amidinated fibrin (Fig. 4, slots 8 and 9). Spermine or spermidine enhanced cross-linking between amidinated fibronectin and amidinated fibrin (Fig. 4, slots 13 and 14).

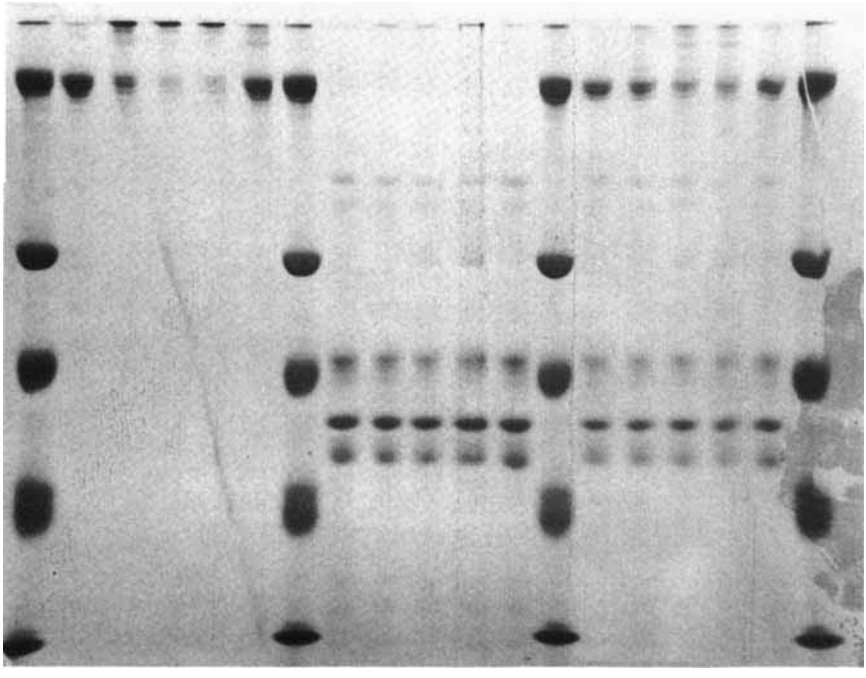

Fig. 4. Effects of spermidine, spermine, and monodansylcadaverine on factor $XIII_a$-mediated cross-linking of amidinated fibronectin and fibrin. The reduced products of 15 different incubations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through a 3%/8% slab gel. Incubations contained factor XIII, 25 μg/ml; thrombin, 1 unit/ml; and 10 mM calcium ion. Incubations 1–5 contained amidinated fibronectin, 720 μg/ml, and 10 mM dithiothreitol. Incubations 6–10 contained amidinated fibrinogen, 1.2 mg/ml. Incubations 11–15 contained amidinated fibronectin, 310 μg/ml, and amidinated fibrinogen, 610 μg/ml. Incubations 1, 6, and 11 contained 10 mM EDTA instead of calcium ion. Incubations 3, 8, and 13 contained 0.67 mM spermidine; incubations 4, 9, and 14 contained 0.67 mM spermine; and incubations 5, 10, and 15 contained 0.63 mM monodansylcadaverine. Incubations 1–5 were at 37°C for 2 h. Incubations 6–15 were at 0° for 2 h. Molecular-weight markers (see Fig. 1) were analyzed on every sixth slot.

DISCUSSION

Vuento and Vaheri demonstrated that polyamines bind to fibronectin during gel filtration and can be used to dissociate fibronectin from insolubilized gelatin [25]. The present experiments demonstrate that polyamines inhibit factor $XIII_a$-mediated cross-linking between fibronectin and collagen. The same concentrations of polyamines had little effect on factor $XIII_a$-mediated cross-linking among fibrin molecules and enhanced factor $XIII_a$-mediated cross-linking among molecules of amidinated fibronectin. Cross-linking of amidinated fibronectin presumably is enhanced by formation of $N^{1,n}$-(di-γ-glutamyl)-polyamine cross-linkages, as described by Schrode and Folk [24]. Monodansylcadaverine, which is the best-known inhibitor of factor $XIII_a$-mediated fibrin-fibrin cross-linking [28, 29], also inhibited fibronectin-fibrin, fibronectin-fibronectin, and fibronectin-collagen cross-linking. The results suggest that polyamines specifically interact with the fibronectinyl-factor $XIII_a$ acyl-enzyme intermediate [30–32] and compete with lysyl residues of

collagen as acyl acceptors in factor $XIII_a$ -mediated cross-linking. In contrast, monodansylcadaverine apparently reacts with both the fibrinyl-factor $XIII_a$ and fibronectinyl-factor $XIII_a$ acyl-enzyme intermediates and is a more general inhibitor of factors $XIII_a$ -mediated cross-linking.

Previous studies suggest that fibrin and collagen compete for the same binding site on fibronectin. Loss of fibronectin into the fibrin clot is less when denatured collagen is present during clotting [7], and α1 (I)-CB7 inhibits cross-linking of the α chain of fibrin to fibronectin [19]. Conversely, fibrinogen inhibits the binding of fibronectin to denatured collagen [7], and fibrin inhibits the cross-linking of ^{125}I-α1 (I)-CB7 to fibronectin [19]. In our experiments, polyamines had a dual effect on fibronectin-fibrin cross-linking. Spermidine and spermine enhanced cross-linking of amidinated fibronectin and amidinated fibrin (Fig. 4), presumably by being incorporated into $N^{1,n}$-(di-γ-glutamyl)-polyamine cross-linkages. However, spermidine partially inhibited cross-linking between unmodified fibronectin and unmodified fibrin (Fig. 3). The α chain of fibrin contains glutaminyl residues which are susceptible to the action of factor $XIII_a$ [13], whereas we have been unable to demonstrate susceptible glutaminyl residues in collagen (unpublished results). We suspect that polyamines compete as acyl acceptors in factor $XIII_a$-mediated cross-linking between fibronectin and fibrin just as between fibronectin and collagen. However, the competition may be partially masked when assayed by polyacrylamide gel electrophoresis because of formation of $N^{1,n}$-(di-γ-glutamyl)-polyamine cross-linkages between reactive glutaminyl residues in fibronectin and reactive glutaminyl residues in fibrin.

Further experiments are needed to determine the significance of the present findings. The concentrations of polyamines and their biosynthetic enzymes increase when enkaryotic cells proliferate [21, 22]. Both tissue transglutaminases and the plasma enzyme are widely distributed [13]. One can think of a number of ways in which the formation of the extracellular fibronectin-collagen matrix could be influenced by polyamines. For instance, transglutaminase-catalyzed incorporation of a polyamine into fibronectin might make fibronectin incapable of associating with collagen. Conversely, polyamines could disrupt noncovalent associations between fibronectin and collagen [25], but not cross-linked fibronectin-collagen complexes. To test such hypotheses, it will be necessary to characterize the biochemistry of the fibronectin-collagen and fibronectin-polyamine interactions in more detail and to correlate biochemical studies with events which take place in cultures of proliferating cells and in proliferating tissues.

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