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THE COMPARATIVE ABILITY OF PLASMA AND TISSUE TRANSGLUTAMINASES TO USE COLLAGEN AS A SUBSTRATE

MAGDALENA M. JELEŃSKA [a], LESZLO FESÜS [b] and MARIA KOPEĆ [a]

[a] *Department of Radiobiology and Health Protection, Institute of Nuclear Research, 03-195 Warsaw, Dorodna 16 (Poland) and* [b] *Department of Clinical Chemistry, University School of Medicine, P.O.B. 40, Debrecen H-4012 (Hungary)*

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Summary

Heat denatured type I and type III calf skin collagen were found to be substrates for guinea pig liver transglutaminase (R-glutaminyl-peptide:amine γ-glutamyl-yltransferase, EC 2.3.2.13) but not for active plasma factor XIII (factor XIIIa). Liver transglutaminase was shown to catalyse incorporation of ^{14}C-putrescine into subunits of denatured collagen of both types, cross-linking of the latter into high molecular weight polymers and their co-cross-linking to fibrin and fibrinogen. Factor XIIIa is inactive in these respects. None of these reactions was catalysed by liver transglutaminase and plasma factor XIIIa when nondenatured collagens both soluble or in the forms of reconstituted fibrils served as substrates. Some cross-linking of cleavage products of collagen type I (obtained by treatment with collagenase from human neutrophiles) was induced by liver transglutaminase and factor XIIIa. The results indicate that although appropriate glutamine and lysine residues for a ε-(γ-glutamine) lysine cross-linked formation are present in collagen, the native conformation of collagen prevents the action of liver transglutaminase and factor XIIIa.

Introduction

The active plasma coagulation factor XIII (plasma transglutaminase, factor XIIIa), as well as tissue transglutaminase (R-glutaminyl-peptide:amine γ-glutamyl-yltransferase, EC 2.3.2.13) catalyse a calcium-dependent acyl-transfer reaction between γ-carboxyamide group of a peptide-bound glutamine residue and various primary amines [1]. When peptide-bound lysine serves as an acyl acceptor inter- or intramolecular cross-links are formed in proteins [2]. Cross-linking

of fibrin was most intensively investigated in this respect. The transglutaminases are widespread in living organisms and several other important biological reactions have been shown to be catalysed by them [2]. The involvement of factor XIIIa in the proliferation of granulation tissue during wound healing [3,4] and the organization occurring in thrombi attached to vessel walls [5,6] have implicated the formation of ϵ-(γ-glutamyl) lysine cross-links in collagen. However, the experimental results published so far are conflicting. In 1975 Soria et al. [7] reported that the presence of placental factor XIIIa in the incubation mixture diminished the proportion of monomeric α-chains in the electrophoretic pattern of collagen and impaired its polymerization. These results were interpreted as indirect evidence for plasma factor XIIIa-mediated cross-linking of collagen subunits. The cross-links could block the sites engaged in noncovalent interactions necessary for collagen polymerization. At the same time Duckert and Nyman [8] reported that collagen can be bound to fibrin due to an active factor XIII-mediated reaction. These authors found an increased amount of ^{125}I-labelled fibrinogen radioactivity bound to collagen in a system containing plasma factor XIII, Ca^{2+} and thrombin, as compared to controls lacking any of these components. The absence of fibrin γ-chain dimers in the electrophoretic pattern of washed, reduced clots formed from plasma with normal factor XIII level to which Ca^{2+} and collagen had been added, was considered as an additional argument for covalent fibrin-collagen binding. However, Hörmann and Stemberger [9] using type I and type III collagen could not confirm either cross-linking of collagen alone or its coupling to fibrin under the influence of factor XIIIa. They also found that monodansylcadaverine was not incorporated into collagen. A diminished formation of fibrin γ-chain dimers in the presence of collagen fibrils could, however, be demonstrated by these authors [9].

The above detailed discrepancies prompted us to re-investigate whether type I and type III collagen and the products of their cleavage, by mammalian collagenase, can serve as substrates for transglutaminases, namely for bovine factor XIIIa and for the tissue enzyme of guinea pig liver. Part of this work has been already reported [10].

Materials and Methods

Type I and type III collagens were isolated from calf skin and purified according to Fuji and Kühn [11]. Soluble native preparations, reconstituted fibrils [12] and denatured samples (60°C, 1 h) of collagen were used in the experiments. Collagen was labelled with ^{125}I using the Bolton and Hunter reagent [13], purchased from the Radiochemical Centre Amersham, U.K. (1.3 mCi/ml). Digestion products of type I collagen were prepared by its treatment with human neutrophile collagenase at the enzyme to substrate protein ratio 1 : 100. Purified collagenase [14] was kindly supplied by Dr. Wojtecka-Łukasik (Institute of Rheumatology, Warsaw). Before use, samples of collagen or its digestion products were dialyzed into 20 mM Tris-HCl (pH 7.5)/10 mM $CaCl_2$/ 1 mM EDTA.

Fibrinogen, free of factor XIII (96—98% clottable), and factor XIII were prepared from bovine plasma [15,17]. Human thrombin (300 U/ml) was a

product of Hoffman La Roche. Guinea pig liver transglutaminase was purified according to the procedure of Connelan et al. [17]. The activity of the enzyme used, determined by the hydroxamate assay [17], was 10.4 U/mg protein.

[^{14}C]Putrescine (60 μCi/μmol) was purchased from the Radiochemical Centre Amersham (U.K.).

Effects of of plasma factor XIIIa and liver transglutaminase on nonradioactive collagen and its digestion products or ^{125}I-labelled collagen were investigated in a system containing: 100 μl collagen (3 mg/ml) or 150 μl digested collagen (2 mg/ml), 5 μl factor XIII or liver transglutaminase and 5 μl 20 mM dithiothreitol. Factor XIII was activated by addition of 3 U thrombin (10 μl) into the incubation system. In some experiments 10 μl fibrinogen (21 mg/ml), or [^{14}C]putrescine (5 μl, 2.5 μCi) were also included. Preliminary experiments on collagen cross-linking dependence on time of incubation with tissue transglutaminase indicated that a plateau is reached after 30 min at 37°C. We actually chose a 1 h incubation time for further experiments. The reaction was terminated by the addition of 10 μl 200 mM EDTA. To control samples EDTA was added before incubation.

The influence of plasma factor XIIIa or liver transglutaminase on the retention of denatured ^{125}I-labelled collagen in fibrin clots, was studied as follows: 50 μl fibrinogen (21 mg/ml), 25 μl factor XIII or liver transglutaminase and 25 μl thrombin were added to 400 μl ^{125}I-labelled collagen (3 mg/ml). The reaction was allowed to proceed at 37°C for 30 min. The clot was isolated and washed on a nylon cloth. The radioactivity of the fibrin film and of the filtrate were separately measured. In some experiments denatured ^{125}I-labelled collagen was cross-linked by liver transglutaminase (as described above) prior to addition to the incubation system.

Disc polyacrylamide gel (4% or 7% with or without 2.5% spacer) electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS) according to Clark [18]. Prior to electrophoresis, samples were denatured at 100°C for 3 min in the presence of 4 M urea/1% SDS/1% β-mercaptoethanol. Quick Scan apparatus (Helena Lab.) was used for densitometric tracing of the stained gels. Duplicate gels were sliced and solubilized in Soluene-350 tissue solubilizer (Packard), and the ^{14}C-radioactivity was measured in a Packard liquid scintillation counter type 3320. The ^{125}I-radioactivity was measured in a γ-counter; well type NaI crystal detector with NK 350 scaler (Gamma).

Results

Incubation with plasma factor XIIIa does not alter the electrophoretic pattern of native, as well as denatured type I and III collagen (Fig. 1). As shown in Fig. 2 the pattern of native type I and III collagen also remains unchanged after incubation with the liver transglutaminase. In contrast, when denatured type I and III collagen were incubated with liver transglutaminase the α-, β- and γ-subunits diminished, while the formation of high-molecular weight polymers could be observed (Fig. 2). The decrease in the amount of α-chains was the most pronounced, indicating that mainly collagen subunits contribute to the formation of polymers during liver transglutaminase action. It should be noted, however, that the polymer bands may consist not only of cross-linked collagen but also,

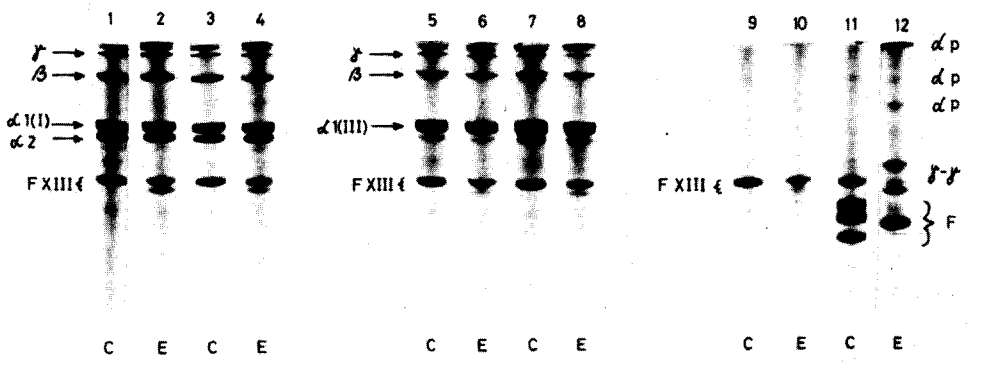

Fig. 1. Electrophoretic pattern of type I and type III collagen after incubation with plasma factor XIIIa. 100 μg factor XIIIa were incubated with 300 μg collagen samples in the presence of 3 U thrombin and 2.4 mM of dithiothreitol at 37°C, for 1 h in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA/10 mM $CaCl_2$. The reaction was stopped by adding 10 μl 200 mM EDTA, then equal vols. of 2% SDS and 2% mercaptoethanol in 8 M urea. After heating (at 100°C for 3 min) 40 μl was analysed by electrophoresis on 7% polyacrylamide gels. Native type I collagen (gels 1, 2) and denatured (60°C, 1 h) type I collagen (gels 3, 4) after incubation with factor XIIIa. Native type III collagen (gels 5, 6) and denatured (60°C, 1 h) type III collagen (gels 7, 8) after incubation with factor XIIIa. Factor XIII incubated alone (gels 9, 10) or with 300 μg fibrinogen (gels 11, 12) in the system described above. C: control, EDTA added before incubation with factor XIIIa. E: active enzyme, EDTA added after incubation with factor XIIIa. α1(I), α2, α1(III), β, γ are subunits of collagen. F: fibrinogen α, β, γ chains, γ-γ(dimers of fibrin γ-chains), αp (polymers of fibrin α-chain).

in part, of the polymerized liver transglutaminase.

Values calculated from the densitometric tracing of the stained gels (Table I), indicate that polymers could not be found in control samples but

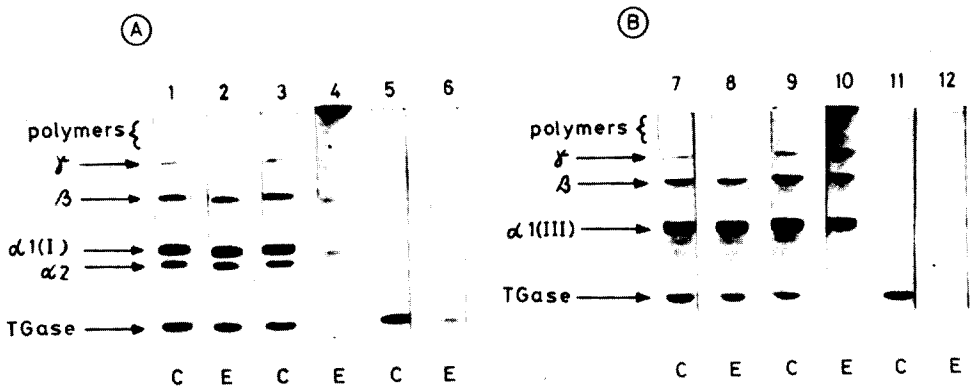

Fig. 2. Electrophoresis pattern of type I (A) and type III (B) collagen after incubation with liver transglutaminase. 40 μg liver transglutaminase were incubated with 300 μg collagen and 2.4 mM dithiothreitol at 37°C, for 1 h in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA/10 mM $CaCl_2$. Handling of samples: see Fig. 1. 40 μl samples were analysed by electrophoresis on 4% polyacrylamide gels. Native type I collagen (gels 1, 2) and denatured (60°C, 1 h) type I collagen (gels 3, 4) after incubation with liver transglutaminase. Native type III collagens (gels 6, 7) and denatured (60°C, 1 h) type III collagen (gels 8, 9) after incubation with liver transglutaminase. Gels 5, 10: liver transglutaminase alone. C: control, EDTA added before incubation with liver transglutaminase. E: active enzyme, EDTA added after incubation with liver transglutaminase. α1(I), α2, α1(III), β, γ are subunits of collagen.

TABLE I

EFFECTS OF LIVER TRANSGLUTAMINASE ON ELECTROPHORETIC DISTRIBUTION OF DENATURED TYPE I AND TYPE III COLLAGEN SUBUNITS

Percentages of total protein recovered in gels particular fractions calculated from densitometric tracing. Experimental conditions are as described in Fig. 2.

Fractions	Type I collagen		Type III collagen	
	control	liver transglutaminase	control	liver transglutaminase
α	59.4	26.4	79.8	39.3
β	26.6	14.8	16.1	19.4
γ	9.7	8.2	3.4	14.1
polymers	4.3	50.6	0.7	27.1

amounted to 50% (type I) and 27% (type III) of total protein in denatured collagen samples incubated with liver transglutaminase. These values were obtained at an enzyme/collagen protein ratio of about 1 to 10 and were the same irrespective of the presence or absence of the applied concentration of [^{14}C]-putrescine (data not shown in Table I). At lower concentrations of the enzyme, cross-linking of collagen was also observed: at the enzyme to substrate ratios of 1 to 30 or 1 to 60, polymers of type I collagen amounted to 25 and 15%,

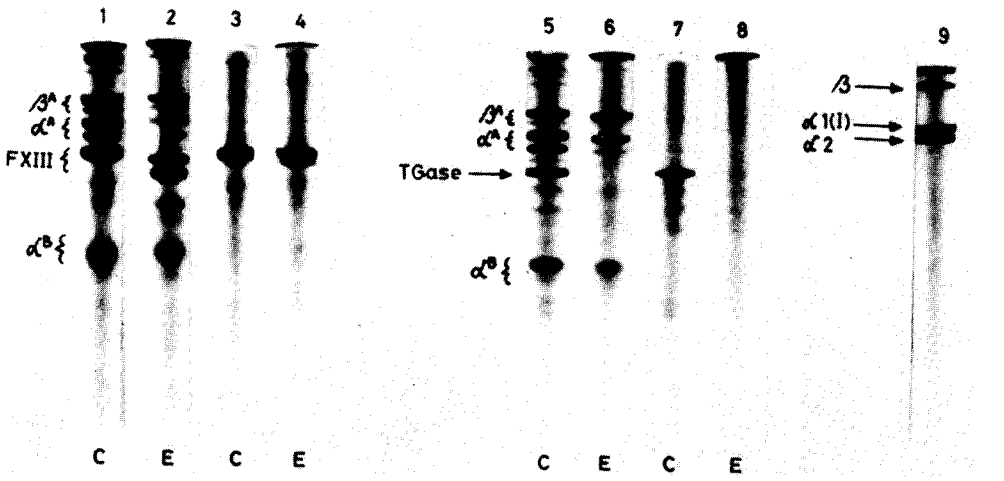

Fig. 3. Electrophoretic pattern of products of cleavage of type I collagen by human neutrophile collagenase, after incubation with plasma factor XIIIa or liver transglutaminase. 70 μg factor XIIIa were incubated with 200 μg cleavage products of collagen in the presence of 3 U thrombin and 2.4 mM dithiothreitol at 37°C, for 1 h in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA/10 mM $CaCl_2$. 25 μg liver transglutaminase were incubated with 200 μg collagen cleavage products and 2.4 mM dithiothreitol at 37°C for 1 h in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA/10 mM $CaCl_2$. Handling of the samples: see Fig. 1. 60 μl samples were analysed by electrophoresis on 7% polyacrylamide gels. Collagen cleavage products after incubation with factor XIIIa (gels 1, 2), factor XIIIa alone (gels 3, 4). Cleavage products (gels 5, 6) after incubation with liver transglutaminase. Gels 7. 8: liver transglutaminase alone. Gel 9, native collagen type I. C: control, EDTA added before incubation with the enzyme. E: active enzyme, EDTA added after incubation with the enzyme. Nomenclature of subunits of collagen cleavage products is given according to Sakai and Gross [20]. α1(I), α2, β are subunits of collagen.

TABLE II

DISTRIBUTION OF [^{14}C]PUTRESCINE IN SUBUNITS OF TYPE I AND TYPE III COLLAGEN AFTER INCUBATION WITH PLASMA FACTOR XIIIa OR LIVER TRANSGLUTAMINASE

Collagen samples (300 μg) were incubated in the presence of [^{14}C]putrescine (2.5 μCi) with 40 μg liver transglutaminase or 100 μg factor XIIIa and 2.4 mM of dithiothreitol in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA/10 mM $CaCl_2$ at 37°C for 1 h. Samples incubated with factor XIIIa contained in addition 3 U thrombin. After incubation and denaturation (see Fig. 1) 40 μl samples were resolved electrophoretically on 4% polyacrylamide gels. Stained gels were sliced and radioactivity measured in a liquid scintillation counter. A and B; the results were expressed in cpm. Values presented as percentage of total activity put on the gel are given in parentheses. C; the results are expressed in percentages of the total radioactivity put on the gel (0.317 μCi). Negative results obtained with native collagen incubated with factor XIII or factor XIIIa were omitted to make this table clearer.

Collagen	Enzyme	Radio-activity put on the gel (cpm)	[^{14}C]putrescine in type I collagen fractions (cpm)				
			α	β	γ	polymers	total
A							
Native	Liver transglutaminase inactive (EDTA)	1 643 750 (100)	822 (0.05)	493 (0.03)	328 (0.02)	986 (0.06)	2627 (0.16)
Native	Liver transglutaminase active (Ca^{2+})	1 603 820 (100)	4491 (0.28)	2406 (0.15)	2406 (0.15)	1603 (0.10)	10 906 (0.68)
Denatured	Factor XIIIa inactive (EDTA)	1 691 205 (100)	3721 (0.22)	1522 (0.09)	1184 (0.07)	1522 (0.09)	7948 (0.47)
Denatured	Factor XIIIa active (Ca^{2+})	1 610 300 (100)	3543 (0.22)	1771 (0.11)	1610 (0.10)	1722 (0.11)	8646 (0.54)
Denatured	Liver transglutaminase inactive (EDTA)	1 621 080 (100)	3242 (0.20)	1135 (0.07)	810 (0.05)	1945 (0.12)	7132 (0.44)
Denatured	Liver transglutaminase active (Ca^{2+})	1 619 010 (100)	51 808 (3.20)	15 704 (0.97)	7933 (0.49)	137 615 (8.50)	213 160 (13.16)
			[^{14}C]putrescine in type III collagen fractions				
B							
Native	Liver transglutaminase inactive (EDTA)	1 690 005 (100)	1183 (0.07)	507 (0.03)	507 (0.03)	508 (0.03)	2705 (0.16)
Native	Liver transglutaminase active (Ca^{2+})	1 660 135 (100)	2988 (0.18)	1660 (0.10)	3486 (0.21)	5810 (0.35)	13 944 (0.84)

Denatured	Factor XIIIa inactive (EDTA)	1 610 195 (100)	1610 (0.10)	1932 (0.12)	1771 (0.11)	1449 (0.09)	6762 (0.42)
Denatured	Factor XIIIa active (Ca^{2+})	1 622 980 (100)	1461 (0.09)	1623 (0.10)	1298 (0.08)	1622 (0.10)	6004 (0.37)
Denatured	Liver transglutaminase inactive (EDTA)	1 685 170 (100)	1685 (0.10)	1348 (0.08)	1348 (0.08)	842 (0.05)	5223 (0.31)
Denatured	transglutaminase active (Ca^{2+})	1 615 240 (100)	21 321 (1.32)	5814 (0.36	20 190 (1.25)	67 840 (4.20)	1 151 65 (7.13)
			[^{14}C]putrescine				
C							
Type I native	Liver transglutaminase inactive (EDTA)		0.05	0.03	0.02	0.06	0.16
Type I native	Liver transglutaminase active (Ca^{2+})		0.28	0.15	0.15	0.10	0.68
Type I denatured	Factor XIIIa inactive (EDTA)		0.22	0.09	0.07	0.09	0.47
Type I denatured	Factor XIIIa active (Ca^{2+})		0.22	0.11	0.10	0.11	0.54
Type I denatured	Liver transglutaminase inactive (EDTA)		0.20	0.07	0.05	0.12	0.44
Type I denatured	Liver transglutaminase active (Ca^{2+})		3.20	0.97	0.49	8.50	13.16
Type III native	Liver transglutaminase (EDTA)		0.07	0.03	0.03	0.03	0.16
Type III native	Liver transglutaminase active (Ca^{2+})		0.18	0.10	0.21	0.35	0.84
Type III denatured	Factor XIIIa inactive (EDTA)		0.10	0.12	0.11	0.09	0.42
Type III denatured	Factor XIIIa active (Ca^{2+})		0.09	0.10	0.08	0.10	0.37
Type III denatured	Liver transglutaminase inactive (EDTA)		0.10	0.08	0.08	0.05	0.31
Type III denatured	Liver transglutaminase active (Ca^{2+})		1.32	0.36	1.25	4.20	7.13

respectively. The decrease in the cross-linked fractions of type III collagen depended on the diminishing enzyme concentrations in a similar way.

The incorporation of [^{14}C]putrescine by plasma factor XIIIa or liver transglutaminase into subunits of type I and III collagen is shown in Table II. The data presented indicate that factor XIIIa does not incorporate radioactivity into native and denatured collagen of either type. Likewise, liver transglutaminase incorporates a negligible amount of [^{14}C]putrescine into native type I (0.52%) and type III (0.68%) collagen. However, the incorporation of [^{14}C]-putrescine into denatured type I and III collagen by liver transglutaminase was significant: 13% (type I) and 7% (type III) of the total [^{14}C]putrescine added, respectively. The highest proportion, amounting to about 60% of the whole radioactivity recovered in gels, was localized in the high-molecular weight polymer bands. This value was corrected by subtracting the amount of label in cross-linked liver transglutaminase. To determine the magnitude of this correction, liver transglutaminase alone was incubated with [^{14}C]putrescine in the presence of Ca^{2+}. It was found that 0.6% of the radioactivity added was incorporated into self-cross-linked liver transglutaminase. Both enzymes did not incorporate [^{14}C]putrescine into reconstituted fibrils of type I and type III collagen.

Effects of plasma factor XIIIa and liver transglutaminase on the products of collagen cleavage by collagenase are illustrated in Fig. 3. It can be seen that incubation with factor XIIIa or liver transglutaminase resulted in a small decrease in intensity of bands corresponding to fragments of the collagen chains.

Further experiments were carried out to establish whether plasma factor XIIIa and liver transglutaminase cross-link collagen to fibrin. The effect of factor XIIIa and liver transglutaminase on binding of collagen to fibrin was

TABLE III

EFFECT OF FACTOR XIIIa AND LIVER TRANSGLUTAMINASE ON RETENTION OF DENATURED ^{125}I-LABELLED COLLAGEN IN FIBRIN CLOTS

1.2 mg of type I (58.0 nCi/mg protein) or type III (58.3 nCi/mg protein) collagen were incubated at 37°C for 30 min, in the presence of 500 μg factor XIIIa or 200 μg liver transglutaminase with 1 mg fibrinogen clotted by 7.5 U thrombin. The clots (after washing) and filtrates radioactivity was measured in a γ-counter. The total radioactivity recovered was in the range of 95—99%.

Collagen	Materials mixed with collagen before clotting with thrombin	% of ^{125}I-labelled collagen radioactivity recovered in clots
Type I denatured	fibrinogen	7.6
	fibrinogen, factor XIIIa	6.2
	fibrinogen, liver transglutaminase	49.0
Type I denatured cross-linked by liver transglutaminase *	fibrinogen, liver transglutaminase	12.1
Type III denatured	fibrinogen	10.8
	fibrinogen, factor XIIIa	8.5
	fibrinogen, liver transglutaminase	36.9
Type III denatured cross-linked by liver transglutaminase *	fibrinogen, liver transglutaminase	13.3

* Collagen subjected to liver transglutaminase (see Fig. 2) prior to addition to the incubation system.

TABLE IV

EFFECT OF FIBRINOGEN AND FIBRIN ON LIVER TRANSGLUTAMINASE-INDUCED CHANGES IN ELECTROPHORETIC DISTRIBUTION OF ^{125}I-LABELLED COLLAGEN SUBUNITS

Denatured samples (300 μg) of type I (22.2 nCi/mg protein) and type III (28.6 nCi/mg protein) collagen were incubated at 37°C for 1 h with fibrinogen (210 μg) or fibrin and 2.4 mM dithiothreitol in the presence of liver transglutaminase in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA/10 mM $CaCl_2$. Fibrin was formed by addition of 3 U thrombin to the incubation medium. After denaturation (see Fig. 1) 20 μl samples were analysed by electrophoresis on 4% polyacrylamide gels with 2.5% spacer gels. Stained gels were sliced and radioactivity measured in a γ-counter. Results are expressed in cpm. Values presented as percentages of total activity are given in parentheses.

Additions to collagen	Type I ^{125}I-labelled collagen radioactivity in fractions (cpm)					Total
	α	β	γ	poly-mers	HMW * polymers on the top of spacer	
A						
Liver transglutaminase inactive (EDTA)	6920 (55.3)	2000 (15.9)	1820 (14.5)	1340 (10.7)	460 (3.6)	12 540 (100)
Liver transglutaminase active (Ca^{2+})	3740 (30.6)	1680 (13.7)	870 (7.1)	5240 (42.9)	700 (5.7)	12 230 (100)
Fibrinogen, liver transglutaminase active (Ca^{2+})	3463 (28.5)	1264 (10.4)	765 (6.3)	3183 (26.2)	3475 (28.6)	12 150 (100)
Fibrinogen, liver transglutaminase active (Ca^{2+}), thrombin	3040 (24.7)	1367 (11.1)	1133 (9.2)	3114 (25.3)	3656 (29.7)	12 310 (100)
	Type III ^{125}I-labelled collagen radioactivity in fractions					
B						
Liver transglutaminase inactive (EDTA)	8060 (57.7)	2840 (20.4)	1240 (8.9)	1430 (10.2)	390 (2.8)	13 960 (100
Liver transglutaminase active (Ca^{2+})	5030 (35.5)	1890 (13.4)	920 (6.5)	5830 (41.2)	480 (3.4)	14 150 (100)
Fibrinogen, liver transglutaminase active (Ca^{2+})	4900 (35.7)	1560 (11.4)	900 (6.5)	3750 (27.3)	2620 (19.1)	13 730 (100)
Fibrinogen, liver transglutaminase active (Ca^{2+}), thrombin	4790 (34.3)	1590 (11.4)	790 (5.7)	4040 (29.0)	2730 (19.6)	13 940 (100)
	% of ^{125}I-labelled collagen radioactivity in fractions					
C						
Type I, liver transglutaminase inactive (EDTA)	55.3	15.9	14.5	10.7	3.6	
Type I, liver transglutaminase active (Ca^{2+})	30.6	13.7	7.1	42.9	5.7	
Type I, fibrinogen, liver transglutaminase, active (Ca^{2+})	28.5	10.4	6.3	26.2	28.6	
Type I, fibrinogen, liver transglutaminase, active (Ca^{2+}), thrombin	24.7	11.1	9.2	25.3	29.7	
Type III, liver transglutaminase inactive (EDTA)	57.7	20.4	8.9	10.2	2.8	
Type III, liver transglutaminase active (Ca^{2+})	35.5	13.4	6.5	41.2	3.4	
Type III, fibrinogen, liver transglutaminase active (Ca^{2+})	35.7	11.4	6.5	27.3	19.1	
Type III, fibrinogen, liver transglutaminase active (Ca^{2+}), thrombin	34.3	11.4	5.7	29.0	19.6	

* HMW, high molecular weight.

examined by measuring of denatured type I and III ^{125}I-labelled collagen trapped into fibrin clots. The data presented in Table III indicate that in the presence of factor XIIIa the amount of collagen retained in fibrin clots did not differ from that in control samples. In contrast, when liver transglutaminase was present, about 42% and 26% (after subtracting the control value) of denatured type I and type III collagen, respectively, were caught in the fibrin clot. Treatment of ^{125}I-labelled collagens with liver transglutaminase, prior to addition to the incubation system, caused a marked fall in percentages of radioactivity retained in clots.

The data presented in Table IV illustrate the effects of the presence of fibrinogen and fibrin on liver transglutaminase-induced changes, in distribution of ^{125}I-labelled collagen subunits in the electrophoretic pattern. It can be seen that inclusion of fibrinogen or fibrin into the incubation system caused a shift of collagen radioactivity to the polymer fractions of very large size, remaining on the top of 2.5% spacer gels. This shift was associated with only a negligible decrease in radioactivity of α-, β- and γ-collagen subunits. However, the fall in accumulation of ^{125}I in polymer fractions, localized on the top of 4% gels, was pronounced. Accumulation of collagen radioactivity in this fraction is the main effect of liver transglutaminase on ^{125}I-labelled collagen in the absence of fibrinogen or fibrin.

Discussion

The results presented here indicate that calf skin collagens of type I and III in the native form do not serve as substrates for active bovine plasma factor XIII and guinea pig liver transglutaminase. Both types of collagen remain resistant to factor XIIIa but become susceptible to the action of the liver enzyme after heat denaturation. Liver transglutaminase has been found to incorporate substantial amounts of [^{14}C]putrescine into denatured collagen subunits, to cross-link them into polymers and to co-cross-link them to fibrin or fibrinogen. Type I denatured collagen appeared to be a somewhat better substrate for liver transglutaminase than type III collagen. The presence of S-S bonds in the latter could be the reason for less accessibility. Birkbichler et al. [19] found that transglutaminase from guinea pig liver can cross-link itself. Our results are consistent with this observation. Thus, it is possible that liver transglutaminase acts not only as a cross-linking enzyme but also as a cross-linker, forming mixed covalently-bound polymers with collagen subunits and other substrates.

Macromolecular products of collagen clevage by mammalian collagenases, TC^A and TC^B, undergo denaturation at temperatures of 33—34°C and 30—31°C, respectively [20]. Therefore, susceptibility of such products at the body temperature to the liver transglutaminase could be assumed. We have prepared products of collagen cleavage by human neutrophile collagenase and subjected them to the action of the transglutaminating enzymes. The appearance of certain amounts polymer in the electrophoretic pattern, after incubation with liver translutaminase and plasma factor XIIIa, can be considered as an indication for accessibility of collagen degradation products to these enzymes and for formation of some cross-linking bonds.

The observation that type I and III collagen in the native form are resistant

to liver transglutaminase but become susceptible, after heat denaturation or partial proteolytic cleavage, is consistent with the results reported for other protein substrates. As stated by Folk and Finlayson [1] "most proteins that do not act as substrates for the enzyme in their native form are converted to substrates by partial digestion with proteases, by oxidization with performic acid or by denaturation". The relatively high specificity and large size of transglutaminases limits their action to glutamine and lysine residues highly exposed in protein structure. Therefore, conformation rather than amino acids sequence determines the formation of ϵ-(γ-glutamyl) lysine cross-linking bonds. Our data indicate that in triplehelical native collagen appropriate glutamine and lysine residues are not exposed and accessible to transglutaminating enzymes. It has recently been demonstrated however, that native collagen could be co-cross-linked to fibronectin by plasma factor XIIIa [22] and by guinea pig liver transglutaminase (Fesüs, L. and Jeleńska, M.M., unpublished observations). Hence, it could be assumed that conformational changes required for the catalytic action of transglutaminases can be achieved in triplehelical collagen in the presence of fibronectin.

The fact that denatured collagens are substrates for liver transglutaminase but not for plasma factor XIIIa can be ascribed to the well recognized differences in their specificities for natural and synthetic substrates. Distinctly different are the effects of plasma factor XIIIa and tissue transglutaminase on the most intensively investigated natural substrate i.e., fibrin. Under the same conditions tissue transglutaminase was reported to incorporate 15 residues of glycine ethyl ester into fibrin, while factor XIIIa only incorporated 7 to 8 [2]. These two transglutaminases have different preferences for fibrin chains and vary in the rate, order and number of introduced cross-linking bonds [1]. A number of simple peptide derivatives susceptible to tissue transglutaminase have been synthetized; benzylocarbonyl-L-glutaminyl-L-glycine is an excellent substrate for the liver enzyme [1]. In contrast, no satisfactory synthetic glutamine substrate is known for plasma factor XIIIa. In general, tissue transglutaminase has a broader substrate specificity than plasma factor XIIIa [1].

Our results are in contrast to those of Soria et al. [7] who, observing the diminishing amounts of subunits of collagen upon incubation with placental factor XIIIa, concluded that it had been cross-linked by the enzyme. This discrepancy might be explained by the presence of some denatured material in the applied commercial preparation of collagen and by contamination of placental factor XIIIa with tissue transglutaminase. Species differences are less likely reasons for the discrepancy in the results obtained. Obviously of great interest is the report of Duckert and Nyman [8] who provided indirect evidence for plasma factor XIIIa-catalysed co-cross-linking of fibrin to collagen. Unfortunately, they have not specified the source of collagen used. According to our data the purified type I and type III collagen can be co-cross-linked to fibrin or fibrinogen only by liver transglutaminase and not by plasma factor XIIIa. An additional condition needed is the conformation of collagen, which could only be coupled to fibrin or fibrinogen in denatured form. It was found that a high percentage of ^{125}I-labelled denatured collagen was retained in the fibrin clots formed in the presence of liver transglutaminase but not in its absence. Previous cross-linking of collagen markedly diminished its subsequent liver transglutami-

nase-mediated retention in the fibrin clots (see Table IV). Sites required for coupling to fibrin were probably blocked in collagen initially cross-linked by liver transglutaminase. Using a 2.5% spacer gel it could be demonstrated that the size of the polymers formed by liver transglutaminase from denatured collagen, in the presence of fibrin or fibrinogen, was higher (could penetrate only the top of the spacer gel) than that of polymers formed when denatured collagen alone was subjected to liver transglutaminase action (these polymers could enter the top of the 4% gel).

In conclusion, according to our data, liver transglutaminase, but not plasma factor XIIIa, catalyses cross-linking of type I and type III collagen and their binding to fibrin. Denaturation of collagen is a necessary requirement for both of these reactions, which limites their pathophysiological significance and explains the fact that ϵ-(γ-glutamyl) lysine cross-links are unusual for collagen. However, transglutaminase activity can be detected in fibroblasts [23] which not only synthetize but also degrade newly synthetized chains of collagen [24]. The possibility of collagen denaturation under in vivo conditions, in particular, in some pathological states, such as burns or inflammation, remains an open question. Hence the pathophysiological role of collagens as substrates for transglutaminating enzyme-mediated reactions might not be restricted to the co-cross-linking to fibronectin.

References

1 Folk, J.E. and Finlayson, J.S. (1977) Adv. Protein Chem. 31, 1—133
2 Chung, S.J. (1972) Ann. N.Y. Acad Sci. 202, 240—255
3 Beck, E., Duckert, F. and Ernst, M. (1961) Thromb. Diath. Haemorrh. 6, 485—491
4 Lee, S.Y. and Chung, S.I. (1979) Thromb. Diath. Haemorrh. 42, 379 (VII. ICTH. Abstr. No. 908)
5 Henry, R.L. (1965) Thromb. Diath. Haemorrh. 13, 35—46
6 Casley-Smith, J.R., Ardlie, N.G. and Schwartz, C.J. (1967) Br. J. Exp. Pathol. 48, 501—506
7 Soria, A., Soria, C. and Boulard, C. (1975) Experientia, 31, 1355—1357
8 Duckert, F. and Nyman, D. (1967) in Collagen-Platelet Interaction (Gaspar, H., ed.), pp. 391—396, F.K. Schattauer Verlag, Stuttgart
9 Hörmann, H. and Stemberger, A. (1976) in Collagen-Platelet Interaction (Gaspar, H., ed.), pp. 397—398, F.K. Schattauer Verlag, Stuttgart
10 Jeleńska, M.M., Fesüs, L. and Kopeć, M. (1979) Thromb. Diath. Haemorrh. 42, 378 (VIIth ICTH. Abstr. No. 900)
11 Fuji, T. and Kühn, K. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 1793—1801
12 Fessler, J.H. (1960) Biochem. J. 76, 452—463
13 Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529—539
14 Wojtecka, E. and Dancewicz, A.M. (1974) Przeglad Lekarski 31, 431—434
15 Kekwick, R.A., Mackay, H.E. and Nance, M.W. (1955) Biochem. J. 60, 671—683
16 Chung, S.J. and Folk, J.E. (1972) J. Biol. Chem. 247, 2798—2807
17 Connelan, J.M., Chung, S.J., Whetzel, N.I., Bradley, L.M. and Folk, J.E. (1971) J. Biol. Chem. 246, 1093—1098
18 Clark, C.C. (1976) in Methods in Connective Tissue Research (Hall, D.A., ed.), pp. 205—226, Joynson-Bruvvers Ltd
19 Birkbichler, P.J., Orr, G.R., Carter, H.A. and Patterson, M.K., Jr. (1977) Biochem. Biophys. Res. Commun. 78, 1—7
20 Sakai, T. and Gross, J. (1967) Biochemistry 6, 518—528
21 Iwanij, V. (1977) Eur. J. Biochem. 80, 359—368
22 Mosher, D.F., Schad, P.E. and Kleinman, H.K. (1979) J. Clin. Invest. 64, 781—787
23 Birckbichler, P.J., Orr, G.R., Conway, E. and Patterson, M.K., Jr. (1977) Cancer Res. 37, 1340—1344
24 Bieńkowski, R.S., Baum, B.J. and Crystal, R.G. (1978) Nature 276, 413—416