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THE DISTRIBUTION OF COLLAGEN:GLUCOSYLTRANSFERASE IN HUMAN BLOOD CELLS AND PLASMA

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Summary

Collagen:glucosyltransferase (UDP-glucose:5-hydroxylysine-collagen glucosyltransferase, EC 2.4.1.66) present in platelets, plasma, granulocytes and lymphocytes has been compared in order to determine whether the platelet enzyme has unique properties or distribution which would support a possible role in platelet-collagen interaction.

The enzyme was purified 5400-fold from human plasma and 4400 from human platelets. The two enzymes were similar in terms of K_m values for reacting with galactosylhydroxylysine (2.75 mM) and UDPglucose (7.4 μM), optimal Mn^{2+} concentration (10—15 mM) and pH optimum (7.0). The enzyme was not detectable in red cells. As in platelets, the enzyme was detected in membrane-bound and soluble forms in lymphocytes and granulocytes. Identical mobilities were obtained after elution following polyacrylamide gel electrophoresis of the enzymes from plasma, platelets, granulocytes and lymphocytes.

These studies do not support a unique role for the collagen:glucosyltransferase of platelets in platelet-collagen interaction.

Introduction

Collagen:glucosyltransferase (UDPglucose:5-hydroxylysine-collagen glucosyltransferase, EC 2.4.1.66) is an enzyme which catalyzes the transfer of glucose residues from UDPglucose to incomplete heterosaccharide chains (galactosyl-

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hydroxylysine residues) that are present in collagen. The enzyme has been reported to be present in a wide variety of tissues, as well as in human blood platelets [1—3], plasma [4,5] and serum [6].

In the case of platelets, it has been suggested that the adhesion of platelets to collagen, or to basement membrane that has been exposed by damage to the vascular endothelium, may be mediated by the formation of a complex between the platelet surface enzyme and incomplete heterosaccharide chain present in collagenous structures [7]. This theory is an extension of the general theory of the possible role of multiglycosyltransferases in cell-cell interaction first proposed by Roseman [8]. The proposed role of these enzymes in cellular physiology implies two principal constraints: first, the enzymic mechanism must be such that the enzyme-acceptor complex can form in the absence of free sugar nucleotides since these are not present in plasma: second, a unique physiological property such as platelet-collagen adhesion might be expected to require an enzyme which is unique either in its properties or in its distribution so as to be capable of conferring the required degree of specificity for the cell-substrate interaction.

We have endeavored to determine whether the collagen:glucosyltransferase of human platelets can meet both of these requirements. We have determined that the enzymic mechanism is ordered BiBi with UDPglucose being the first substrate to bind [9,10]. This mechanism in platelets is similar to that found in chick embryos [11] and indicates that the enzyme-acceptor complex will not form in the absence of the sugar nucleotide. However, the fact that the K_m value of the enzyme for the α_1 chain of collagen is three orders of magnitude less than for free galactosylhydroxylysine [9], together with the fact that the chick embryo enzyme can bind to denatured collagen and to insolubilized α_1 chains in the absence of the substrates required for transferase activity [12], suggests that the enzyme may be recognizing sequences in collagen other than the acceptor site itself. Thus, the kinetic studies alone do not permit a decision to be taken as to the possible role of the platelet glucosyltransferase as a collagen-binding protein in platelet adhesion.

We now address ourselves to the second of the constraints required of an enzyme conferring specific properties on a physiological reaction; namely, the uniqueness of the enzyme and its distribution within the cellular and humoral compartments of blood.

Materials and Methods

Soluble and membrane-bound forms of collagen:glucosyltransferase activity were determined as previously described using galactosylhydroxylysine as acceptor [9]: one unit of activity is defined as the amount of enzyme required for synthesis of 1 nmol of glucosylgalactosylhydroxylysine/h at 37°C. Fresh plasma, or fresh frozen plasma, was obtained from the Washington Regional Red Cross Blood Center.

Separation of blood cells. The buffy coat from one unit of freshly drawn heparinized blood (approx. 40 ml) was removed and placed in a Falcon plastic conical centrifuge tube. The cell suspension was gently agitated in the presence of ADP (final concentration $2 \cdot 10^{-4}$ M) to aggregate the platelets and the

platelets were then centrifuged out in an International Clinical Centrifuge at 200 × *g* for 5 min.

The supernatant cell suspension was then diluted 1 : 1 with Hank's balanced salt solution (calcium and magnesium free, without phenol red; Grand Island Biological Co., Grand Island, NY). 20 ml volumes of the above suspension were layered on 15-ml cushions of 'lymphocyte separation medium' (density 1.077 g/ml; Litton Bionetics, Kensington, MD) in Falcon 'Nalgene' centrifuge tubes. The gradients were centrifuged in the Sorvall RC 2-B centrifuge, HB-4 rotor, at 365 × *g* for 30 min. The lymphocyte-rich bands at the interface were removed and pooled, diluted with Hank's solution and washed twice at 365 × *g* for 30 min to remove any contaminating platelets.

In certain experiments, blood which had been anticoagulated with citrate/phosphate/dextrose was used and the ADP aggregation step was omitted.

The granulocyte-erythrocyte pellets from the above separation were resuspended in Hank's solution (1 : 1) and pooled and 20-ml volumes were layered onto 15-ml cushions of a Ficoll-Hypaque solution which consisted of 10 parts of 50% Hypaque plus 20 parts of 97% Ficoll (specific gravity = 1.119 g/ml; osmolality = 443 mosM. Adjusting the osmolality to 300 mosM, as recommended in the original work [13], gave poorer results in our hands). The gradients were centrifuged at 800 × *g* for 30 min in the International Clinical Centrifuge. The granulocyte-rich interface was diluted 1 : 4 with Hank's solution and washed twice by centrifugation at 800 × *g*. Differential counts were performed using Wright's stain. The isolated lymphocytes and granulocytes were sonicated for four times 10 s at 0°C and the homogenate was then subjected to 20 strokes in the Dounce homogenizer. The homogenates were then centrifuged at 120 000 × *g* for 90 min at 4°C to yield soluble and particulate fractions.

Purification of plasma enzyme. Fresh platelet-poor plasma (250 ml) was dialyzed overnight against twice 4 l of Tris-HCl buffer (50 mM, pH 7.2) containing 0.1 mM dithiothreitol, 1 mM EDTA and 50 mM sodium chloride. The dialyzed plasma was centrifuged (10 000 × *g* for 15 min) to remove the resulting precipitate and 200 ml of the supernatant was pumped onto a column (45 × 2.5 cm) of DEAE-cellulose (Whatman DE 52) at a rate of 50 ml/h and the column was then washed with the dialyzing buffer until the absorbance was less than 0.1 *A* unit. A gradient of NaCl from 50 to 300 mM with a total volume of 1500 ml was then applied and fractions (10 ml) were collected and monitored for absorbance at 280 nm and for enzyme activity (Fig. 1). The peak of enzyme activity was pooled and concentrated by ultrafiltration (Amicon XM-100A-62MM). The concentrate was fractionated by precipitation with ammonium sulfate and the enzyme activity was obtained between 0 and 42% saturation. The precipitate was removed by centrifugation at 20 000 × *g* for 10 min, dissolved in 5 ml of Tris-HCl starting buffer and dialyzed against the same buffer. Further purification of the enzyme was achieved by affinity chromatography on UDP-hexanolamine-Sepharose prepared as described by Barker et al. [14] in a manner analogous to that used for the purification to homogeneity of the chick embryo enzyme [15,16]. An aliquot of the partially purified enzyme preparation (5 ml, 9.5 mg protein) was applied at a flow rate of 3 ml/h to a column (bed volume, 3 ml) of UDP-hexanolamine-Sepharose in

Tris-HCl buffer (50 mM, pH 7.2) containing 15 mM Mn^{2+}. Inactive protein was eluted with the buffer and with 20 μM UDPglucose (three times the K_m). Active enzyme was eluted as a single fraction with 100 μm UDPglucose (15 times the K_m) and bovine serum albumin was added to this fraction to a final concentration of 0.1% to enhance stability.

Affinity chromatography on UDP-hexanolamine-Sepharose was also carried out under similar conditions for the futher purification of the soluble platelet enzyme following chromatography on DEAE-cellulose.

Electrophoretic separation of enzymes. The standard anodic gel system of Ornstein [17] and Davis [18] was used with a 2.5% stacking gel at pH 8.9 and a 7% running gel at pH 9.5 in Tris-HCl/glycine buffer.

Soluble fractions were prepared from homogenates of platelets, lymphocytes and granulocytes, as described, and subjected to precipitation at 42% ammonium sulfate to effect a partial purification (approx. two-fold). The precipitate was then dialyzed extensively against Tris-HCl/glycine gel-running buffer at 4°C overnight: the plasma enzyme was prepared similarly. 100 μl sample was put on each gel, and electrophoresis was carried out at 2.5 mA/gel for 15 min, then continued at 1.25 mA/gel for one more hour. Upon completion of the electrophoresis, the gel was immediately sliced (without prior freezing) into 2.5-mm slices and crushed in the presence of 100 μl of 0.05 M Tris-HCl, 1 mM EDTA and 0.1 mM dithiothreitol buffer. After soaking for 20 h at 4°C, the mixture was centrifuged at 45 000 $\times g$ for 20 min and 50 μl of the clear supernatant was assayed by the usual assay procedure.

Results

Enzyme purification

Plasma enzyme. Approximately 400-fold purification of the collagen: glucosyltransferase activity present in plasma could be achieved by a combination of chromatography on DEAE-cellulose and ammonium sulfate precipitation (Table I). Under the conditions described, most of the applied protein was eluted in the initial washing of the column (Fig. 1) while the retained protein was eluted by the gradient as a series of peaks emerging ahead of the single peak of enzyme activity. While it is probable that a higher degree of purification could be achieved by further variations in the gradient, the inherent instability of the purified enzyme made it desirable to work as quickly as possible using relatively steep elution gradients.

TABLE I

PURIFICATION OF COLLAGEN:GLUCOSYLTRANSFERASE FROM PLASMA

Fraction	Spec. activity (units/mg protein)	Purification	Yield (%)
Platelet-poor plasma	0.08	1	100
DEAE eluate after ultrafiltration	18	220	45
$(NH_4)_2SO_4$ precipitation	33	406	36
Affinity chromatography	440	5400	23

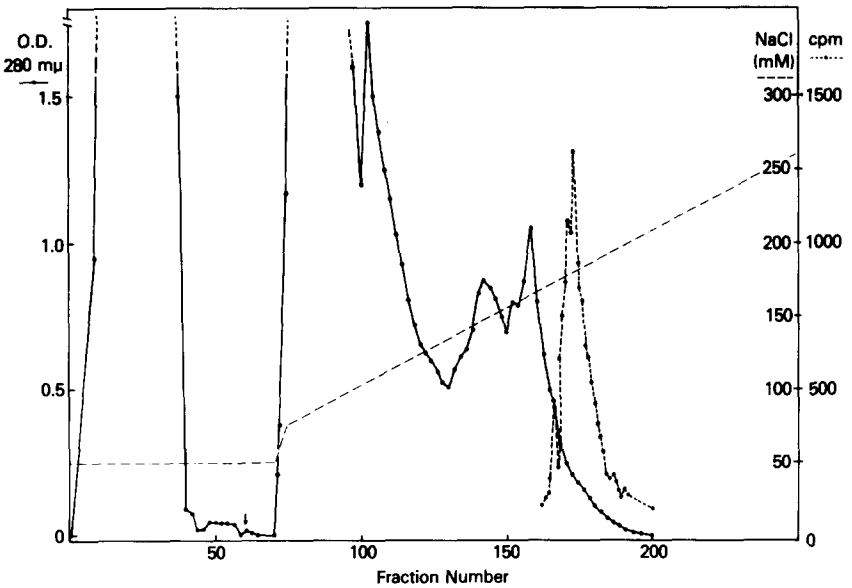

Fig. 1. Chromatographic purification of collagen:glucosyltransferase of plasma. •———•, protein (A_{280nm}); •· · · · · ·•, enzyme activity; · · · · · · · · ·, elution gradient (conductivity).

Enzyme constants were determined at this stage of purification. The K_m value for the reaction with UDPglucose was determined to be 7.4 μM and 2.75 mM for galactosylhydroxylysine. The optimum Mn^{2+} concentration was in the range 10—15 mM and the pH optimum was about 7.0: these values are similar to those for the platelet enzyme [9].

A further purification of approximately 13-fold was obtained by affinity chromatography on UDP-hexanolamine-Sepharose resulting in an overall purification of 5000-fold and an overall recovery of activity 23%.

Platelet enzyme. We have previously used DEAE-cellulose chromatography, as described above, for the 80-fold purification of soluble collagen:glucosyltransferase from a platelet homogenate [9]. When the partially purified platelet enzyme was subjected to affinity chromatography on UDP-hexanolamine-Sepharose, a further 120-fold purification was achieved giving an overall purification from the platelet homogenate of 4300-fold (Table II).

Electrophoretic comparison of platelet and cellular enzymes. The enzyme constants indicated that the platelet and plasma enzymes were similar. Because of the difficulty in obtaining adequate amounts of homogeneous preparations of lymphocytes and granulocytes to obtain the enzyme constants, this approach was not feasible for these two cell types. In these cases, the soluble fractions from the homogenate were partially purified and concentrated by single steps of $(NH_4)_2SO_4$ precipitation as described for the platelet and plasma enzymes. The soluble enzyme fractions from plasma and the three cell types were then subjected separately to gel electrophoresis and assayed by the standard system following elution from the gel. Under these circumstances collagen:glucosyltransferase from plasma, platelets, granulocytes and lymphocytes all ran as single peaks with identical electrophoretic mobilities (Fig. 2). These results,

TABLE II

PURIFICATION OF SOLUBLE COLLAGEN:GLUCOSYLTRANSFERASE FROM PLATELETS

Fraction	Spec. activity (units/mg protein)	Purification	Yield (%)
Platelet-poor cytosol	1.38	1	100
DEAE eluate after ultrafiltration	38	28	90
Affinity chromatography	6000	4350	29

together with the kinetic comparison of the platelet and plasma enzymes, suggest that the enzymes from these four sources are identical.

Distribution of membrane-bound and soluble forms. Previous studies [9] had shown that the platelet enzyme exists in both membrane-bound and soluble

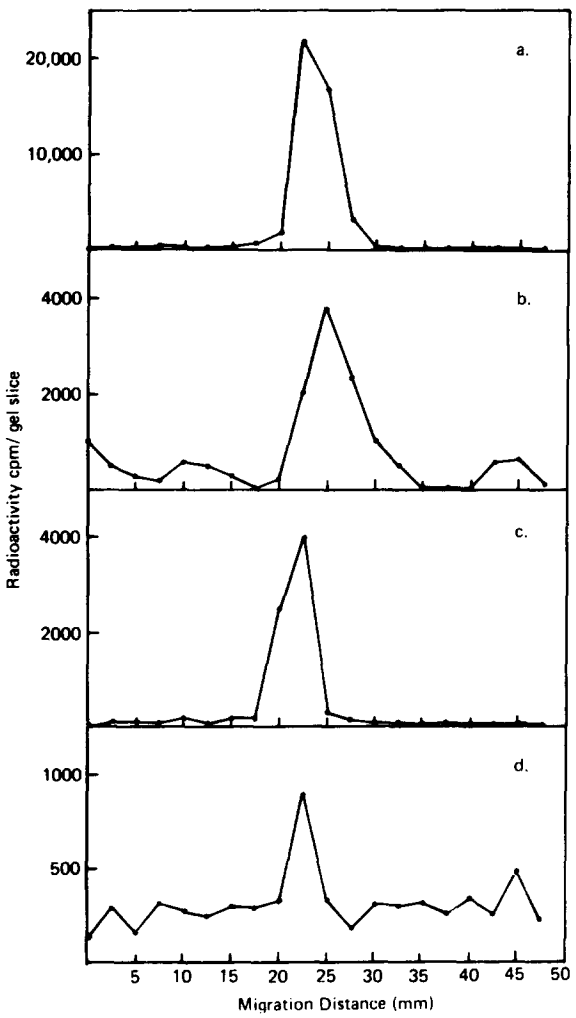

Fig. 2. Gel electrophoretic comparison of collagen:glucosyltransferase from (a) platelets; (b) plasma; (c) lymphocytes, and (d) granulocytes.

TABLE III

DISTRIBUTION OF ENZYME IN SOLUBLE AND MEMBRANE-BOUND COMPARTMENTS OF BLOOD CELLS

n.d., not determined.

	Intact cells (units/10^9 cells)	Homogenate (units/mg protein)	Membrane bound/total	Specific activity in homogenate (units/mg protein)
Platelets	0.45	2.38	1:5	1.4
Granulocytes	2.3	5.4	1:2	0.1
Lymphocytes	3.2	15.8	1:5	0.8
Erythrocytes	0.01	0.02	n.d.	n.d.

forms. It was considered necessary to determine the ratio in other types of blood cells in order to elucidate the exposure of this enzyme on the surface of blood cells. For this purpose, individual cell types were isolated as described in Materials and Methods and the enzyme activity was measured in the intact cells and following cell homogenization. These results show that there is essentially no activity in red blood cells. However, both the total and surface-located enzymic activity found in granulocytes and lymphocytes were in approximately the same range as that found in platelets (Table III).

Discussion

The data presented here provide information on the distribution of collagen: glucosyltransferase in blood cells as well as on the partial purification of the platelet and plasma enzymes. Collagen:glucosyltransferase of chick embryo extract has been purified 30 000—40 000 fold to yield a homogeneous protein by two different procedures involving multiple steps of affinity chromatography [15,16].

The data on enzyme distribution suggest that identical forms of collagen: glucosyltransferase occur in plasma and in all of the major cell types in blood with the exception of erythrocytes. The enzyme in plasma and the platelet enzyme are similar in enzyme constants and the soluble enzymes from all four sources are electrophoretically identical. In the case of granulocytes and lymphocytes, as with the platelet, the enzyme occurs in both membrane-bound and cytoplasmic forms and, in fact, the ratio of membrane-bound and soluble activities is similar in each of the three cell types examined. Contamination of the cell preparations with plasma is unlikely in view of the extensive dilution and washing involved in the gradient separations.

These observations appear to sound the deathknell to any possibility that platelet collagen:glucosyltransferase plays a role in platelet adhesion to subendothelium, as originally proposed [7]. Thus, our previous studies on the enzyme mechanism indicate that the carbohydrate portion of collagen cannot be the primary site of the interaction [9,10] while the present studies provide no evidence for unique properties of the platelet enzyme that would explain the unique features of platelet adhesion. Furthermore, it may be noted that exposed subendothelium will be constantly washed by plasma and thus is,

presumably, saturated with the plasma form of the enzyme.

Finally, it has been shown that fibrillar collagen is not a substrate for the enzyme from chick embryo [19] or from platelets [3,20]. This is a necessary, but not sufficient, condition to eliminate it from consideration as having a role in platelet adhesion. However, in further experiments [3], it was shown that fibrillar collagen does not inhibit incorporation into galactosylhydroxylysine and we have confirmed these observations. Thus, fibrillar collagen is not a substrate itself, nor does it compete with the substrate for the enzyme.

The role of the enzyme in plasma, or in other blood cells, is difficult to envision since none of these elements is known to be involved in collagen biosynthesis although each of them probably plays some role in the repair of endothelial damage and in wound healing. Collagen:glucosyltransferase has been shown to decrease with age both in human skin [21] and in normal platelets [22], as well as being reduced in diabetic platelets.

It is possible that the plasma form arises from cellular breakdown and metabolism [6] and it may provide a useful mechanism for understanding cellular processes. However, the unique contributions of individual cell types are difficult to establish in view of the widespread presence of this enzyme in all cell types in which it has been sought.

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