Collagen-Mediated Platelet Aggregation

EFFECTS OF COLLAGEN MODIFICATION INVOLVING THE PROTEIN AND CARBOHYDRATE MOIETIES

DAVID PUETT, BETTY KAY WASSERMAN, JOHN D. FORD, and LEON W. CUNNINGHAM

From the Department of Biochemistry, Vanderbilt University, Nashville, Tennessee 37232

ABSTRACT In an effort to elucidate the nature of the collagen-platelet interaction, the effects of collagen modification on platelet aggregation have been studied. We have shown that purified rat skin (salt) soluble collagen is effective at about 20 nM in mediating platelet aggregation in human platelet-rich plasma. This concentration is somewhat greater than that required of several skin insoluble collagens (ca. 10 nM). Both the $\alpha 1$(I) and $\alpha 2$ chains from rat skin soluble collagen produced platelet aggregation, but only at concentrations of about 13 μM and 55 μM, respectively. In contrast, heat-denatured collagen and chains (e.g., 65 μM $\alpha 1$(I) and 160 μM $\alpha 2$) failed to induce platelet aggregation and to inhibit platelet aggregation by native collagen.

Glycopeptides were prepared from human skin insoluble collagen by extended digestion with bacterial collagenase and trypsin, and were purified by gel filtration into two classes. One class of higher molecular weight contained sialic acid, glucosamine, galactosamine, fucose, mannose, galactose, and glucose, and the other of lower molecular weight consisted primarily of a mixture of galactose and galactosyl-glucose units *O*-glycosidically linked to hydroxylysine-containing peptides. We found that, after the residual tryptic activity contaminating the higher molecular weight fraction was inhibited, neither of the glycopeptide classes produced nor inhibited native human skin insoluble collagen-mediated platelet aggregation at the highest concentration examined (ca. 1–2 mg glycopeptide per ml of platelet-rich plasma).

Highly purified samples of the hydroxylysyl glycosides, hydroxylysylgalactose and hydroxylysylgalactosyl-glucose (Hyl-Gal and Hyl-Gal-Glc, respectively), were prepared from human urine and labeled at galactose using galactose oxidase followed by reduction with tritiated borohydride. Binding studies with platelet-rich plasma showed that, at concentrations greater than 50 nM, Hyl-Gal gives apparent binding to platelets, but there was no evidence of Hyl-Gal-Glc binding to platelets at concentrations up to 250 nM. At concentrations several hundredfold higher than the equivalents present in the minimum concentration of rat skin soluble collagen required for platelet aggregation, neither Hyl-Gal (at 29 μM) nor Hyl-Gal-Glc (at 18 μM) caused platelet aggregation or inhibited platelet aggregation by native collagen. Also, at a concentration of 85 μM (which represents a concentration about two thousandfold higher than the equivalents in the minimum concentration of soluble collagen required for platelet aggregation) the Gal-Glc-containing 36 residue rat skin soluble collagen $\alpha 1$(I)-cyanogen bromide #5 peptide had no platelet aggregating or inhibiting activity.

Modification of at least 90% of the rat skin soluble collagen carbohydrate by mild periodate oxidation had no effect on the platelet aggregating activity. Human skin insoluble collagen was reacted with periodate under the same conditions, and this had no demonstrable effect on its ability to induce platelet aggregation. This indicates that the normal carbohydrate side chains of these collagens are not required for the platelet interaction that produces the release of ADP and other metabolic constituents and leads to aggregation.

Thus, collagen-platelet interactions appear to involve at least two distinct binding sites on the platelet plasma membrane. One is a protein binding site that activates platelet aggregation and has high specificity and affinity for the collagen triple-helical fold or perhaps even for a particular amino acid sequence in the triple helix.

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The second site, which may be in close proximity to the first, is a carbohydrate binding site of lower affinity and may be a platelet glycosyltransferase(s). This site, however, is incapable of activating platelet aggregation. Although binding of very low molecular weight collagen glycopeptides at this latter site alone does not lead to platelet aggregation, extremely high concentrations may inhibit collagen-mediated platelet aggregation by interfering with the binding of intact collagen or derivatives at the protein binding site. Thus, we conclude that under physiological conditions, where the criteria of high specificity and affinity are met, the collagen carbohydrate is not a determining factor in collagen binding to platelets, but, rather, the effect is mediated primarily by the protein moiety.

INTRODUCTION

It is now well established that one of the physiological roles of platelets involves binding to subendothelial tissue after vascular injury (1–6). This binding is followed by the release of ADP, 5-hydroxytryptamine, and other substances from the bound platelets, thus causing additional circulating platelets to adhere to the original deposition. These aggregating platelets undergo rather dramatic morphological changes (7, 8) and in the presence of thrombin rapidly fuse to form a "hemostatic plug." Hence, platelet aggregation is the first step in hemostasis; fibrin formation is initiated only after this has occurred.

Clearly, an understanding of the nature of the initial binding event is of both clinical and basic biochemical interest. For example, the elucidation of this normal physiological mechanism involving a macromolecule-cell interaction may provide a rigid framework for the design of specific therapeutic agents that inhibit platelet aggregation and, thus, would be of potential use in preventing or perhaps disrupting arterial thromboses. Recent evidence indicates that collagen is the connective tissue component to which platelets adhere (9–12). It was initially shown by Butler and Cunningham (13, 14) that soluble collagen contains only galactose and galactosyl-glucose units *O*-glycosidically linked to the hydroxyl group of hydroxylysine. The insoluble collagen fraction was later shown by Cunningham and Ford (15) to contain, in addition to the above carbohydrate units, complex oligosaccharides composed of amino sugars, sialic acid, mannose, glucose, and galactose. The demonstrated presence of various glycosyltransferases on the platelet plasma membrane (16–18) has led to the proposal (18–20) that the collagen carbohydrate side-chain represents the "recognition site" and that the binding involves an enzyme-substrate (or inhibitor) type interaction, e.g., platelet-glucosyltransferase complexed with collagen-gal. This hypothesis is a specific example of Roseman's (21) generalized theory regarding intercellular interactions and Cunningham's (22) suggestions pertinent to the role of glycoproteins in cellular recognition.

The purpose of this work is to examine the nature of the platelet-collagen interaction using well characterized collagen samples and collagen derivatives. Specifically, we are interested in determining if both the normal collagen carbohydrate and the triple helical structure are necessary for this interaction or if it is mediated mainly through the protein moiety.

METHODS

Materials. The following materials were obtained from Worthington Biochemical Corporation (Freehold, N. J.); *Clostridum histolyticum* collagenase (CLS 8GF, 125 U/mg), *Dactylium dendroides* galactose oxidase (GAO IAA, 33 U/mg and GAO OLA, 26 U/mg), Galactostat and Glucostat Reagent Kits, lima bean trypsin inhibitor (LBI 9FA), and TPCK-trypsin (TRTPCK 9IA, 210 U/mg). Catalase was a Sigma product (beef liver, 2 × crystallized, lot No. 41C-8070) with a reported activity of 33.3 Sigma U/mg. Tritiated potassium borohydride and sodium borohydride were purchased from the Amersham-Searle Corporation (Arlington Heights, Ill.) and had stated activities of 145 mCi/mg and 79 mCi/mg, respectively.

Collagen extraction and purification. RSSC[1] was obtained from lathyritic male Sprague-Dawley rats using the method of Bornstein and Piez (23). Guinea pig skin (salt, acid, and citrate) soluble collagen was prepared as described elsewhere (14), and bovine skin (acid) soluble collagen was kindly given to us by Dr. Edward J. Miller. Human infant skin (acid) soluble collagen was prepared by extraction with 0.5 M acetic acid, followed by dialysis against 10 mM Na_2HPO_4 to precipitate the collagen fibrils. The rat, human, and guinea pig insoluble skin collagens were prepared by the method of Veis et al. (24), as modified by Cunningham and Ford (15). The human insoluble tendon collagen was isolated and purified using the method of Segrest and Cunningham (25). The chick sternal cartilage and the bovine articular cartilage (pepsin solubilized) collagens were kindly provided by Dr. Edward J. Miller (26, 27).

Preparation of platelet-rich plasma. Whole blood was obtained from healthy adults who were not taking oral contraceptives and had not ingested acetylsalicylic acid for at least 7 days. Blood (10–90 ml) was collected using an 18 gauge needle and sterile plastic syringes containing sodium citrate as an anticoagulant (1:9, 3.2% sodium citrate: blood). The blood was transferred to plastic centrifuge tubes, and the white cells and erythrocytes were removed by centrifugation for 8–16 min at 180 *g* using the Sorvall GLC-1 centrifuge. The contents comprising the upper one-third (approximately) of the centrifuge tube, i.e., PRP,

[1] *The abbreviations used are:* CNBr, cyanogen bromide; HSIC, human skin insoluble collagen; Hyl-Gal, hydroxylysylgalactose; Hyl-Gal-Glc, hydroxylysylgalactosylglucose; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RSSC, rat skin (salt) soluble collagen; α1(I)-CNBr #5, a 36 amino-acid residue cyanogen bromide glycopeptide from the α1(I) chain of collagen (cf. reference 59 for a full description).

were used within 3–4 h for the binding and aggregation studies. Platelet concentration was determined using a Coulter Counter, and these were always in the normal range, ca 300,000 platelets per mm^3. The material remaining from the original centrifugation was centrifuged for 8 min at 37,000 *g* (Sorvall RC2-B, SS-34 rotor) to separate the cells from the PPP. All of the above operations were conducted at room temperature.

Isolation and tritiation of the hydroxylysyl glycosides. The highly purified hydroxylysyl glycosides, Hyl-Gal and Hyl-Gal-Glc, were isolated from human urine as described earlier (25, 28). These compounds were tritiated at C-6 of galactose, using purified galactose oxidase (29) followed by reduction with tritiated borohydride.

About 2 mg of Hyl-Gal were dissolved in 1 ml of 50 mM NaCl, 50 mM phosphate buffer, pH 7.0. Then, 0.1 ml (12.5 U) of galactose oxidase in 25 mM phosphate buffer, pH 7.0, was added, and the mixture was incubated for 25 h at 37°C in a toluene atmosphere. The reduction was then carried out in the hood. 1 ml of 50 mM NaCl, 0.2M phosphate buffer, pH 7.8 was added to the digest to increase the pH. An aliquot (0.5 ml) from a solution of cold potassium borohydride (12 mg/5 ml of 10 mM KOH) was added to an ampoule containing 12.8 μmoles of tritiated potassium borohydride (100 mCi), and this was then transferred to the digest. The mixture was stirred for 10 min and then acidified with 50 μl of glacial acetic acid under a stream of nitrogen. The solution was chromatographed on a calibrated 2.5 × 38.5 cm Bio-Gel P-2 column (located in a hood) that was equilibrated and developed with 0.1 M pyridine acetate, pH 5. The Hyl-Gal fraction was pooled, lyophilized, and then chromatographed on a 0.9 × 19 cm Dowex-50 column as described earlier (28). The tritiated Hyl-Gal fraction was pooled, lyophilized, and desalted using the Bio-Gel P-2 column.

Hyl-Gal-Glc was labeled using conditions similar to those described for Hyl-Gal. Notable differences included the use of tritiated sodium borohydride, 25 U of galactose oxidase, the addition of 4 μg of catalase (30), and incubation for 48 h. The labeled Hyl-Gal-Glc was then chromatographed as described above for Hyl-Gal.

Preparation of collagen chains and peptides. HSIC (1 g) was digested with collagenase and trypsin exactly as described elsewhere (15). After the 2-day digestion, the soluble portion was chromatographed on a 2.5 × 96 cm Sephadex G-25 (coarse) column equilibrated and developed with 0.1 M pyridine-acetate, pH 5. The effluent was monitored both by ninhydrin and orcinol analysis (31).

The RSSC α1(I) and α2 chains, as well as the mixture of CNBr peptides, were generously provided by Dr. Hector Aguilar. The individual chains were prepared according to Piez et al. (32) and Bornstein and Piez (23). The α1(I) chains as prepared by this method are contaminated with some β11 chains; however, we rechromatographed the fraction on CM-cellulose in order to reduce the β11 contamination. The CNBr peptides were prepared as described by Butler and Ponds (33). No attempt was made to fractionate these. The highly purified α1(I)-CNBr #5 peptide from RSSC was kindly provided by Dr. William T. Butler.

Preparation of carbohydrate-modified collagen. The carbohydrate moieties of RSSC were modified by periodate oxidation, using essentially the conditions specified by Spiro (34) for renal glomerular basement membrane. RSSC was dissolved in 0.9% NaCl or water to give a solution of 2 mg/ml. The solution was then made 50 mM in sodium acetate buffer, pH 4.5, and a concentrated solution of sodium *m*-periodate in saline was added to give a final concentration of 24 mM (the final protein concentration was about 90% of the original value). The incubation flask was wrapped in aluminum foil, and incubation proceeded for 25–27 h with occasional stirring. The reaction was terminated by the addition of excess ethylene glycol, although platelet aggregation experiments were conducted both before and after the addition of ethylene glycol. A collagen control was always prepared in which saline was added in place of periodate. All of the above steps were at 5°C, and all solutions were maintained in the dark until addition to the PRP in the aggregometer. Before chemical analysis the mixture was dialyzed against distilled water and lyophilized. A limited amount of HSIC was also reacted under identical conditions. HSIC was first homogenized (Thomas-B37429 homogenizer), then stirred for several hours and finally centrifuged for 5 min at 180 *g*. The supernate was then used in the periodate oxidation.

RSSC was incubated with galactose oxidase, using a modification of the conditions reported by Spiro (30) for renal glomerular basement membrane RSSC was dissolved in either distilled water or 0.9% NaCl at 5°C to a final concentration of 2 mg/ml. The solution was then made 50 mM in sodium phosphate buffer, pH 6.8±0.2, followed by the addition of lyophilized galactose oxidase (0.1 mg enzyme per mg collagen) and catalase (1 mg enzyme per 300 mg collagen). Originally, the incubation was for 24–27 h at 30°–35°C; however, this resulted in pronounced aggregation, i.e., visible clumps, such that the collagen could be centrifuged out at low speed. We also performed the incubation for 7 h at 25°C, and whereas this resulted in some opalescence, clarification of the solution was readily effected by lowering the pH to 4.6–4.8. Occasionally, excess sodium borohydride was added to the mixture and incubated for 45 min before the pH was lowered.

Collagen-mediated platelet aggregation. Aggregation studies were performed with 1 ml of PRP (stirring at 900 rpm) at 37°C using the Peyton Aggregation Module (Peyton Associates, Inc., Buffalo, N. Y.) with a Bausch and Lomb Inc. 10 mV VOM-5 recorder. The instrument was adjusted for 100% transmission with 1 ml of PPP and for 0% transmission with 1 ml of PRP. Generally, collagen (or collagen derivatives) was added in a volume of 50 μl, although occasionally volumes of 5–100 μl were used. The transmittance of the samples was followed with time and displayed on the recorder (*ca.* 4 inches full scale and a chart speed of 1 inch/min). Aggregation invariably registered 100% transmission, and particulate formation was always confirmed by visual observation.

Binding of labeled hydroxylysyl glycosides to platelets. The binding studies were performed with 1 ml of PRP and various concentrations of hydroxylysyl glycosides in buffer at a volume of 100 μl. Controls of PPP were present for every sample. The samples and controls were incubated for 30 min at 37°C with occasional stirring. These were then centrifuged for 50 min (5°C at 12,350 *g*), and aliquots of the supernates were diluted with Aquasol and counted in a Packard Liquid Scintillation Spectrometer.

Carbohydrate and amino acid analysis. Neutral sugars were qualitatively analyzed as the alditol-acetate derivatives using the GLC method of Lehnhardt and Winzler (35), sometimes with slight modifications. For example, hydrolysis was occasionally performed in 2 N HCl for 4 h, at 100°C. Arabinose was then added as an internal standard, and the sample was dried on a rotary evaporator. The residue was dissolved in water and applied to the standard ion exchange tandem columns (35).

Total neutral sugars of the HSIC collagenase-trypsin glycopeptides were determined using the automated orcinol method described elsewhere (31). Total sialic acid of these glycopeptides was analyzed using established method (36, 37). Glucosamine was measured on the 15 cm column of the amino acid analyzer after hydrolysis (in evacuated ampoules) in 4 N HCl for 6 h at 110°C.

Glucose and galactose of RSSC and carbohydrate-modified RSSC were quantitatively determined using the Glucostat and Galactostat Kits after hydrolysis and ion exchange chromatography as described above. The amounts of glucose and galactose in RSSC were measured using 145 and 68 mg of collagen, respectively. These levels permitted three different concentration determinations for each carbohydrate.

Amino acid analysis of the glycopeptides was performed on a Beckman 120 Amino Acid Analyzer after hydrolysis (in evacuated ampoules) in 6 N HCl for 20 h at 110°C (38). Hydroxyproline was determined on a separate run at 30°C.

The protein concentrations of the soluble collagens were determined from dry weight measurements on the lyophilized, salt-free powder. Concentrations of the insoluble collagens were estimated using the micro-Biuret method (39) on the supernates after homogenization and centrifugation (180 *g*); RSSC and chick sternal cartilage collagen were used as standards.

RESULTS

Collagen-mediated platelet aggregation. In order to achieve optimum reproducibility in the platelet aggregations, particular care was taken to ensure that no large collagen particules were present. For example, RSSC was completely dissolved by stirring in 0.9% NaCl (or H_2O) at 5°C for 48 h at a protein concentration of about 2 mg/ml. This solution was normally diluted twofold before the addition of small aliquots to PRP. The insoluble collagens (ca. 2 mg/ml) were well dispersed by homogenization and centrifugation at 180 *g*; only the slightly opalescent supernates (ca. 0.6 mg/ml) were used. The collagen solutions were generally stored at 5°C; however, they were usually warmed to room temperature before addition of PRP. Similar platelet aggregations were obtained if sodium acetate (to 50 mM, pH 6.8) was added to either the RSSC or the HSIC stock in 0.9% NaCl or H_2O, although lag periods varied from 1 to 3 min. We found that about 2–3 μg of HSIC in 0.9% NaCl was required to produce aggregation although about 10–30 μg were routinely used since this level gave better reproducibility. Additional insoluble collagens from rat skin, guinea pig skin, and human tendon were also screened and found to be effective at about the same or slightly higher levels than HSIC. RSSC was somewhat less effective in that about 6 μg in 0.9% NaCl was required for aggregation; however, 10–30 μg were generally used. Guinea pig and human infant soluble collagens were also effective, as were the chick sternal cartilage and bovine articular cartilage collagens. Concentrations of collagen required for aggregation are somewhat dependent upon pH, temperature, and perhaps time of storage in solution.

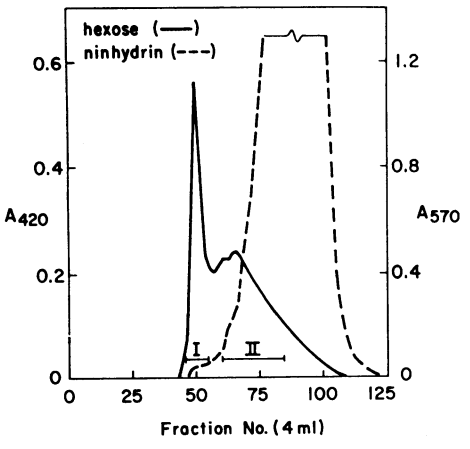

FIGURE 1 Gel filtration of the collagenase-trypsin digest of HSIC on a 2.5 × 96 cm column of Sephadex G-25 equilibrated and developed with 0.1 M pyridine acetate, pH 5.0, at 25°C. The effluent was monitored for both orcinol-positive and ninhydrin-positive material.

Effects of collagen modification—heat denaturation and chain separation. Several collagens (HSIC, RSSC, and rat skin insoluble collagen) were denatured by heating in boiling water for 10–15 min. As expected (12), denatured collagen levels much higher than normally used to produce aggregation by native collagen were completely ineffective.

Purified α1(I) and α2 chains of RSSC (23, 32) were incubated in 0.9% NaCl at 37°C to obtain maximum solubility. We found that under these conditions α1(I) chains would aggregate platelets, but only at levels of about 1.3 mg. Lower amounts were found ineffective; also, the lower levels failed to inhibit HSIC induced aggregation. After heat denaturing these chains at 50°C, they were no longer effective even at levels of 6.5 mg. The α2 chains in 0.9% NaCl at 25°–27°C did not aggregate platelets at 2.7 mg, although 5.5 mg of lyophilized α2 chains were effective. Also, there was no inhibition by the α2 chains at 2.7 mg since aggregation proceeded normally upon addition of HSIC. Heat-denatured α2 chains were also ineffective in aggregation and inhibition of native collagen-mediated aggregation even at levels of 15.8 mg. Both the RSSC CNBr peptides (630 μg) and the purified α1(I)-CNBr #5 peptide (330 μg) failed to aggregate platelets and to inhibit aggregation by native collagen.

Effects of collagen modification—digestion by collagenase and trypsin. Fig. 1 shows the gel filtration elution profile of collagenase-trypsin-digested HSIC. The peaks were pooled as indicated and lyophilized. Based on the dry weights of the three pools, the recovery was 80%. The amino acid and carbohydrate analyses of pools I

TABLE I

Amino Acid and Carbohydrate Analyses of Pools I and II from the Collagenase-Trypsin Digest of Human Skin Insoluble Collagen*

Residue	I	II
Hyl	4.7	3.9
Lys	12.0	33.3
His	4.5	2.5
Arg	11.3	20.3
Hyp	13.2	88.9
Asp	37.4	42.6
Thr	16.2	12.2
Ser	23.8	24.8
Glu	46.9	65.9
Pro	61.4	87.8
Gly	153.6	225.1
Ala	94.2	64.0
Cys	0	0
Val	38.6	22.9
Met	2.6	0.6
Ile	12.1	7.4
Leu	20.0	12.8
Tyr	14.8	1.9
Phe	8.1	4.0
Glucosamine‡	4.9	0.7
Sialic acid§	0.7_7	—
Hexose‖	4.4	0.7_6

* Reported as micromoles of residue per 100 mg of either pool I or II for the amino acids and glucosamine, and as mg/100 mg for sialic acid and hexose.

‡ Small amounts of galactosamine were also found in pool I.

§ The sialic acid content of pool I represents an approximate upper limit; trace amounts of sialic acid were detected in pool I.

‖ From GLC analysis the galactose:mannose:fucose ratios in pool I are approximately 1:1:0.1:0.04. In pool II, the galactose:glucose ratio is about 4:3 and trace amounts of mannose and fucose are present.

and II are given in Table I. The first glycopeptide peak to elute, i.e., pool I, contains glucosamine, fucose, sialic acid, mannose, galactose, glucose, and trace amounts of galactosamine, whereas the second hexose positive peak, i.e., pool II, contains mainly galactose and glucose (some carry-over of pool I into pool II is indicated by the presence of glucosamine in the latter). The large ninhydrin-positive peak represents amino acids and small peptides. Each of the major peaks shown in Fig. 1 is known to be heterogeneous (15).

Pools I and II were dissolved in 0.9% and checked for platelet aggregation and inhibition. It was found that at levels of 10 μg to 1.0 mg, pool I neither aggregated platelets nor inhibited aggregation by HSIC. The latter studies were conducted after incubation of PRP with pool I for 5 min at 37°C; in fact, there was no effect of pool I on the lag time after addition of HSIC. Between 1 and 2 mg a decrease in optical density was noted; however, much of this was reversible after a few minutes. Again, there was no noticeable effect on aggregation upon addition of HSIC. Above 2 mg pool I gave what appeared to be a normal aggregation. Recognizing that pool I probably contains residual collagenase and trypsin from the preceding digestion, we added lima bean trypsin inhibitor to a portion of pool I at a level sufficient to inhibit all the trypsin in the original digest. As shown in Fig. 2 *a*, pool I was rendered ineffective in platelet aggregation, although occasionally extremely small particles were visually observed in the PRP. Also, even after incubation of PRP with 6.6 mg of pool I (plus trypsin inhibitor), HSIC produced normal aggregation. Trypsin inhibitor alone, ca. 2 mg, neither caused aggregation nor inhibited aggregation by HSIC. Based on the yield of pool I from the known amount of collagen that was digested, 6.6 mg of pool I represents about 154 mg of collagen equivalents. These data strongly suggest that the platelet aggregation caused by pool I can be attributed to residual tryptic activity. Supporting this, it has been shown that low levels of trypsin can lead to platelet nucleotide release and subsequent aggregation (40); we found that the addition of 100 μg of trypsin of PRP produced a gel very rapidly. In contrast, Hovig (41) found that the incubation of washed platelets with trypsin prevented subsequent aggregation with either collagen or ADP.

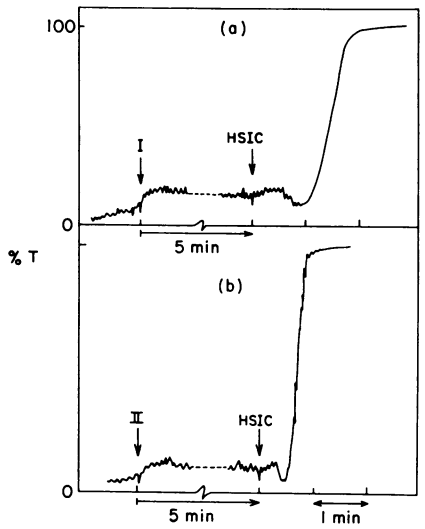

FIGURE 2 Collagen (HSIC)-mediated platelet aggregation profiles showing the absence of aggregating and inhibiting activity by pools I and II (% transmission vs. time with 1 ml of PRP at 37°C). (*a*) The first arrow denotes the addition of 1.65 mg of pool I (with trypsin inhibitor), and the second arrow denotes the addition of 4 μg of HSIC. (*b*) The first arrow indicates the addition of 1.0 mg of pool II, and the second arrow denotes the addition of 4 μg of HSIC.

TABLE II

Carbohydrate Analysis of Rat Skin Salt Soluble Collagen before and after Periodate Oxidation

Collagen	Galactose	Glucose
	% (W/W)	% (W/W)
Control	0.20	0.13_3
Periodate treated	0.00_3*	0.02_7*

* This represents an upper limit since the result is based on rather low absorbance values and artifacts; e.g., light scattering may make significant contributions. GLC analysis indicated greater than 84% destruction of galactose and greater than 93% destruction of glucose.

Pool II (4 μg to 2 mg) neither aggregated platelets nor inhibited their aggregation by HSIC as shown in Fig. 2*b* for 1.0 mg of pool II. Likewise, 2 mg of the large ninhydrin positive peak in Fig. 1 had no effect on platelet aggregation. As shown in Fig. 2, pools I and II did not produce aggregation during the 5 min incubation; other experiments (data not presented) confirmed there was no aggregation even after 30 min incubation.

Effects of collagen modification—alteration of the carbohydrate moiety. Carbohydrate analyses are given in Table II for normal and periodate-treated RSSC. In agreement with the earlier findings (13, 14, 28), the present GLC results show that galactose and glucose are the major carbohydrates present in soluble collagen. We finding that galactose and glucose comprise about 0.2 and 0.13% (w/w), respectively, of RSSC. It can be seen from Table II that periodate oxidation modifies most of the galactose and glucose units of RSSC.

Periodate-oxidized RSSC was found to aggregate platelets in a fashion similar to normal RSSC as shown in some typical aggregation profiles in Fig. 3 *a* and 3 *b*. The dependence of the extent of aggregation on RSSC (normal and periodate oxidized) concentration is present in Fig. 4. It can be seen that within experimental error periodate-oxidized RSSC aggregates platelets with the same effectiveness as unmodified RSSC. This was checked both before and after the addition of ethylene glycol, which alone did not produce platelet aggregation. To obtain maximum solubility, the periodate oxidized RSSC solution was maintained at 5°C (in the dark) until addition to the aggregometer. A control was always present under the same conditions(the addition of RSSC at 5°C gave a normal platelet aggregation although the lag times were generally somewhat longer than 25°C). We also reacted HSIC with periodate and found that the product still produced platelet aggregation. It was noted that extended incubations of RSSC with periodate (e.g., several days) yielded a derivative that was much less effective in platelet aggregation. Presumably, this is due to secondary reactions occurring at the protein moiety.

Alteration of the carbohydrate by incubation of RSSC with the galactose oxidase-catalase system of Spiro (30) for 27 h at either 30°C or at 35°C was also explored. This treatment decreased the solubility of collagen, but the suspension was still active in platelet aggregation. Incubation for 7 h at 25°C resulted in the modification of only 19% of the galactosyl groups as determined by the Galactostat method. Under these latter conditions, the modification probably involves mainly the non-glucosyl-linked galactosyl groups (which comprise about 35% of the total RSSC galactose) since

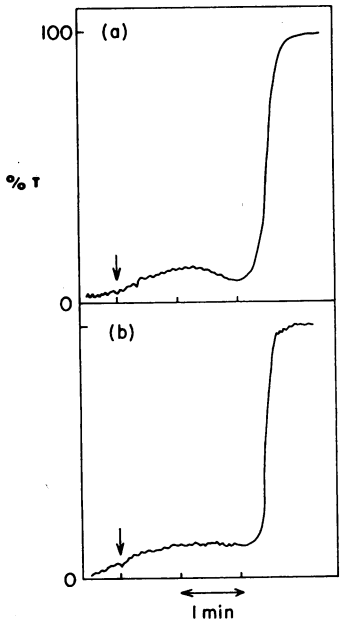

FIGURE 3 Collagen-mediated platelet aggregation profiles of normal and carbohydrate modified RSSC. (*a*) The arrow denotes the addition of 13.6 μg of RSSC in 0.9% NaCl. (*b*) The arrow indicates the addition of 6.8 μg of periodate oxidized RSSC.

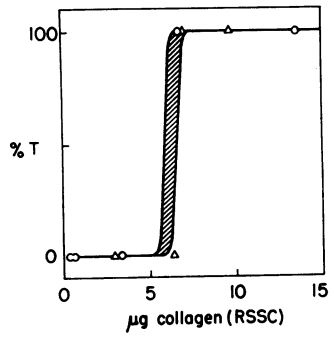

FIGURE 4 The percent transmission obtained from the aggregometer tracing as a function of the added amount of RSSC for normal (○) and periodate oxidized (△) collagen. The conditions were the same as given in Fig. 3.

Spiro (30) found that only 15% of the galactose (present as the disaccharide) of intact bovine renal glomerular basement membrane could be oxidized by galactose oxidase after 48 h at 37°C. The partially modified collagen (i.e., with 19% oxidation of the galactosyl units) aggregated platelets regardless of whether the digest was added at pH 6.8±0.2 (slightly opalescent solution) or at pH 4.7±0.1 (optically clear solution), although in the latter case there was a longer lag period. A control of galactose oxidase and catalase in the same buffers failed to produce aggregation. Although the 27 h incubation may increase the extent of galactose oxidation, it is unlikely that more than about 35–50% of the galactose would be modified in RSSC since the galactose of the disaccharide in intact protein is not readily oxidized (30). In any case, the product is fully active in platelet aggregation.

Studies involving the hydroxylysyl glycosides. The purity of the labeled hydroxylysyl glycosides was established by their R_f on a calibrated Dowex 50 column (28) and by amino acid analysis. The latter was also used quantitatively to determine concentrations. We found that Hyl-Gal (10–29 μM) and Hyl-Gal-Glc 18 μM) failed to produce platelet aggregation. These upper limits represents at least a several hundredfold increase over the amount of carbohydrate present in the minimum level of RSSC required for platelet aggregation. Also, phase microscopy indicated that there was no gross change in platelet morphology; moreover, relative to PRP there was no additional clumping. Spectroscopic measurements were made on the supernates of centrifuged PRP (control, hydroxylysyl glycoside treated and collagen treated); however, no conclusions could be drawn since all three samples gave similar spectra over the wave length interval 240–700 nm. We found that the absorption spectrum (400–700 nm) of PRP was only slightly altered by the addition of the hydroxylysyl glycosides (an apparent decrease in the optical density was also registered on the aggregometer). There appeared to be no inhibition of collagen-mediated aggregation since the subsequent addition of HSIC gave normal aggregation.

Whereas the hydroxylysyl glycosides neither produce platelet aggregation nor inhibit aggregation, it is possible that they bind to platelets. The results of our binding studies using PRP are summarized in Fig. 5 where the extent of binding r, i.e., the number of molecules of hydroxylysyl glycoside bound per platelet, is plotted as a function of the logarithm of the unbound hydroxylysyl glycoside concentration over the range of about 0.2 nM to 9 μM Hyl-Gal and 0.4 nM to 0.25 μM Hyl-Gal-Glc. The brackets indicate the estimated counting and dilution errors associated with each measurement. We are using the term "binding" in an operational sense,

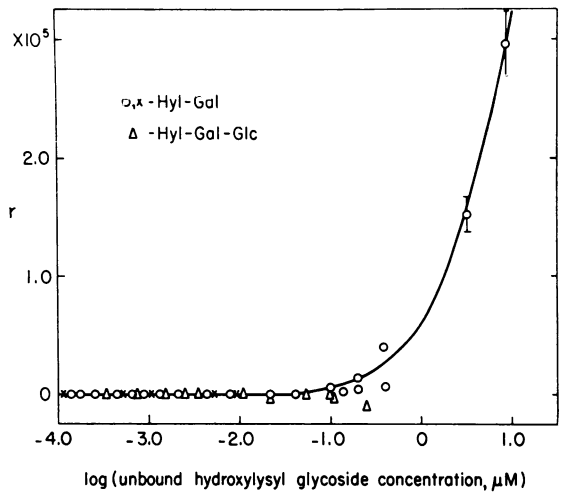

FIGURE 5 The extent of binding, r (molecules of bound hydroxylysyl glycoside per platelet), versus the logarithm of the unbound concentration. The measurements were made using tritiated Hyl-Gal and Hyl-Gal-Glc in the presence of 1 ml of PRP and the conditions given in Methods. The ×'s denote experiments with Hyl-Gal in the presence of UDP-Glc (0.1 μM). The solid line is theoretical and was plotted according to equation 1 with the parameters given in the text.

namely the measured differences in radioactivity between samples of PRP and PPP incubated with identical amounts of the tritiated hydroxylysyl glycosides. Clearly, this does not distinguish binding at platelet membrane receptors from what may be cellular uptake via diffusion.

Although there is binding of Hyl-Gal above ca. 50 nM (represents approximately 50 collagen equivalents and about twice that present in the minimum amount of collagen required for aggregation) there is no indication of Hyl-Gal-Glc binding up to 250 nM. Above 250 nm Hyl-Gal-Glc, we obtained an apparent "negative binding," perhaps due to a platelet-dependent fluorescing factor. If so, the extent of Hyl-Gal binding may be even higher. In any case, 250 nM Hyl-Gal-Glc represents about 125 collagen equivalents and roughly six times that present in the minimum collagen concentration necessary for platelet aggregation. Binding studies on washed platelets from outdated platelet concentrates also indicated some binding of Hyl-Gal, thus showing that a serum factor is not required for this interaction. Many of the binding studies involving Hyl-Gal were also performed in the presence of UDP-Glc; as can be seen in Fig. 5, this had no effect on binding.

The solid line in Fig. 5 is theoretical and was plotted according to the equation below. This relationship follows from the law of mass action (42) and gives the dependence of r on the number of identical, noninteracting

binding sites per platelet, n, and the binding constant, k,

$$r = nk(L)/[1 + k(L)] \quad (1)$$

In this equation (L) denotes the unbound ligand (either Hyl-Gal or Hyl-Gal-Glc) concentration. As shown in Fig. 5, the binding results for Hyl-Gal can be described in terms of a single class of sites (ca. 650,000 per platelet) and a binding constant of $k = 10^5$ M^{-1}. (In Fig. 5 we have plotted r as a function of log (L) rather than a Scatchard plot $[r/(L)$ vs. $r]$, in order to emphasize the essential absence of binding over a wide range of hydroxylysyl glycoside concentration.)

DISCUSSION

Platelet aggregation by normal collagen. It has been reported that although neither native bone nor tendon collagen will produce platelet aggregation (19, 43), other insoluble collagens as well as soluble collagens are effective (10, 12). Our results clearly demonstrate that purified RSSC and other soluble collagens are effective in extremely small amounts in platelet aggregation. Although even slightly smaller amounts of purified insoluble skin collagens are effective, the use of soluble collagen is preferred for both research and clinical purposes since the reproducibility of measurements is greatly improved and, also, because soluble collagens are chemically better characterized than are insoluble collagens.

The various skin collagens used in this study are of the $\alpha 1(I)_2$ $\alpha 2$ class (44), whereas the chick cartilage collagen (26, 44) and the bovine articular cartilage collagen (45) are of the $\alpha 1(II)_3$ class. Interestingly, both classes of collagen are active in platelet aggregation.

Platelet aggregation by protein-modified collagen. Purified $\alpha 1(I)$ and $\alpha 2$ chains (23, 32) are both active in inducing platelet aggregation as also recently shown by Katzman et al. (46). The finding, however, that relative to native RSSC, large amounts of the individual collagen chains are required for platelet aggregation indicates that there is little intrinsic activity in the separated chains. Also, the difficulty in obtaining purified chains without some contamination by crosslinked chains or partially renatured material is well established (23), and, thus, some caution is in order regarding these results. Based on the lack of activity in heat-denatured collagen, others have concluded that the collagen-fold is essential for aggregation (12, 47). We have extended this to show that high concentrations of heat-denatured collagen and α-chains do not inhibit the aggregation caused by native collagen. This indicates that there is little interaction of the heat-denatured material with platelets, at least at the "aggregation" binding sites for native collagen.

Zucker and Borrelli (10) originally reported that purified *Clostridium histolyticum* collagenase destroyed the ability of both human subcutaneous tissue and mouse tail tendon to aggregate platelets, and later Wilner et al. (12) confirmed that collagenase inactivated the aggregating ability of human skin insoluble collagen. Chesney et al. (47) found that tadpole collagenase inactivated acid-soluble guinea pig skin collagen. This latter enzyme cleaves each of the three collagen chains in nearly the same position producing two triple helical fragments. (48–51). On the other hand, Houck and Chang (52) have reported that a rat cutaneous collagenase (53) yields some peptides from RSSC that can produce platelet aggregation.

Our results on the glycopeptide fractions of HSIC after extensive bacterial collagenase and trypsin digestion demonstrate that at the milligram level, these collagen fragments neither aggregate platelets nor inhibit native collagen-mediated platelet aggregation after the residual trypsin activity has been destroyed. In contrast, Jamieson et al. (19) found that a carbohydrate-rich fraction isolated from a collagenase digest of bovine tendon collagen was able to inhibit collagen aggregation. The source of the collagenase was not stated, and the active fraction was not characterized. Thus, it has not been demonstrated whether this glycopeptide fraction is of the pool I type or the pool II type (containing hydroxylysyl glycosides), as suggested by these investigators. Also, incompletely digested collagen and, perhaps, residual proteolytic activity may be responsible for the observed effects.

These various results on the platelet aggregation activity of collagen after collagenase digestion can largely be rationalized on the basis that certain fragments of collagen, containing a particular amino acid sequence(s) and perhaps some ordered structure reminescent of the conventional collagen-fold (with perhaps the ability to aggregate into microfibrils), can adhere to platelets and induce the release reaction. Thus, various collagenases that exhibit different specificities (54) may destroy collagen activity either by causing chain unwinding by extended proteolysis and drastically reducing the affinity for the (platelet membrane) collagen binding site (see below) or by cleaving an essential amino-acid sequence.

Katzman et al. (46) have recently shown that the $\alpha 1(I)$-CNBr #5 peptide from chick skin collagen at a concentration of about 250 μM will aggregate human platelets. At a concentration of 85 μM the RSSC $\alpha 1(I)$-CNBr #5 peptide neither produced platelet aggregation nor inhibited platelet aggregation by native collagen. These results raise interesting questions on species specificity and on the mechanism of action of platelet aggregation by intact collagen. The latter finding is consistent with our observation that the mixture of CNBr peptides from RSSC has no platelet aggregating or inhibitory activity. Heat-denatured $\alpha 1(I)$ chains at 65

μM neither produced nor inhibited native collagen-induced platelet aggregation.

Platelet aggregation by carbohydrate-modified collagen. Chesney et al. (47) reported that galactose oxidase-modified acid-soluble guinea pig skin collagen was not effective in producing platelet aggregation. After reduction with borohydride, however, the collagen was again rendered active in platelet aggregation. These results imply that an intact and accessible collagen galactosyl unit is essential for human platelet aggregating activity in guinea pig skin collagen. However, the derivative was not characterized and, thus, it can only be assumed that galactose was the group affected. Our results demonstrating that the platelet aggregating activity of galactose oxidase-catalase-modified RSSC is unchanged from that of RSSC are not surprising since under conditions which optimized the solubility of the derivative, only 19% of the RSSC galactose was modified. The use of more drastic conditions (e.g., higher temperature and extended digestion times) yielded a derivative that was quite insoluble but was, nevertheless, active in platelet aggregation.

Our results on periodate-oxidized RSSC and HSIC clearly demonstrate that the normal carbohydrate sidechains of these collagens are not required for platelet aggregation. In contrast, Kang et al. (55) reported the periodate oxidation inactivated insoluble chick skin collagen. Assuming that none of the common side reactions associated with periodate oxidation (56) occurred, these results indicate a striking species specificity with regard to the collagen carbohydrate. Alternatively, collagen carbohydrate may simply not be a critical element in the highly specific, physiological role of collagen in platelet aggregation.

We found that Hyl-Gal (29 μM) and Hyl-Gal-Glc (18 μM) neither produce nor inhibit the native collagen-mediated aggregation of platelets. At high concentrations (e.g., about 20,000 μM), Katzman et al. (46) demonstrated that Hyl-Gal-Glc inhibits aggregation of platelets by the chick α1(I) chains, although Hyl-Gal-Glc itself did not induce platelet aggregation. The binding sites we found for Hyl-Gal may represent the platelet membrane glycosyltransferases (16, 18, 19); however, our data suggest that these do not represent the binding sites which are responsible for platelet aggregation.

Another consideration in evaluating these apparently conflicting results (other than that of the different collagens used) is that collagen microfibrils may enhance platelet aggregation as suggested by Jaffe and Deykin (57). The carbohydrate moieties of collagen have been implicated in fibril formation (58), and, conceivably,

TABLE III

Summary of Platelet Aggregation Studies Using Collagen and Collagen Derivatives*

Collagen or derivative	Platelet aggregation activity (PAA)	Minimum weight relative to soluble collagen for PAA	Minimum moles relative to soluble collagen for PAA
Native soluble collagen	+	1	1
Periodate oxidized soluble collagen	+	1	1
Native insoluble collagen	+	0.5	0.5
α1(I)	+	220	650
α2	+	920	2,750
Heat-denatured collagen	—	>490	>490
Heat-denatured α1(I)	—	>1,100	>3,200
Heat-denatured α2	—	>2,630	>7,900
CNBr peptides-soluble collagen	—	>105	—
α1(I)-CNBr #5	—	>55	>4,250
Hyl-Gal	—	>1.4	>1,420
Hyl-Gal-Glc	—	>1.4	>880
Insoluble collagen high mol wt glycopeptide fraction	—	>1,100	—
Insoluble collagen low mol wt glycopeptide fraction	—	>330	—

* Soluble collagen refers to RSSC and insoluble collagen refers to HSIC. The chains and CNBr peptides are from RSSC.

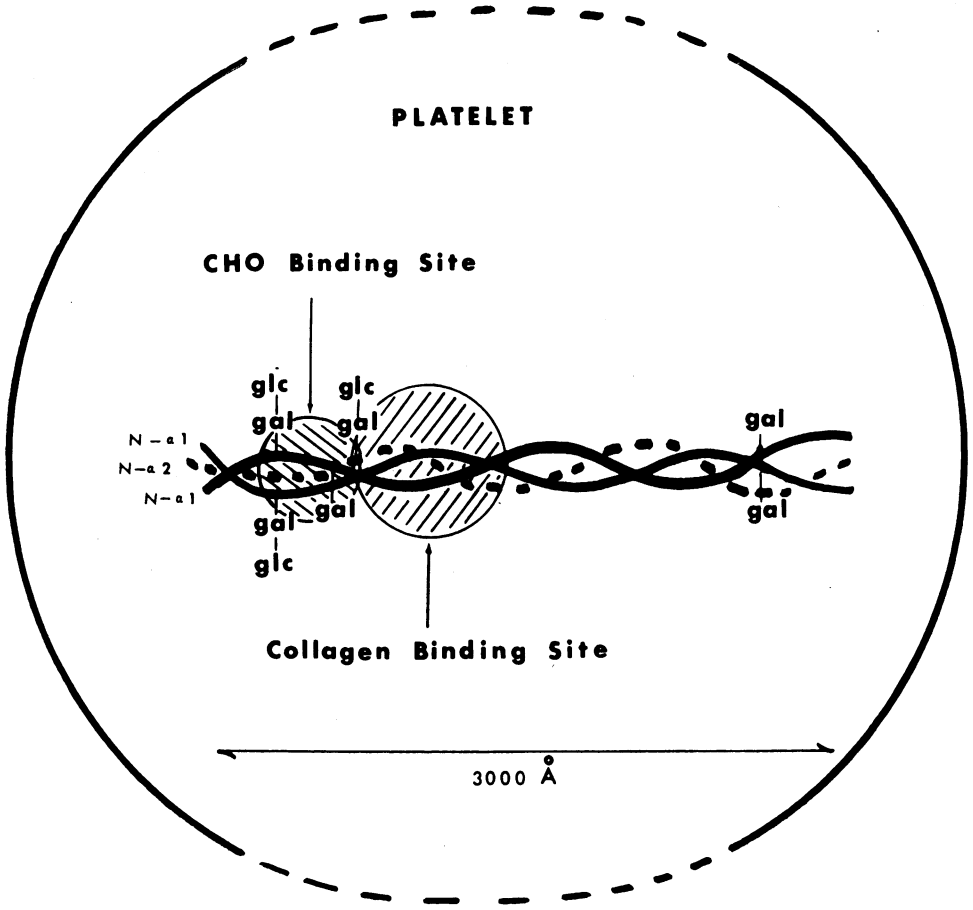

FIGURE 6 Schematic drawing of the collagen-platelet interaction as explained in the text.

different types of modification could yield derivatives with differing tendencies for fibril formation.

Collagen-platelet interactions; general considerations. Our results on platelet aggregation by collagen and various derivatives are summarized in Table III. We have also listed the minimum concentrations (relative to RSSC) required for platelet aggregation activity, or the maximum levels of derivatives we tried which were ineffective. These data show that purified α1(I) and α2 chains are much less effective than RSSC, but that heat-denatured collagen and chains, as well as low molecular weight derivatives, are ineffective in producing platelet aggregation even at the highest concentrations we used.

These data, along with the results of Katzman, Kang, and Beachey (46, 55) have led us to develop the tentative scheme shown schematically in Fig. 6 to summarize our present understanding of collagen-platelet interactions. We propose the presence of at least two distinct sites on the platelet plasma membrane. One is a binding site that leads to platelet aggregation. This site is believed to have high specificity and affinity for the native triple-helical collagen polypeptide moiety or perhaps even for a particular amino-acid sequence in the collagen fold. The affinity of this collagen-platelet interaction is strongly dependent on collagen conformation. As the degree of ordered structure decreases, as in chains or particular peptides, much higher concentrations of the derivatives are required to produce platelet aggregation.

In close proximity to this site is a binding site for carbohydrate units. The platelet receptor in this case may be the glycosyl transferases (16–20). The affinity between this site and the collagen carbohydrate is much less than that of the other site and the collagen protein. Since both the chick skin α1(I)–CNBr #5 peptide, which can produce platelet aggregation (46, 55), and the majority of the carbohydrate are near the amino-terminal region of soluble skin collagen (59, 60), the two proposed binding sites may both have some specificity for this same general area of this collagen molecule. Thus, in certain limiting conditions such as extremely high concentrations, the binding of low molec-

ular weight collagen carbohydrate derivatives at the carbohydrate binding site may inhibit the binding by collagen or various collagen derivatives, e.g., chains and peptides. Also, in the case of collagen chains and peptides lacking a high degree of ordered structure but containing carbohydrate, the carbohydrate binding site interaction may provide sufficient stabilization to enable the nonstructured polypeptide moiety to assume an active conformation at the aggregation site. Binding at the carbohydrate site only it, however, neither sufficient nor necessary for platelet aggregation. Thus, we envision these two sites as being distinct in terms of physiological responses.

We conclude that under physiological conditions where the criteria of high specificity and affinity are presumably met, the collagen carbohydrate is not a determining factor in collagen binding to platelets, but, rather, the effect is mediated primarily by the native protein moiety.

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