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Research Article

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Platelet Interaction with Modified Articular Cartilage

ITS POSSIBLE RELEVANCE TO JOINT REPAIR

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ABSTRACT During studies concerned with the platelet-collagen interaction, it was observed that platelets did not adhere to bovine or human articular cartilage and that cartilage did not induce platelet aggregation *in vivo* or *in vitro*. To study the mechanism responsible for this observation, the role of proteoglycans was examined. Purified cartilage collagen proved to be fully active as a platelet aggregant. Addition of small amounts of proteoglycan subunit (PGS) blocked platelet aggregation, whereas chondroitin sulfate, a major glycosaminoglycan component of cartilage matrix, impaired platelet aggregation only at concentrations which resulted in a marked increase in viscosity. Moreover, PGS abolished aggregation of platelets by polylysine but did not prevent aggregation by ADP, suggesting that PGS may block strategically placed lysine sites on the collagen molecule. Treatment of fresh articular cartilage with proteolytic enzymes rendered the tissue active as a platelet aggregant. *In vivo* experiments demonstrated that surgical scarification of rabbit articular cartilage does not result in adhesion of autologous platelets. Treatment of rabbit knee joints with intraarticular trypsin 1 wk before the injection of blood resulted in adhesion and aggregation of platelets on the surface of the lesions. Since there is evidence from other studies that some degree of cartilage healing may take place after initiation of an inflammatory response, it is postulated that induction of platelet-cartilage interaction may eventuate in cartilage repair.

INTRODUCTION

The role of collagen in platelet adhesion and aggregation has been a subject of intensive research for more than a decade (1-3). In general, these studies

have been aimed at the elucidation of hemostasis or the pathogenesis of atherosclerosis (for review see 4). The possibility that platelets may also serve a crucial function in connective tissue repair has sometimes been raised but has never been pursued in depth. It is generally recognized that irreversibly aggregated platelets form a firm substrate for the deposition of fibrin as well as for the proliferation of fibroblasts. The nature of the intermembranous bond between individual platelets or the mechanism whereby platelets adhere to collagen is therefore of great interest from many points of view. Yet, despite several attractive theories which have been advanced in recent years (5-9), in essence the process has remained poorly understood. On the basis of currently available evidence, it seems likely that a minimal structural unit consisting of rigidly spaced polar groups is necessary for platelet adhesion and subsequent aggregation to occur (10-13). To this should be added that, *in vivo*, the reactivity of a given connective tissue with platelets may not only be a reflection of the spacing and incidence of the active sites on the collagen molecule, but also of the type, concentration, and steric conformation of the surrounding mucopolysaccharides which may block these sites. Thus, we observed that platelets do not adhere to cartilage and that minced cartilage does not induce platelet aggregation *in vitro* (14). This corroborates clinical experience that intact or lacerated cartilage does not support thrombus formation. On the other hand, after complete extraction of the proteoglycan matrix, cartilage collagen induced platelet aggregation. This process appeared to involve the formation of periodic bridges between the platelet membranes and the dense bands of the collagen fibrils. Re-addition of defined moieties of proteoglycans re-established the inertness of cartilage collagen *vis-à-vis* platelets (14). Since it seemed possible that a modification of the cartilage matrix would permit platelets to interact with cartilage

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collagen *in vivo* and thereby set the stage for cartilage repair, a study was undertaken in rabbits to demonstrate that platelets would also adhere to cartilage enzymatically treated in the living animal. The experiments involving the action of platelets with native and modified articular cartilage *in vitro* and *in vivo* form the subject of this report.

METHODS

Platelet-rich plasma (PRP)¹ was prepared from normal subjects with plastic disposable syringes containing 1 vol of 3.8% trisodium citrate for each 9 vol of blood collected. For some experiments, the blood was collected in heparin as described before (15). The specimens were centrifuged at room temperature at 250 *g* for 7 min, after which the PRP was removed with a Pasteur pipette. Platelet counts ranged between 200,000 and 400,000/mm³. Platelet-poor plasma (PPP) was prepared similarly except that the anticoagulated blood was centrifuged for 20 min at 1,200 *g*. The interaction of various collagen preparations and control compounds with platelets was measured turbidimetrically with the help of an aggregometer (Chrono-Log Corp., Havertown, Pa.) by a modification of the method of Born and Cross (16). The instrument was adjusted for 100% light transmission with 0.4 ml PPP and for 0% light transmission with 0.4 ml PRP. Generally, the test compounds were added in volumes of 0.1 ml after equilibration after the addition of 0.05 ml 1% calcium chloride. Alternatively, the experiments were performed in the absence of exogenous calcium chloride. ADP was obtained from Sigma Chemical Co., St. Louis, Mo., and D,L-polylysine from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y. When native cartilage was used in aggregometer studies, 12–15 pieces measuring approximately 1 mm³ suspended in 0.1 ml Hanks' solution were added to PRP.

Preparation of proteoglycans. Proteoglycans and collagen were prepared from bovine proximal humeral articular cartilage. The isolation, chemical, and physical characterization of the proteoglycan aggregate and subunit species used in this study were described in detail previously (17, 18). Briefly, proteoglycans were extracted from bovine articular cartilage in 3 M MgCl₂, 0.15 M potassium acetate, pH 6.3, at 5°C. Proteoglycan aggregate was reassociated by dialysis against 0.15 M potassium acetate, pH 6.3, at 5°C, then isolated by equilibrium density gradient centrifugation under associative conditions. Fraction 6 from this gradient, recovered at densities greater than 1.69 g/ml, contained 16S proteoglycan subunit and 70S proteoglycan aggregate in approximately equal amounts. Proteoglycan subunit was isolated from the mixture of aggregate and subunit by equilibrium density gradient centrifugation under dissociative conditions in 4 M guanidine hydrochloride, 3.0 M cesium chloride. The proteoglycan subunit is referred to as PGS.

Preparation of purified articular cartilage collagen (CC). For the complete removal of the extractable proteoglycan, fresh diced bovine articular cartilage was extracted by slow stirring at 5°C, first in 3 M MgCl₂ 0.01 M MES (2-[N-morpholino] ethane sulfonic acid), pH 6.3, for 48 h, then sequentially with 3 M GuHCl, 0.01 M MES, pH 6.3,

for 48 h as detailed previously (17). The cartilage residue was suspended in water (1 g/10 ml) and rehydrated by slow stirring at 5°C for 16 h. Aliquots of the residue were then frozen under liquid nitrogen and freeze-powdered under liquid nitrogen with a Spex mill (Spex Industries, Inc., Metuchen, N. J.). When needed, 10 g of the finely powdered collagen was suspended in 500 ml of water, stirred at 5°C for 16 h, then filtered through a fine nylon mesh to remove large particles. The collagen was then again collected by centrifugation (5,000 rpm for 30 min) and freeze dried.

Preparation of chondroitin sulfate. Proteoglycan dissociatively extracted from bovine nasal cartilage was degraded with 0.2 N NaOH at room temperature for 24 h. The solution was neutralized with HCl and 1% potassium acetate was added. The degraded proteoglycan was precipitated with 3 vol of cold ethanol, collected by centrifugation, washed with ethanol, followed by ether, and dried in a vacuum. The product was degraded sequentially with pepsin, trypsin, and papain, as previously described (19). Fractions of chondroitin sulfate and keratan sulfate were separated by a stepwise 5% ethanol fractionation in 0.8% BaCl₂. The chondroitin sulfate fraction isolated in 25% ethanol was converted to the K⁺ salt and used in the studies described here.

Viscosity measurements. Solutions were prepared by dissolving samples dried to constant weight in 0.15 M NaCl, 0.02 M N-Tris hydroxymethyl-2-aminoethane sulfonic acid, pH 7. Addition of solvent to dry samples and all dilutions were made by weight. Viscosity measurements were made with an Ubbelohde type viscometer (Cannon Instrument Co., State College, Pa.) in a constant temperature water bath maintained at 20±0.01°C with a mercury in glass type thermoregulator (Arthur H. Thomas Co., Philadelphia, Pa., model 9655G), and a Beckman differential thermometer (Beckman Instruments, Inc., Fullerton, Calif.).

Preparation of whole fresh wet cartilage. Bovine articular cartilage was collected and added immediately to ice-cold 0.15 M sodium chloride. Specimens of human articular cartilage were prepared from limbs amputated for a variety of reasons. The cartilage was diced in 1-mm cubes, washed and used as such, or aliquots were incubated at 37°C overnight in the following solutions: (a) Trypsin: 1 mg/ml (0.1%) in 0.15 M NaCl, 0.01 M CaCl₂, 0.05 M Tris buffer at pH 8.0; (b) Papain: 1 mg/ml in 0.15 M NaCl, 0.005 M cysteine, 0.005 M EDTA, 0.05 M MES at pH 6.0; (c) Pepsin: 1 mg/ml in 0.15 M NaCl, 0.05 M MES at pH 3.8.

Electron microscopy. For ultrastructural studies, 2–5-ml aliquots of PRP were incubated with fresh cartilage, purified CC proteoglycans, or other agents in the same proportions as they were used for the turbidimetric assays. Fixation was accomplished in suspension by the addition of equal amounts of 3% phosphate-buffered glutaraldehyde at 37°C when gross aggregation had begun in a manually agitated test tube. When no aggregation ensued the specimens were fixed after a minimum of 15 min of incubation with the compound tested. Such specimens were centrifuged at 1,200 *g* for 5 min to pellet the formed elements before fixation. To improve contrast of the platelet surface coat and the inter-platelet bridges, ruthenium red at a concentration of 1 mg/ml was added to the fixatives essentially as described by Luft (20). Postfixation was achieved in 2% osmium tetroxide for 2 h. Dehydration and embedding in Epon (Shell Chemical Co., New York) was carried out as in previous reports (21). Thin sections were cut with an LKB ultratome equipped with a diamond knife (LKB Instruments, Inc., Rockville, Md.). A Siemens Elmiskop

¹Abbreviations used in this paper: CC, cartilage collagen; CS, chondroitin sulfate; MES, 2-(N-morpholino) ethane sulfonic acid; PGS, proteoglycan subunit; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

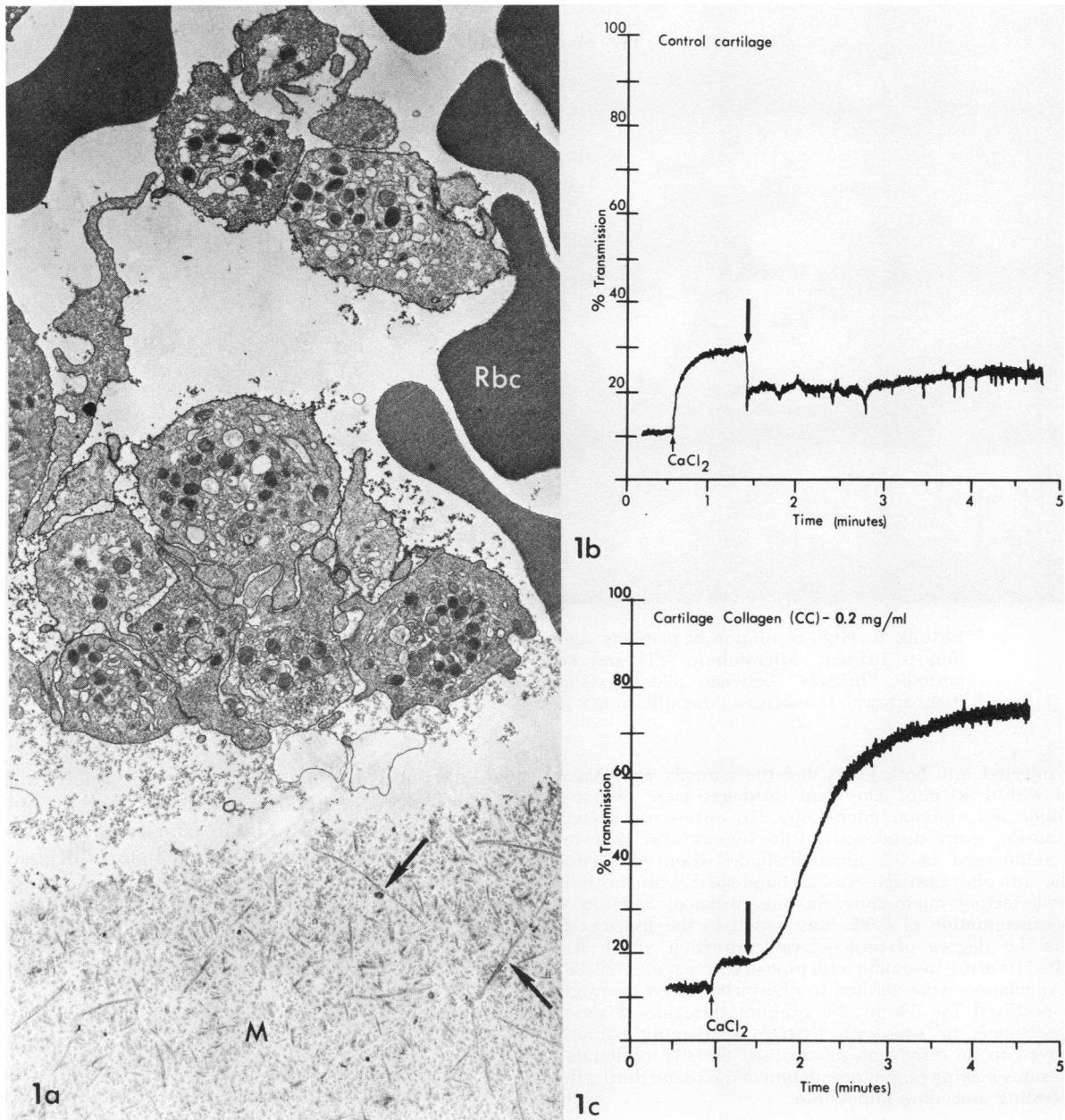


FIGURE 1 (a) Electron micrograph of platelets sedimented onto fresh articular cartilage. Although the platelets have been deformed by centrifugation, they are not degranulated and show no adherence to the cartilage. Rbc, erythrocytes; M, cartilage matrix; Arrows indicate collagen fibrils. Magnification $\times 8,000$. (b) Aggregometer curve obtained with fresh control cartilage shows that no aggregation is taking place. (c) Curve obtained with collagen extracted from same cartilage. Arrows indicate when substances were added.

I electron microscope (Siemens Corp., S. Iselin, N. J.) was the instrument used for all studies.

In vivo experiments. The procedure for creation of the joint cartilage lesions has become standard in a number of laboratories. In brief, adult New Zealand rabbits weighing about 4 kg underwent arthrotomy of both knees under ether anesthesia. The patella was retracted medially exposing the articular surface of the femur. Longitudinal

scarifications were made into the articular cartilage with a no. 15 scalpel blade. The wound was irrigated with normal saline, closed with interrupted sutures of no. 4 chromic catgut or nylon. The wounds were allowed to heal up to 2 wk after which the experimental joint of each animal was injected with 5 mg trypsin in saline whereas the control joint of the same animal was only given saline. 1 wk later, 0.5 ml of freshly collected heparinized autologous blood

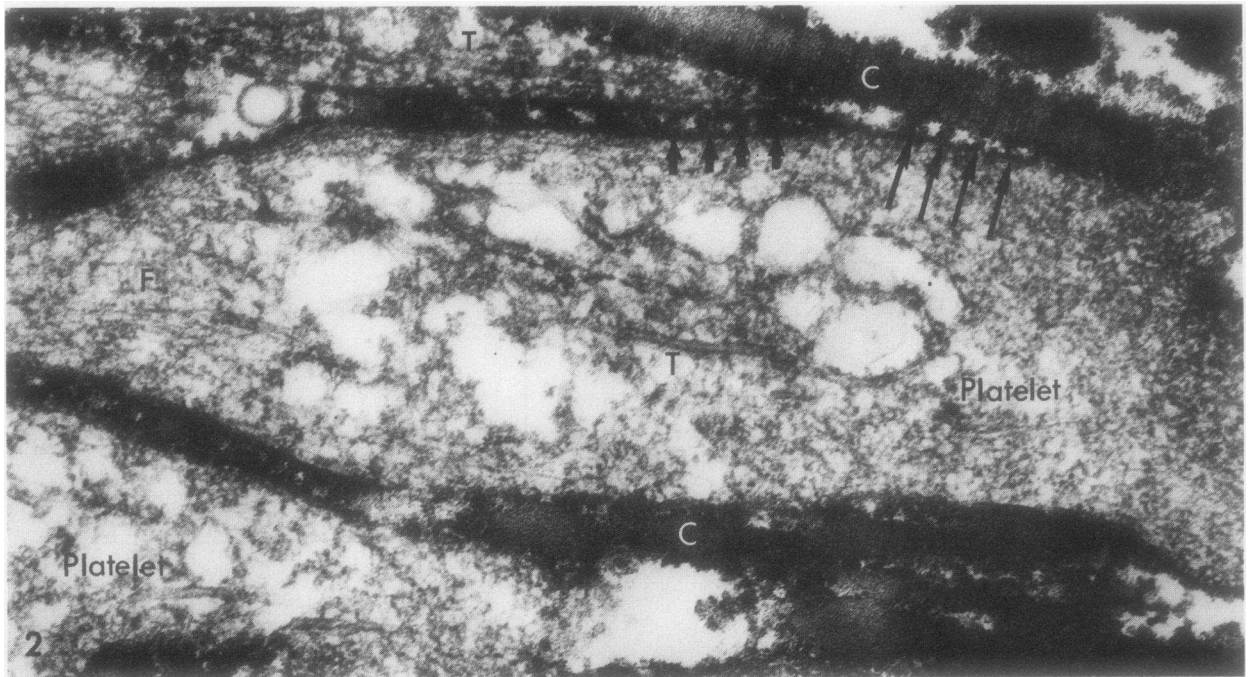


FIGURE 2 High resolution of platelets aggregated with CC as shown in Fig. 1c. Degranulation is evident. Microtubules (T) and actin filaments (F) (21) remain. Arrows indicate periodic "bridges" between platelets (short arrows) and between platelets and collagen (long arrows). C, collagen. Magnification $\times 72,000$.

was injected into both joints and the animals were sacrificed within 30 min. The joint cartilages were prepared for light and electron microscopy. To ensure satisfactory fixation the entire distal ends of the femora were removed and submerged in 3% glutaraldehyde where dissection of the articular cartilage was accomplished with the help of a dissecting microscope. In one instance, Safranin O, at a concentration of 0.6%, was added to the fixatives to assess the degree of proteoglycan extraction which had resulted from the treatment with proteolytic enzymes (22, 23). The specimens were washed in phosphate buffer overnight and postfixed for 2 h in 2% osmium tetroxide. Fixation of specimens *in situ* with fixatives containing Safranin O proved to be disadvantageous since the dye obliterated the lesions making proper orientation of the tissue during the embedding procedure impossible.

RESULTS

Finely minced fresh articular cartilage did not aggregate platelets, nor did platelets adhere to this tissue even when the cartilage and platelets were pelleted together by centrifugation (Figs. 1a and b). On the other hand, when proteoglycans were completely extracted, the collagen residue (CC) which had retained its tertiary fibrillar structure supported platelet aggregation (Fig. 1c). Moreover, on electron microscopy, it became evident that the "bridges" which form between the membranes of platelets aggregated by other agents (15) could also be resolved between

platelets and collagen, and that the periodicity of the "bridges" corresponded to the dense banding of the collagen fibrils (arrows, Fig. 2). The addition of small amounts of PGS to the CC 5 min before incubation with platelets inhibited the platelet collagen interaction as evidenced by the turbidimetric as well as morphologic studies (Figs. 3 and 4). It is noteworthy that purified proteoglycans alone did not cause platelet aggregation even when high concentrations amounting to 2 mg of PGS were used. Since PGS markedly increased the viscosity of the suspending medium, the possibility existed that platelets were prevented from aggregating on this basis alone. Two studies were performed to exclude this possibility. Platelets were incubated with PGS after which an amount of ADP was added known to induce aggregation in the absence of PGS. As is seen in Fig. 5, a 20-fold increase in the minimum concentration of PGS which had impaired aggregation of platelets with collagen did not prevent aggregation of platelets with ADP. Secondly, chondroitin sulfate (CS), the major glycosaminoglycan component of PGS, was tested for its effect on the platelet collagen interaction. Fig. 6 shows the relative viscosity curves of PGS and CS. Only when the calculated viscosity of CS was 10 times as high as that of PGS and when its concentration was almost 200-fold that of PGS

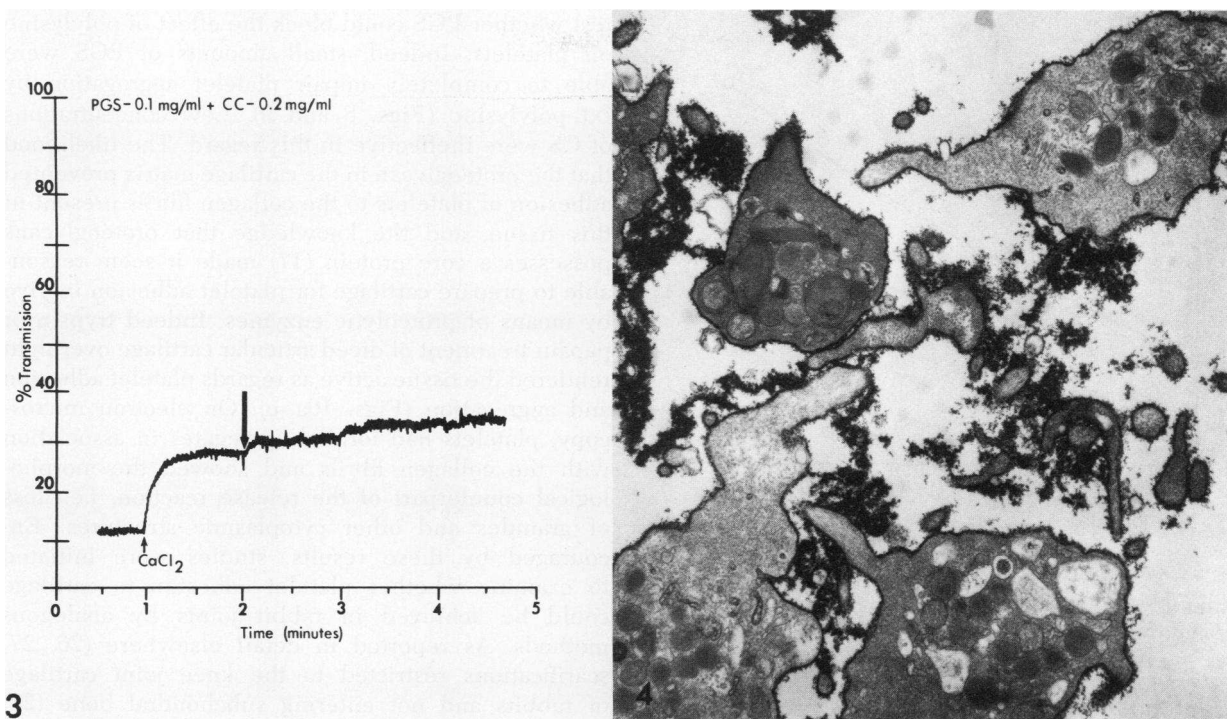


FIGURE 3 Aggregometer curve obtained when proteoglycan was added to CC. No aggregation ensued.

FIGURE 4 Appearance of platelets sedimented with CC and PGS used in the experiment illustrated in Fig. 3. Platelets are not aggregated or degranulated. Black material between platelets represents collagen fibrils and PGS fixed in the presence of ruthenium red. Magnification $\times 14,000$.

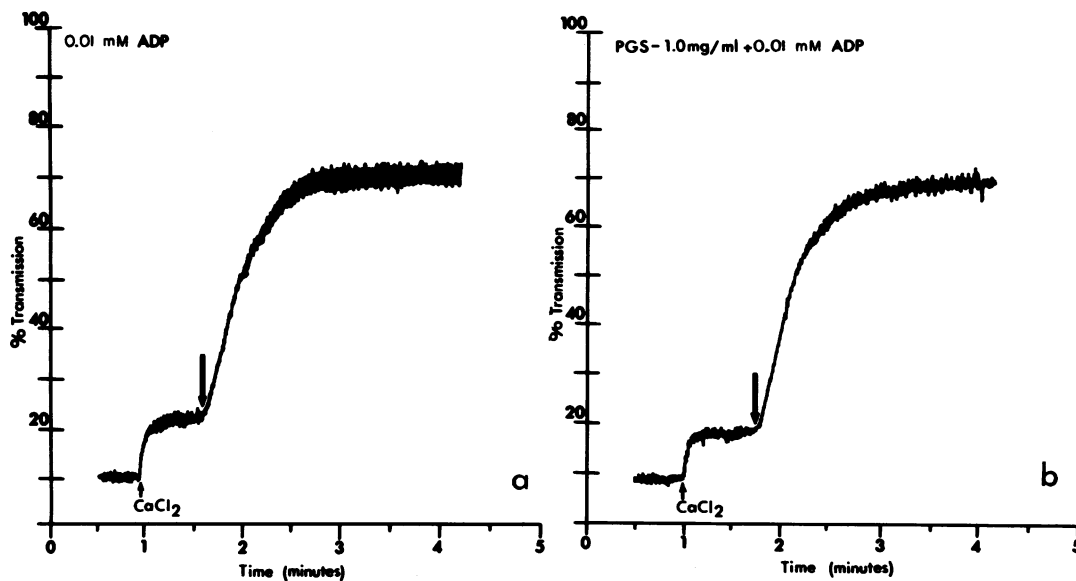


FIGURE 5 (a) Control platelets aggregated with ADP. (b) An aggregometer curve demonstrating that aggregation of platelets by ADP is not impaired in the presence of a concentration of PGS (1 mg/ml) five times higher than that required to inhibit aggregation with collagen.

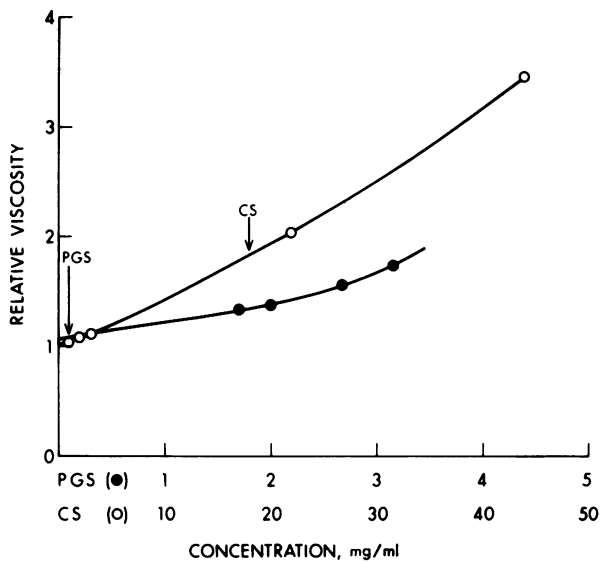


FIGURE 6 Relative viscosity curves of proteoglycan aggregate and chondroitin sulfate.

did inhibition of aggregation by CS become noticeable (Fig. 7). It is thus likely that PGS is more specific than CS in blocking the critical sites on the collagen molecule which interact with platelets. Since it had been suggested that free lysine groups on the collagen molecule may play an important role in triggering the platelet reaction (5) and since it had also been demonstrated that polylysine is a potent platelet aggregant (24, 25), it seemed of interest to

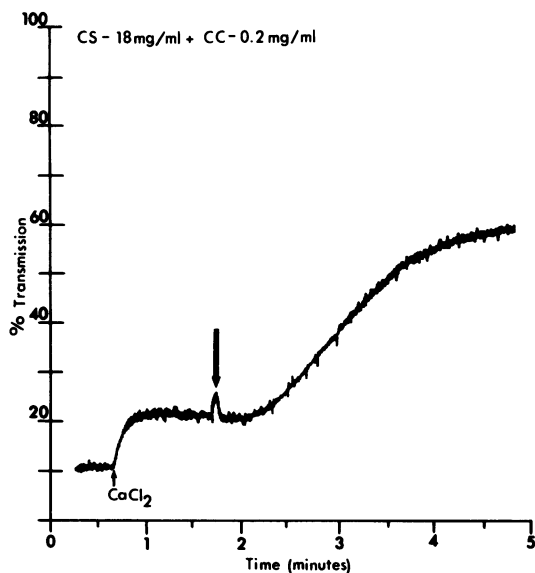


FIGURE 7 Aggregometer curve showing that 200-fold concentration of CS causes delayed and incomplete aggregation. This effect may be due to slow release of endogenous ADP.

test whether PGS could block the effect of polylysine on platelets. Indeed, small amounts of PGS were able to completely impair platelet aggregation by D,L-polylysine (Figs. 8 and 9). Low concentrations of CS were ineffective in this regard. The likelihood that the proteoglycan in the cartilage matrix prevented adhesion of platelets to the collagen fibrils present in this tissue, and the knowledge that proteoglycans possesses a core protein (17) made it seem reasonable to prepare cartilage for platelet adhesion *in vivo* by means of proteolytic enzymes. Indeed trypsin or papain treatment of diced articular cartilage overnight rendered the tissue active as regards platelet adhesion and aggregation (Figs. 10a-c). On electron microscopy, platelets had formed aggregates in association with the collagen fibrils and showed the morphological counterpart of the release reaction, i.e., loss of granules and other cytoplasmic structures. Encouraged by these results, studies were initiated to examine whether platelet adhesion to cartilage could be achieved in rabbit joints by analogous methods. As reported in detail elsewhere (26, 27) scarifications restricted to the knee joint cartilage of rabbits and not entering subchondral bone (28) showed no evidence of inflammation or repair no matter how long after arthrotomy such lesions were examined.² In the present study, no platelets or fibrin were seen to adhere to the cartilage surface 48 h after the intraarticular injection of 0.5 ml of heparinized whole blood, although a few morphologically unaltered erythrocytes and leukocytes were present in the space created by the lesion (Figs. 11a-c). On the other hand, joints which had been treated with intraarticular trypsin 8-12 days before the injection of blood showed not only adhesion of numerous platelets to the surface of the cartilage lesion, but in many areas platelets had aggregated and undergone degranulation (Fig. 12). Leukocytes which were probably derived from the injected blood were sometimes seen in association with such platelet aggregates.

DISCUSSION

The observations recorded here have theoretical as well as practical implications. As regards the platelet collagen interaction, it has become generally recognized that a multimeric or tertiary structure of collagen is a prerequisite for platelet aggregation (10-13) even though such polymerization may take place in reaction mixtures which, at the outset, contain only glycopeptide subfragments (29). The corollary, that this requirement is not met by collagen preparations or tissues which fail to aggregate platelets, has

² Rosenberg, L. Unpublished observations.

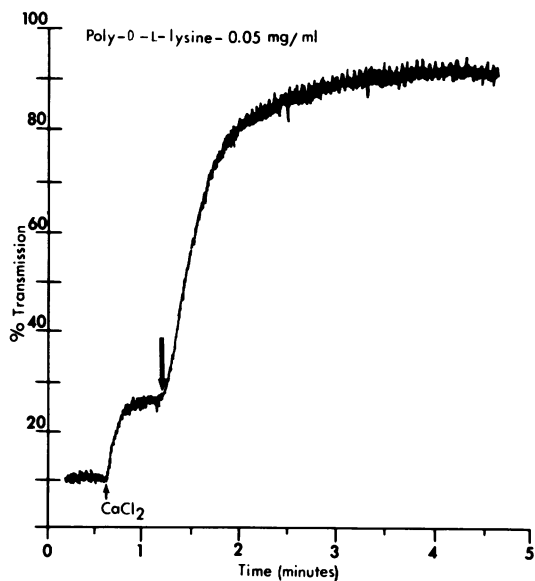


FIGURE 8 Aggregation of platelets with D,L-polylysine.

been tacitly assumed. Since all phases of the present study have been carried out with fibrillar collagen confirmed by electron microscopy, a discussion of inhibitors which may interfere with collagen polymerization and therefore preclude platelet aggregation is not relevant. On the other hand, few investigators have given consideration to other molecules present in connective tissue matrices which even at low concentration could block collagen sites which interact with the platelet membrane. As has been shown in this study, small amounts of some protein polysaccharide moieties can block collagen aggregation of platelets whereas others, such as CS do not. Therefore, the reason why some connective tissues or basement membranes are more reactive with platelets than others could be attributable to the type and/or location of the protein polysaccharides rather than

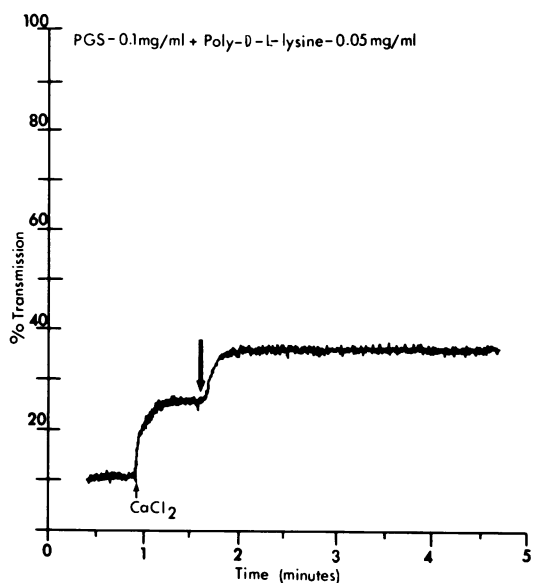
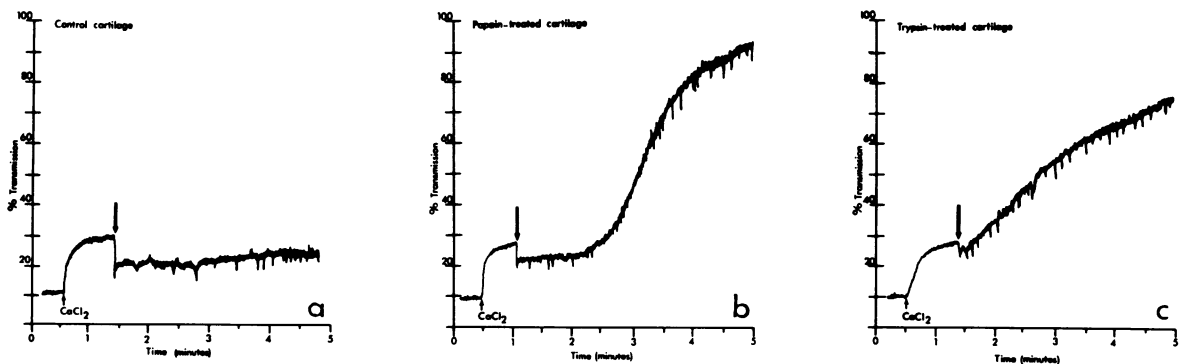
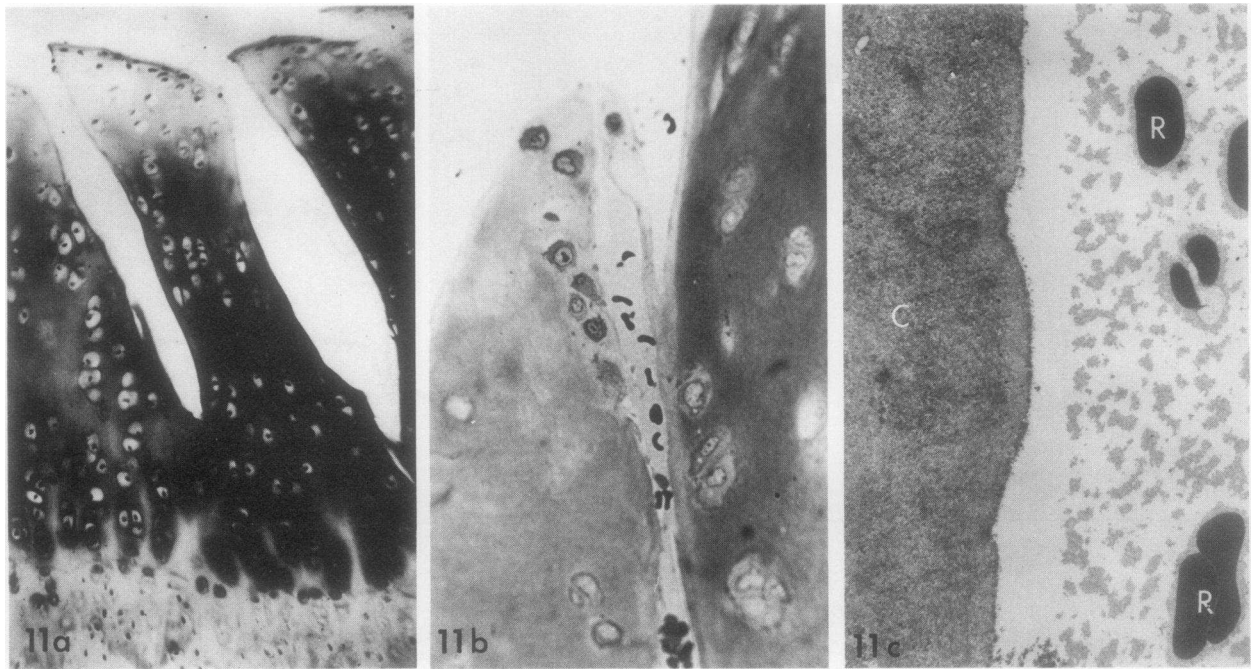


FIGURE 9 Complete inhibition of platelet aggregation by D,L-polylysine in the presence of 0.1 mg/ml PGS which is reminiscent of the inhibitory effect of PGS on platelet aggregation by collagen.

to the nature or degree of multimerization of the collagen contained therein. In any case, proteoglycan blockage may be effective even when the platelet aggregant is a single chain polypeptide as has been exemplified by the observation reported here that small amounts of PGS can prevent platelet aggregation by polylysine (Fig. 9). One wonders whether cyanogen bromide peptides of collagen which purportedly did not reassociate (30) would also be inhibited by PGS. At any rate, the observations make it seem unnecessary to postulate a platelet receptor which is specific for collagen. The architecture of the intact collagen fibril may merely provide suitably spaced polar groups (be they positive or negative)



FIGURES 10a-c Comparison of curves obtained with (a) untreated control cartilage showing no platelet aggregation, (b) cartilage treated with papain showing complete aggregation after a 2-min lag phase, and (c) curve obtained with trypsin-treated cartilage.



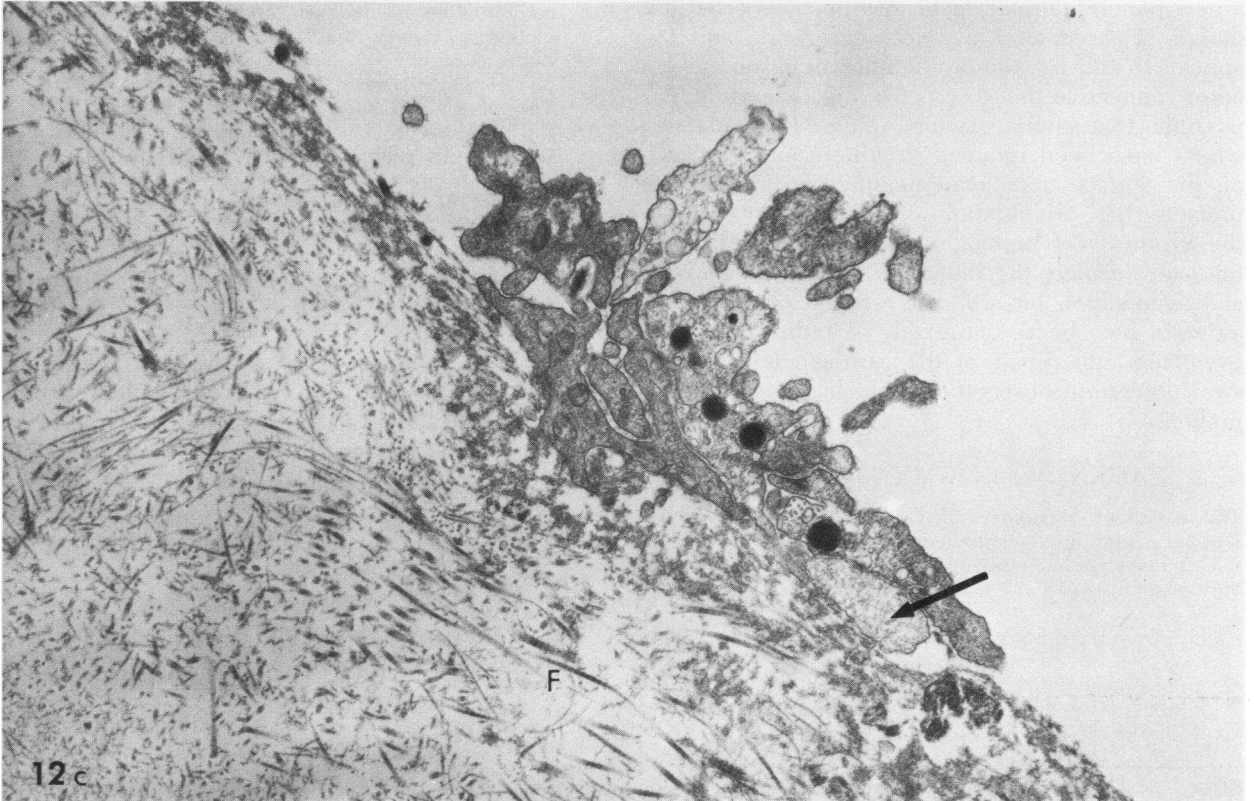
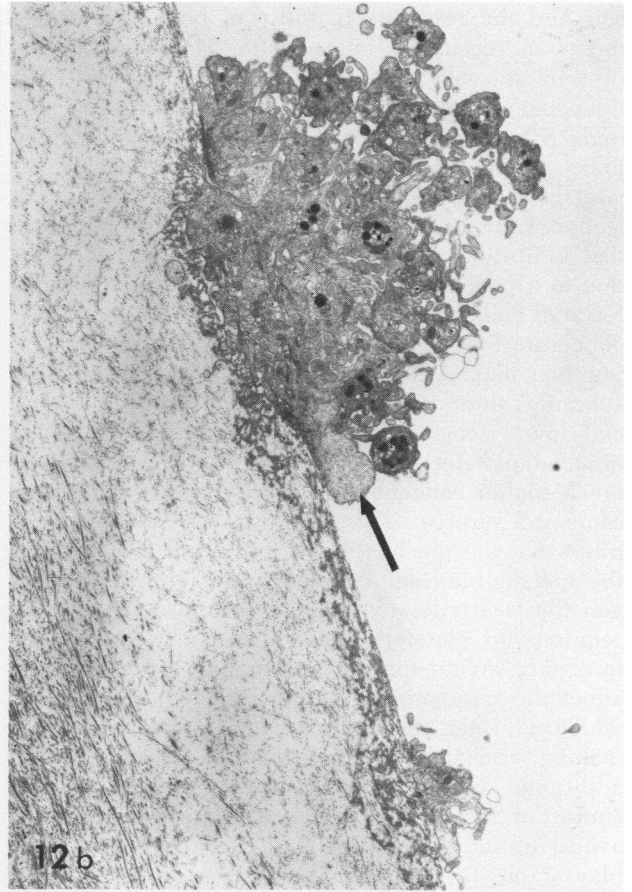
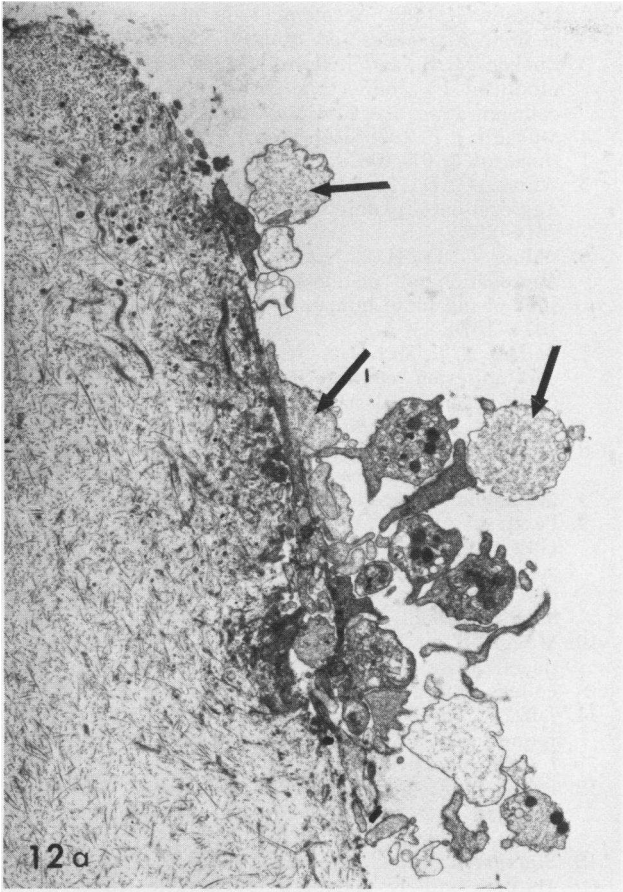
FIGURES 11a-c Illustrations of surgically induced articular cartilage lesion. (a) Conventional photomicrograph of a lesion 8 mo after surgery stained with Safranin O. The grooves remained patent and no repair is evident. (b) Phase photomicrograph of a thick section through the lesion from a joint which had been injected with autologous blood. Erythrocytes are seen in the groove, but no thrombus has formed. (c) Electron micrograph of the lesion illustrated in Fig. 11b verified the absence of adhering platelets. R, Erythrocytes; C, Cartilage. Magnification $\times 2,400$.

able to trigger platelet release. In this context the identical spacing of bridges between aggregated platelets and those between platelet membranes and collagen fibrils (Fig. 2) may be significant. It should also be noted that the location of the bridges corresponds to the dense bands of the collagen fibril, the presumed site of polar residues in most models of collagen structure (31). It will be of interest to examine whether the number of bridges corresponds to the number of sites on the platelet membrane which need to be cross-linked covalently or ionically, as the case may be, for the release phenomenon to occur. In the final analysis, the mechanisms that induce platelet aggregation may be analogous to those which take place when various ligands or multivalent antibodies cross-link sites on the lymphocyte membrane causing this cell to undergo transformation. It is possible that in the case of platelets, the number of

sites which need to be cross-linked for release to occur may be found by an analysis of the precise nature and binding site of PGS to collagen. For that purpose, ultrastructural studies will have to be carried out utilizing smaller tagged subunits of proteoglycan or its core protein rather than the aggregate used in the present experiments.

The practical aspects of the studies described in this communication were designed to explore conditions which may promote healing of articular cartilage. After injury to most other tissues an inflammatory response ensues which, broadly speaking, consists of adhesion and aggregation of platelets, conversion of fibrinogen to fibrin, and the invasion of fibroblasts and other cells. When the injury is limited to articular cartilage, no inflammatory response takes place. The reason for the lack of such response has long been attributed to the absence of blood ves-

FIGURES 12a-c Several areas of the surface of the articular cartilage lesions obtained from a rabbit knee joint which had been treated with intra-articular trypsin 1 wk before the injection of autologous blood. Platelets are seen adherent in many areas. The majority of platelets have undergone degranulation (arrows). Occasionally, fairly large thrombi have formed in intimate association with the cartilage (12b). The cartilage matrix seems to have been partially extracted (12c). Periodicity of fibrils (F) is seen at higher magnification (12c). Magnification: 12a, $\times 6,000$; 12b, $\times 4,000$; 12c, $\times 11,000$.



sels and the resulting inability of blood-borne elements to reach the injured site. However, in the present as well as in many previous studies (27), it has been clearly demonstrated that cartilage lacerations (Figs. 11a and b) do not support platelet or fibrin adhesion even when blood is present in the joint as a result of arthrotomy or intraarticular injection. In the initial phases of this work, we believed that inhibition of platelet adhesion to cartilage was due to a physical effect, i.e., contact between platelets and collagen fibrils was prevented by the dense concentration of proteoglycan aggregates covering the collagen fibrils. The finding that proteoglycan subunit solutions at extremely low concentrations and low viscosities completely inhibited collagen-induced platelet aggregation while CS solutions at much higher concentrations and viscosities did not, came as a surprise. This observation raises the possibility that the mechanism of inhibition may involve the specific binding of proteoglycan subunit to collagen fibrils at sites close to or identical to the sites required for platelet adhesion. As suggested in the foregoing, treatment with proteolytic enzymes may affect the core protein of the proteoglycan subunit which may interact with the collagen molecule, thereby making crucial sites accessible to platelets. One additional observation deserves mention in the context of this discussion. During platelet aggregation a host of active substances are released, known to play a role in the inflammatory response. Recently a new platelet-derived factor has been reported (32) that is released after platelet aggregation and that appears to enhance the proliferation of fibroblasts and other connective tissue cells (33, 34). Therefore, it is possible that similar factors released by platelets which have been induced to adhere and aggregate on the surface of enzyme-modified cartilage could promote the proliferation of chondrocytes and/or the synthesis of proteoglycans by these cells. The first step, namely the induction of platelet adhesion and aggregation on articular cartilage in the living animal, has been achieved. Whether this will eventuate into repair of the cartilage lesions must await long-term observations which are currently in progress.

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