Brief Definitive Report

RELEASE OF HEPARAN SULFATE FROM ENDOTHELIAL CELLS

Implications for Pathogenesis of Hyperacute Rejection

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In normal blood vessels, heparan sulfate proteoglycan on the surface of endothelial cells functions to maintain an anticoagulant environment by localized activation of antithrombin III (1, 2), a potent inhibitor of thrombin generation. Heparan sulfate proteoglycan also renders endothelial surfaces relatively impermeable to transit of blood cells and plasma proteins (3, 4). Intravascular coagulation and extravasation of blood elements are prominent pathological features of hyperacute rejection (5) and other diseases thought to be mediated by the binding of antibodies to endothelium and the consequent activation of complement. We therefore used an in vitro model to test the hypothesis that binding of human natural antibodies to xenogeneic endothelium might lead to a change in cell-associated proteoglycan, which in turn might eventuate the pathological picture of hyperacute rejection (6–11).

Materials and Methods

Source of Sera. Serum samples from normal individuals were pooled or used individually. Antibody reactivity with cultured porcine endothelial cells was assayed by ELISA (12). Serum was obtained from two individuals deficient of C2 (13) and pooled serum from which Factor B had been immunoabsorbed was obtained from Cytotech (San Diego, CA). The natural antibody titers of complement-deficient sera were comparable to those of normal human sera. Porcine serum had no reactivity and one human serum had very markedly decreased reactivity with cultured endothelial cells (12). Purified C2 (Cordis) used at 2,000 U/ml reconstituted C2-deficient serum to low normal complement activity (CH50).

Endothelial Cell Cultures, Biosynthetic Labeling, and Extraction of Proteoglycans. Porcine aortic endothelial cells were cultured in DME with 20% vol/vol heat-inactivated FCS and antibiotics (14). Endothelial cell identity was based on the ability to take up acetylated low density

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lipoprotein (15). Cell monolayers were labeled with [^{35}S]sulfate (100 μCi/ml) in DME (0.124 μM sulfate) for 16 h, washed, then incubated at 37°C in RPMI 1640 containing 25% vol/vol pooled fresh human serum. The supernatant was saved and the cell fractions were then extracted with 4 M guanidine HCl, 0.05 M sodium acetate, 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 0.5 mg/ml 1,10-phenanthroline, 1 mg/ml benzamidine, 1 mM iodoacetamide, pH 5.8, at 4°C for 24 h. Extracts and supernatant fractions were exhaustively dialyzed in 3,500 dalton exclusion tubing against 0.5 M sodium acetate, 0.1 M sodium sulfate, 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, and 10 mM PMSF. Percent proteoglycan release was determined as: 100 × [(cpm medium)/(cpm medium + cpm cells)].

For determination of ^{51}Cr release, endothelial cell monolayers in 2-cm^2 wells were washed with RPMI 1640 and then labeled with ^{51}Cr (2 μCi/well) for 2–3 h at 37°C. Cells were then washed with RPMI 1640 and incubated in human serum as described above. The supernatant fraction (*A*) was removed, cells (*B*) were extracted with 1 N NaOH, and the radioactivity was counted. The control cells were incubated in RPMI alone and supernatants (*C*) were counted. Percent cytotoxicity was determined as: 100 × [(A − C)/(A + B − C)]. Values shown represent the mean of four determinations. In some experiments, endothelial cells were exposed to human serum for 1 h, washed, and then incubated in [^{3}H]thymidine (1μCi/ml) or [^{3}H]uridine (4μCi/ml) for 16 h. Cell fractions were then washed and extracted as described above.

Characterization of Endothelial Cell Proteoglycans. Porcine endothelial cell extracts and supernatant fractions prepared as described above were dialyzed into 6 M urea, 0.1 M NaCl, 0.05 M Tris, 0.2% CHAPS with protease inhibitors, pH 7.0, applied to DEAE-Sephacel columns and eluted with a 0.1 M NaCl to 2.0 M NaCl gradient in the same buffer (14). Fractions representing eluted peaks were pooled and applied to a 100 cm × 0.6 cm Sepharose CL-4B column, equilibrated, and eluted with 4 M guanidine HCl in 0.05 M NaAc (V_o 19.6 ml; V_t 49 ml). Samples were also studied by electrophoresis in 0.6% agarose–1.6% polyacrylamide gels from which fluorograms were prepared (16). Samples of cell and supernatant fractions were digested with heparitinase or with chondroitinase ABC in the presence of protease inhibitors (17) before electrophoresis.

Results and Discussion

Exposure of porcine endothelial cells to human serum caused release of ~5% of labeled proteoglycans at 4 min and >50% at 1 h (Fig. 1). Loss of heparan sulfate proteoglycan from endothelial cells at these times did not reflect irreversible changes leading to cell death as endothelial cells prelabeled with ^{51}Cr and then exposed to fresh human serum released proteoglycan before any detectable release of ^{51}Cr-labeled material (Fig. 1). Further, endothelial cells exposed to fresh human serum for 1 h and then washed and placed back in culture incorporated the same amounts of [^{3}H]uridine (246, 225 cpm) and [^{3}H]thymidine (10,067 cpm) during a subsequent 16-h period, as did controls [^{3}H]uridine (255,568 cpm), [^{3}H]thymidine (11,125 cpm). After continued exposure of endothelial cells to human serum for 4 h, release of ^{51}Cr was observed (Fig. 1), and incorporation of [^{3}H]thymidine decreased (data not shown).

Purification and analysis of endothelial cell proteoglycans after treatment of cultured cells with human serum for 1 h revealed that the smaller of two heparan sulfate proteoglycans normally associated with porcine endothelial cells (2, 18, 19) was no longer present (Fig. 2). The supernatant contained fragments of this proteoglycan that, based on elution from molecular sieve columns and electrophoretic migration, consisted of glycopeptides or glycosaminoglycan chains. The released proteoglycan and fragments were shown to be heparan sulfate, as they were sensitive to digestion by heparitinase (Fig. 2) but not chondroitinase ABC (data not shown). These results

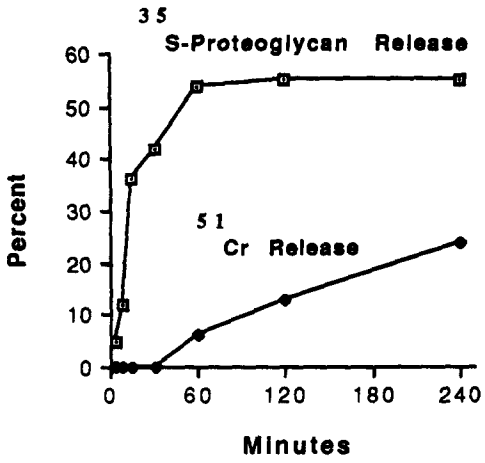

FIGURE 1. Release of [^{35}S]sulfate-labeled proteoglycans and ^{51}Cr from cultured porcine aortic endothelial cells exposed to human natural antibodies and complement in human serum.

suggest that release is mediated by enzymatic cleavage of the protein core and/or of glycosaminoglycan chains. In contrast, supernatant from endothelial cells exposed to human serum for 4 h contained intact proteoglycan, possibly reflecting vesiculation or cell lysis as well as proteoglycan fragments (Fig. 2 *D*).

The cleavage and release of endothelial cell proteoglycans appeared to be triggered by the binding of natural antibodies to endothelial cells and activation of complement. Proteoglycan release was observed with pooled human sera (Fig. 1) and with sera obtained from individuals manifesting natural antibody reactivity with porcine endothelial cells. In contrast, serum from one individual with a very low natural antibody titer did not cause proteoglycan release (Table I). That the critical component lacking in this serum in terms of its inability to mediate release of heparan sulfate is the absence of the natural antibodies is evidenced by the ability of that serum to mediate release from endothelial cells precoated with natural antibodies (Table I). In addition, porcine serum that contained no detectable anti-porcine endothelial antibodies caused no release.

The role of complement activation was evaluated by exposing cultured porcine endothelial cells to human sera deficient in complement (Table I). Heat-inactivated human serum or human sera deficient in C2 did not cause release of labeled proteoglycans. Repletion of the C2-deficient serum with purified C2 to low normal complement activity enabled that serum to mediate proteoglycan release comparable to that seen for normal individuals. Factor B-depleted serum did cause proteoglycan release. These results indicated that cleavage and release of endothelial proteoglycans depended upon activation of the classical complement pathway.

The loss of heparan sulfate proteoglycan from endothelial cells mediated by natural antibodies and complement may induce a series of events that could well contribute in a major way to two of the key features of hyperacute rejection: interstitial hemorrhage and intravascular thrombosis. It seems reasonable to suggest that the same mechanism, i.e., loss of heparan sulfate proteoglycan, may be involved in other diseases such as hemolytic uremic syndrome or Kawasaki disease, which may be mediated by anti-endothelial cell antibodies (20, 21). The presence of heparan sul-

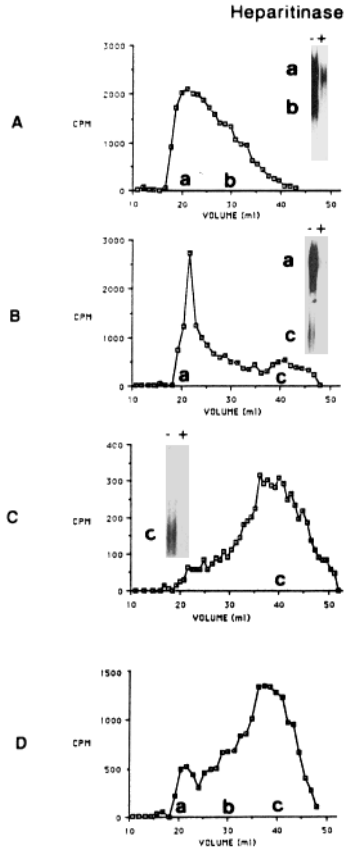

FIGURE 2. Analysis by gel filtration chromatography and gel electrophoresis of [^{35}S]sulfate-labeled porcine endothelial cell proteoglycans after treatment of monolayers with human serum. (*A*) Proteoglycans from porcine aortic endothelial cells, exposed to heat-inactivated (56°C, 30 min) human serum, eluted from Sepharose CL-4B columns as overlapping peaks denoted *a* (K_{av} 0.04) and *b* (K_{av} 0.35). Sensitivity of corresponding electrophoretic bands to heparitinase (designated +) is shown. (*B*) Proteoglycans from endothelial cells exposed to fresh human serum were in peak/band *a* and in a minor, late eluting peak/band *c* (K_{av} 0.73), whereas peak/band b was absent. (*C*) Supernatant of endothelial cells briefly exposed (15 min) to human serum contained molecules in the form of glycopeptides and/or glycosaminoglycans (peak/band *c*) sensitive to digestion by heparitinase. (*D*) Supernatant from monolayers exposed to human serum for 4 h contained proteoglycan fragments as in *C* and larger, apparently intact proteoglycans (peaks *a–c*).

TABLE I

Role of the Classical Pathway in Release of Proteoglycans from Cultured Porcine Endothelial Cells by Human Serum

Serum	[^{35}S]Proteoglycan released percent*
Five individual sera	27–57 (x = 43.2)
Five heat-inactivated sera	2.1–3.8 (x = 2.9)
Natural antibody deficient serum #1	7.1
Normal heat-inactivated serum + natural antibody-deficient serum #1	45.0
C2-deficient serum 1	3.5
C2-deficient serum 2	12.7
C2-deficient serum 2 + purified C2	30.7
Factor B–depleted serum	49.4
Porcine serum	5.1

* The percent release of [^{35}S]sulfate-labeled proteoglycans from cultured porcine endothelial cells was determined after incubation of labeled endothelial cells with medium containing 25% serum for 60 min as described in Materials and Methods. In one experiment, endothelial cells were incubated in normal heat-inactivated serum (which by itself caused no release at 60 or 120 min), washed, and then incubated with natural antibody-deficient serum 1. C2 alone caused no release.

fate proteoglycan on endothelial cell surfaces is involved in binding of extracellular superoxide dismutase (22, 23), which protects against injury by oxygen radicals (24). The loss of heparan sulfate proteoglycan from the endothelial cells might render the cells subject to oxidant-mediated cell injury, as occurs in organ preservation (reperfusion) injury. The understanding of the mechanisms leading to loss of heparan sulfate proteoglycan may allow the devising of strategies to prevent the sequelae of that loss.

Summary

Heparan sulfate proteoglycan associated with endothelial cells in normal blood vessels inhibits intravascular coagulation and egress of blood cells and plasma proteins, key features of hyperacute rejection. It was shown herein that exposure of cultured porcine endothelium to human serum as a source of natural antibodies and complement caused cleavage and release of 5% of endothelial cell proteoglycans within 4 min and >50% within 1 h. Proteoglycan release depended on activation of the classical complement pathway and preceded irreversible cell injury. These findings suggest that loss of endothelial cell proteoglycan may be a critical step in the pathogenesis of hyperacute rejection and in diseases involving humoral injury to endothelial cells.

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