Oxidative degradation of rat mast-cell heparin proteoglycan

Dean D. METCALFE,* Helen L. THOMPSON,* Seymour J. KLEBANOFF† and William R. HENDERSON, Jr.†‡

*Mast Cell Physiology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, U.S.A., and †Department of Medicine, University of Washington School of Medicine, Seattle, WA 98195, U.S.A.

The susceptibility of rat mast-cell heparin to oxidative degradation was examined. Heparin as a component of intact mast-cell granules (MCG) was degraded following ingestion by normal human neutrophils. In contrast, neutrophils from patients with chronic granulomatous disease (CGD), which do not respond to stimulation with respiratory-burst activity, exhibited a greatly diminished ability to degrade phagocytosed MCG heparin. MCG-associated heparin also was cleaved by H_2O_2 plus Fe^{2+} (Fenton's reagent). Isolated heparin proteoglycan (average M_r approx. 750 000) was rapidly cleaved to smaller molecules similar in size to commercial pig heparin upon exposure to Fenton's reagent. This cleavage was inhibited by catalase and by the hydroxyl-radical ($\text{OH}^\bullet$)-scavenger mannitol, but not by superoxide dismutase (SOD). The cleavage products retained approx. 26% of the anticoagulant activity of the native molecule. The heparin proteoglycan was also cleaved by acetaldehyde/xanthine oxidase/ FeSO_4 , a system that generates superoxide ($\text{O}_2^{\bullet-}$), H_2O_2 and $\text{OH}^\bullet$. Whereas the cleavage at relatively high iron ion concentrations was inhibited by catalase and mannitol but not by SOD, at lower iron ion concentrations the cleavage was inhibited by catalase, mannitol and SOD. These findings suggest the involvement of $\text{OH}^\bullet$, which at high Fe^{2+} concentrations is generated by Fenton's reagent (H_2O_2 plus Fe^{2+}), and at low iron ion concentrations is generated by the iron-ion-catalysed interaction between $\text{O}_2^{\bullet-}$ and H_2O_2 (Haber–Weiss reaction). These studies suggest that oxygen radicals generated by activated phagocytes may contribute to the degradation *in vivo* of both solubilized and granule-associated proteoglycan heparin.

INTRODUCTION

Native high- M_r heparin proteoglycan has been identified in rat [1], mouse [2] and human [3] mast cells. This heparin is released into the extracellular space following both immunological and non-immunological mast-cell activation [4], where it remains firmly bound to proteinases and certain other proteins as a constituent of the mast-cell-granule (MCG) matrix. It expresses both anticoagulant [1,3,5] and anti-complementary [6,7] activities. Heparin subsequently undergoes degradation *in vivo*. In one study ^{35}S -labelled ox heparin glycosaminoglycan was injected either intraperitoneally or intravenously into rats; within 24 h 50% of the radioactivity was detected in the urine, where it was recorded in lower- M_r degradation products [8]. Among the cells responsible for heparin degradation are cells capable of phagocytosis. ^{35}S -labelled heparin in membrane-free MCG has been shown to be phagocytosed and degraded by mouse and guinea-pig macrophages and neutrophils [9,10], rat fibroblasts [11] and bovine endothelial cells [12], with free [^{35}S]sulphate being released into the extracellular fluid.

The biochemical pathways responsible for heparin degradation are incompletely understood. The catabolism of glycosaminoglycans in mammalian organisms may require the sequential intracellular action of endoglycosidases, exoglycosidases and sulphatases, which are widely distributed in mammalian tissues [13,14]. Endoglycosidases cleave glycosaminoglycan side chains at internal positions and thus increase the number of target sites for exo-enzymes. Endoglycosidases capable of degrading heparin-like molecules have been demonstrated in mouse mastocytoma tissues [15] and human platelets [16,17].

An alternative mechanism by which some phagocytes may degrade heparin is through the generation of oxidants. Neutrophils, eosinophils and mononuclear phagocytes respond to stimulation with an increase in O_2 consumption (respiratory burst), and much, if not all, of the extra O_2 consumed is

converted into toxic O_2 metabolites [18]. The initial product formed is the superoxide anion ($\text{O}_2^{\bullet-}$), which is converted into H_2O_2 in a dismutation reaction (reaction 1) as follows:

H_2O_2 in some phagocytes can react with peroxidase and a halide to form potent oxidants with toxic activity. H_2O_2 can also react with ferrous salts (Fenton's reagent) to form hydroxyl radicals ($\text{OH}^\bullet$) (reaction 2). When the iron ion concentration is limiting, reduction of the Fe^{3+} formed is required for continued $\text{OH}^\bullet$ generation. This can be accomplished with $\text{O}_2^{\bullet-}$ (reaction 3), with the overall reaction (4) being the iron-ion-catalysed interaction of $\text{O}_2^{\bullet-}$ and H_2O_2 to form $\text{OH}^\bullet$ (Haber–Weiss reaction):

The oxidants formed by phagocytes have been shown to kill micro-organisms and inactivate soluble chemical mediators of inflammation such as chemotactic factors, α_1 -proteinase inhibitor and leukotrienes [18]. The depolymerization of hyaluronic acid by reactive oxygen species [19–24] and the partial degradation of heparin by ascorbic acid [25] suggest that the degradation of heparin in mammalian tissues might be accomplished in part by free radicals and other oxidants generated by phagocytes. In support of this hypothesis, we report here that normal human neutrophils extensively degrade mast-cell-granule (MCG) heparin after MCG phagocytosis. Chronic-granulomatous-disease (CGD) neutrophils, which lack respiratory-burst activity, have markedly diminished heparin-degrading activity. Further, isolated heparin proteoglycan is rapidly cleaved to molecules of lower M_r by hydroxyl radicals generated by Fenton's reagent or the iron-ion-catalysed Haber–Weiss reaction.

Abbreviations used: CGD, chronic granulomatous disease; MCG, mast-cell granules; RPMC, rat peritoneal mast cells; SOD, superoxide dismutase.

‡ To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Chondroitin ABC lyase from *Proteus vulgaris*, superoxide dismutase (SOD) from bovine erythrocytes (12300 units/mg) and BSA were obtained from Miles Laboratories (Elkhart, IN, U.S.A.); acetaldehyde was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); Azure A and pig intestinal heparin (170 i.u./mg) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); carrier-free $\text{H}_2^{35}\text{SO}_4$ was from New England Nuclear Corp. (Boston, MA, U.S.A.); fetal bovine serum, penicillin (10000 i.u./ml) and streptomycin (10000 $\mu\text{g}/\text{ml}$) were from GIBCO (Grand Island, NY, U.S.A.); Sepharose 4B and Sephadex G-25 were from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.); Dowex AG 1-X2 (100–200 mesh; Cl^- form) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.); catalase (bovine liver; 60000 units/mg) was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); CHAPS and xanthine oxidase (bovine milk; 10 mg/ml, approx. 0.4 unit/ml) were from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). The catalase and xanthine oxidase were dialysed against deionized water, and all salt solutions were passed twice over a Chelex 100 ion-exchange resin to remove trace-metal ions. Where indicated, the catalase was heated at 100 °C for 15 min, and the SOD was autoclaved at 120 °C for 30 min.

Methods

Neutrophil isolation. Neutrophils were isolated from the venous blood of either normal subjects or CGD patients by single-step isopycnic banding on Percoll [26]. Briefly, 4 ml of human venous blood, anticoagulated with heparin, was layered on to discontinuous Percoll gradients, made from solutions of density 1.088, 1.079 and 1.070 g/ml. Final adjustment of density was facilitated by measurement of refractive index at 22 °C with a Bausch and Lomb refractometer (Fisher Scientific, Silver Spring, MD, U.S.A.). After centrifugation at 700 g for 25 min at 22 °C, the majority of the neutrophils were found in the band at the density 1.070/1.079 interface (Percoll band 3). Purity was greater than 97% neutrophils, and viability was greater than 95% as determined by Trypan Blue dye exclusion. Neutrophil yields from each gradient were $3.8 \times 10^6 \pm 1.2 \times 10^6$ cells.

^{35}S -labelling *in vivo* and isolation of rat peritoneal mast-cell (RPMC) granules. For radiolabelling with [^{35}S]sulphate *in vivo*, male Sprague–Dawley rats (200–250 g) were injected intraperitoneally with 250 μCi of [^{35}S]sulphate in 0.2 ml of Hanks balanced salt solution (Biofluids, Rockville, MD, U.S.A.) on days 1, 3 and 5. On day 7 RPMC were harvested by peritoneal lavage, the erythrocytes were removed by mild hypo-osmotic lysis and the mast cells were purified over 0–38% (w/v) albumin gradients [27]. The average yield of RPMC was 10^6 cells/rat with greater than 97% purity, as determined by Toluidine Blue staining [28]. Intact ^{35}S -labelled MCG were prepared from purified RPMC by a previously described procedure [29] in which the mast-cell membrane, but not the secretory-granule membrane, is disrupted by sonication. Membrane-bound ^{35}S -labelled MCG containing perigranular membranes were separated from cell debris over 0.34 M-sucrose by centrifugation. More than 95% of the total radioactivity placed on the sucrose gradient was consistently shown to be present in the granule-rich fractions [11]. Membrane-free ^{35}S -labelled MCG, which were prepared by resuspending purified membrane-bound ^{35}S -labelled MCG in sterile distilled water, were used in the experiments reported in the present paper. Such granule preparations, which have been shown to be largely devoid of perigranular membranes

by electron microscopy [29], contained all of the ^{35}S -labelled material of the intact membrane-bound granule preparation. Samples of granule suspensions were diluted and stained with aq. 0.05% Toluidine Blue, and the granule number was estimated by light microscopy [11].

Phagocytosis of ^{35}S -labelled MCG by neutrophils. Neutrophils were prepared for phagocytosis experiments by placing 0.5 ± 10^6 neutrophils per 35 mm-diam. tissue-culture dish in 1 ml of Hepes-buffered RPMI 1640 medium (Biofluids) containing 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml. ^{35}S -labelled MCG preparations were overlaid in 1 ml of conditioned medium to give a granule/neutrophil ratio of 100:1. On the assumption that a mast cell contains 1000 secretory granules [30], this granule/neutrophil ratio corresponded to a theoretical mast-cell/neutrophil ratio of 1:10. Experimental and control cultures were incubated for 30 min (see Figure legends) in a humidified atmosphere of 5% CO_2 in air at 37 °C. Control cultures consisted of an identical culture system in the absence of neutrophils.

After incubation of ^{35}S -labelled granules with neutrophils, the culture medium containing non-adherent neutrophils was removed and centrifuged at 400 g for 5 min at 4 °C. The supernatant (medium) was saved, and the non-adherent neutrophil pellet was combined with the adherent neutrophil fraction (see below). The adherent neutrophils were washed three times with 0.5 ml of 1.0 M-NaCl to remove extracellular ^{35}S -labelled granules. The medium and first salt wash of the adherent neutrophils were combined as pool 1, and the second two salt washes of the adherent neutrophils were combined as pool 2 and maintained separately. The radioactivities in these two pools were added to determine extracellular compartment radioactivity. In every case more than 97% of the radioactivity was found in pool 1. To avoid dilution, determination of molecular size of extracellular ^{35}S -labelled material was performed only on pool 1. To obtain intracellular ^{35}S -labelled material, adherent neutrophils were removed from the culture plates with the aid of a cell scraper, and the plates were washed with 0.5 ml of 1 M-NaCl to remove residual neutrophils. Cell-surface ^{35}S -labelled material was not characterized individually, as this has been found to represent less than 2% of the total radioactivity present [11]. To solubilize intracellular ^{35}S -labelled material, the combined neutrophil fractions (non-adherent neutrophil pellet plus adherent neutrophils) were sonicated for 20 s. Residual cell debris was pelleted at 2000 g for 5 min, and the supernatant, containing more than 99% of the intracellular radioactivity, was retained for characterization of ^{35}S -labelled intracellular material.

The apparent M_r distribution of ^{35}S -labelled material within the original ^{35}S -labelled granules or within either the extracellular or intracellular compartments at various time intervals was established by filtration of representative portions of material over a column (1 cm $\times$ 60 cm) of Sephadex G-50 equilibrated in 0.1 M-LiCl at 4 °C.

^{35}S -labelling *in vitro* and extraction of RPMC. For radiolabelling with [^{35}S]sulphate *in vitro*, 2×10^7 – 5×10^7 mast cells were placed in 20–50 ml of Spinner's medium (Microbiological Associates, Bethesda, MD, U.S.A.), supplemented with 0.4–1.0 mCi of [^{35}S]sulphate, 50 i.u. of penicillin/ml, 100 μg of streptomycin/ml and 0.6% L-glutamine. After incubation of mast-cell cultures for 18 h at 37 °C under 5% CO_2 in air, the cells were centrifuged at 400 g for 5 min, washed three times in Hanks balanced salt solution and resuspended in 0.1 ml of a solution of 0.5% CHAPS, pH 6.0, containing 0.1 M-6-aminohexanoic acid, 0.5 M-EDTA, 5 mM-benzamidine hydrochloride, 2 mM-iodoacetamide and 0.1 M-sodium acetate, followed by the addition of 0.9 ml of 4 M-guanidinium chloride [31]. Heparin was

extracted by sonication for 20 s with a Sonicator Cell Disruptor (Heat Systems Ultrasonic, Plainview, NY, U.S.A.). The cell debris was sedimented at 10000 *g* for 30 min, and the supernatant containing ^{35}S -labelled heparin proteoglycan was removed.

Isolation of mast-cell heparin. The extract of 2×10^7 – 5×10^7 mast cells from 20–50 rats was applied to a column (1 cm $\times$ 5 cm) of Dowex AG 1-X2 equilibrated with 1 M-NaCl in 10 mM-Tris/HCl buffer, pH 6.0, and eluted in stepwise fashion with 1 M-, 3 M- and 4 M-NaCl in the same buffer [32]. Portions of each eluate were analysed for radioactivity, protein (by absorbance at 280 nm) and uronic acid (by the carbazole reaction) [33,34]. The 1 M-NaCl effluent and 1 M-NaCl wash contained more than 98 % of the total protein and at least 90 % of the radioactivity, but were essentially without uronic acid. The material eluted with 3 M-NaCl consistently contained a small amount of protein and approx. 5–10 % of the [^{35}S]sulphate. Proteoglycan, with approx. 15–20 μg of uronic acid, was recovered in the 3 M-NaCl eluate per 10^6 mast cells. The 4 M-NaCl eluate had no significant radioactivity or uronic acid. The 3 M-NaCl eluates were dialysed, freeze-dried and incubated with chondroitin ABC lyase [35] to remove small amounts of chondroitin sulphate (15 %). After the treatment with chondroitin ABC lyase, the extract was dialysed against distilled water at 4 °C for 12 h and rechromatographed over a second Dowex AG 1-X2 column to remove the chondroitin ABC lyase and products of the enzymic digestion. The column was washed with 1 M-NaCl, and the chondroitin ABC lyase-resistant proteoglycan was eluted with 3 M-NaCl, dialysed against distilled water at 4 °C for 18 h and freeze-dried. This material could be completely degraded by purified heparinase [36]. Two measures were used to assess the amount of heparin: (1) the change in absorbance at 510 nm after the formation of a heparin–Azure A metachromatic complex [4,37], and (2) the anticoagulant activity determined from a partial thromboplastin time assay, with commercial pig heparin as a standard [38].

Degradation of heparin by cell-free $\text{OH}^\bullet$ -generating systems. The components of the reaction mixture (see legends to Figures and Tables) in a total volume of 0.5 ml were added to 12 mm $\times$ 75 mm polystyrene tubes, with either H_2O_2 or acetaldehyde added last. In general, the conditions employed were those previously found to be optimal for $\text{OH}^\bullet$ formation [39]. The tubes were incubated at 37 °C in a shaking water bath for the periods indicated; the reaction was stopped with the addition of 385 μg of catalase. The suspensions were then immediately frozen in acetone/solid CO_2 and stored at –70 °C until further chromatographic analysis.

Following the incubation of the [^{35}S]proteoglycan heparin with the cell-free reaction mixture components, the products were separated by chromatography on Sepharose 4B columns (1 cm $\times$ 60 cm) with 2 M-NaCl as the eluent at a flow rate of 1.5 ml/h at 4 °C. The M_r distribution of the ^{35}S -labelled molecules was further assessed by h.p.l.c. on a 7.5 mm $\times$ 300 mm Spherogel TSK 3000 SW column (10 μm particle size) equilibrated in 0.1 M-Tris/sulphate buffer, pH 7.5 [40].

RESULTS

Degradation of MCG heparin by neutrophils

^{35}S -labelled RPMC granules were incubated with human neutrophils for 30 min and the degradation of the extracted labelled heparin was assessed by filtration over Sephadex G-50. As shown in Fig. 1(a), ^{35}S -labelled heparin from control granules chromatographed over Sephadex G-50 as a sharp peak between fractions 11 and 22. After incubation with human neutrophils, however, the ^{35}S -labelled material in the supernatant appeared as

Fig. 1. Degradation of rat MCG-associated ^{35}S -labelled heparin by human neutrophils

^{35}S -labelled MCG were incubated with neutrophils from a normal volunteer (a) and from a CGD patient (b) for 30 min at 37 °C. Heparin degradation was assessed by chromatography over Sephadex G-50 of extracellular radioactive material (●—●) and radioactive material extracted from neutrophil cell pellets at 30 min (○---○), and the patterns were compared with that of control extracts prepared from MCG comparably incubated in the absence of neutrophils (□---□). Fractions (1 ml) were collected and assayed for radioactivity. V_0 (fraction 12) and V_t (fraction 40) were determined with Blue Dextran and [^{35}S]sulphate respectively.

two peaks, in fractions 14–20 and 20–30 (Fig. 1a), indicating a cleavage to lower- M_r fractions. ^{35}S -labelled material was also recovered in the neutrophil cell pellets after solubilization and sonication; this cell-associated material chromatographed on Sephadex G-50 between fractions 20 and 40 (Fig. 1a).

In contrast, no degraded ^{35}S -labelled material was observed in the supernatant fractions after incubation of ^{35}S -labelled RPMC granules for 30 min with CGD neutrophils (Fig. 1b). This supernatant material chromatographed on Sephadex G-50 in the same fractions (11–21) as did that from control ^{35}S -labelled MCG (Fig. 1b). Approx. 50 % of the ^{35}S -labelled material extracted from the CGD-neutrophil cell pellets chromatographed with the ^{35}S -labelled material extracted from the control MCG, and the remainder chromatographed in fractions 22–28, corresponding to material of lower M_r (Fig. 1b). The much greater conversion of ^{35}S -labelled material from the MCG into lower- M_r material by normal neutrophils compared with CGD neutrophils suggested a role for respiratory-burst oxidants in the degradation of MCG heparin proteoglycan.

Degradation of MCG-associated heparin by Fenton's reagent

To assess the ability of $\text{OH}^\bullet$ to degrade MCG-associated heparin, isolated [^{35}S]sulphate-labelled MCG were incubated in the presence and in the absence of Fenton's reagent for 30 min at 37 °C. The heparin was solubilized in 1 M-NaCl, and portions

Fig. 2. Degradation of rat MCG-associated ^{35}S -labelled heparin by Fenton's reagent

^{35}S -labelled MCG incubated alone (●—●) or in the presence of $0.1\text{ mM-H}_2\text{O}_2 + 50\text{ }\mu\text{M-FeSO}_4$ (○—○) for 30 min at 37°C were solubilized in 1 M-NaCl and chromatographed over Sepharose 4B as described in the Materials and methods section.

were chromatographed over Sepharose 4B for size assessment. As shown in Fig. 2, the granule heparin proteoglycan incubated in buffer alone chromatographed as a high- M_r molecule in fractions 16–20. After treatment with Fe^{2+} and H_2O_2 , ^{35}S -labelled material appeared in fractions 26–30, indicating cleavage of heparin proteoglycan in MCG to lower- M_r fragments.

Degradation of heparin proteoglycan by Fenton's reagent

^{35}S -labelled heparin proteoglycan that had been incubated in acetate buffer, pH 5.5, for 5 min (Fig. 3a) chromatographed as a high- M_r polydispersed molecule with an estimated average M_r of 750 000, as has been previously reported [1,41]. The addition of either $0.1\text{ mM-H}_2\text{O}_2$ (Fig. 3b) or $50\text{ }\mu\text{M-FeSO}_4$ (Fig. 3c) alone did not affect the size of the heparin proteoglycan; however, when both H_2O_2 and FeSO_4 were added, the heparin proteoglycan was cleaved to molecules of lower M_r , which appeared in fractions 30–36 of the chromatogram (Fig. 3d). The degradation of heparin by H_2O_2 plus FeSO_4 was prevented by the addition of catalase, which degrades H_2O_2 (Fig. 3e), and was greatly diminished but not abolished by the addition of 0.1 M-mannitol , an $\text{OH}^\cdot$ scavenger (Fig. 3f). In the presence of mannitol, molecules of intermediate size were found. Heparin degradation by Fenton's reagent was not inhibited by the addition of SOD ($50\text{ }\mu\text{g/ml}$), heated catalase ($60\text{ }\mu\text{g/ml}$) or mannitol (10 mM) (results not shown). The Fenton's-reagent-induced degradation of heparin was dependent on the Fe^{2+} concentration in the reaction mixture. Progressively less degradation of the heparin occurred as the Fe^{2+} concentration was decreased from 50 to $0.05\text{ }\mu\text{M}$ (results not shown).

To determine the time course of heparin degradation by Fenton's reagent, ^{35}S -labelled proteoglycan was incubated for periods from 0 to 60 min with $0.1\text{ mM-H}_2\text{O}_2$ and $50\text{ }\mu\text{M-Fe}^{2+}$, with the reaction terminated by the addition of catalase. The heparin degradation products 1 min after interaction with Fenton's reagent were intermediate in size, appearing in Sepharose 4B fractions 26–34. At 5 min the breakdown products were smaller, appearing in fractions 30–38. No further decrease in the size of the reaction products occurred when the incubations were extended for periods up to 60 min. The degradation products generated by Fenton's reagent were next compared with intact rat heparin proteoglycan and rat heparin glycosaminoglycans.

Fig. 3. Heparin degradation by the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ system: effects of catalase and mannitol

^{35}S -labelled RPMC heparin ($2\text{ }\mu\text{g}$; 10000 d.p.m.) was incubated at 37°C for 5 min in $0.2\text{ M-sodium acetate buffer}$, pH 5.5, alone (a) or containing $0.1\text{ mM-H}_2\text{O}_2$ (b), $50\text{ }\mu\text{M-FeSO}_4$ (c), $0.1\text{ mM-H}_2\text{O}_2 + 50\text{ }\mu\text{M-FeSO}_4$ (d), $0.1\text{ mM-H}_2\text{O}_2 + 50\text{ }\mu\text{M-FeSO}_4 + \text{catalase}$ ($60\text{ }\mu\text{g/ml}$) (e) or $0.1\text{ mM-H}_2\text{O}_2 + 50\text{ }\mu\text{M-FeSO}_4 + 0.1\text{ M-mannitol}$ (f). The mixtures were then chromatographed over Sepharose 4B.

When these heparin moieties were chromatographed over tandem TSK 3000 SW–TSK 4000 SW h.p.l.c. columns, intact heparin proteoglycan was eluted at 16–20 min, heparin glycosaminoglycans (generated by β -elimination) were eluted at 18–28 min and Fenton's-reagent-generated heparin fragments were eluted at 28–38 min (Fig. 4). These data confirm that the rat heparin fragments produced by Fenton's reagent are smaller than rat heparin glycosaminoglycans. The M_r of the heparin degradation products from incubation with Fenton's reagent was next compared with that of commercial pig heparin by h.p.l.c. (Fig. 5). Degradation products from both 5 min and 60 min incubations with Fenton's reagent chromatographed similarly, showing broad biphasic peaks that overlapped with the elution pattern of commercial heparin, which has an estimated M_r range of 5000 – 26000 [41].

Fig. 4. H.p.l.c. elution profiles of rat heparin proteoglycan, rat heparin glycosaminoglycans and Fenton's-reagent-generated rat heparin-degradation products

^{35}S -labelled RPMC heparin was incubated at 37°C for 30 min in the absence (—) and in the presence (.....) of 0.1 M-NaOH, or with 0.1 mM- H_2O_2 plus $50\text{ }\mu\text{M}$ - FeSO_4 (—) in 0.2 M-sodium acetate buffer, pH 5.5. These heparin moieties were examined by h.p.l.c. on tandem Spherogel TSK 3000 SW-TSK 4000 SW columns ($7.5\text{ mm} \times 30\text{ cm}$). The columns were equilibrated in 0.1 M-Tris/sulphate buffer, pH 7.5, containing 4 M-guanidinium chloride and 0.1 M- NaSO_4 , and samples were eluted in this solvent at a flow rate of 0.7 ml/min. The h.p.l.c. eluent radioactivity was continuously monitored with a radioactive-flow detector (Flo-one/Beta, model IC; Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.). V_0 and V_t were determined with Blue Dextran and [^{35}S]sulphate respectively.

Rat heparin proteoglycan has been reported to have 10–30% of the anticoagulant activity of commercial bovine heparin [1,5,42]. In our studies the rat proteoglycan heparin had 46 units of anticoagulant activity/mg standardized by metachromasia with Azure A relative to commercial pig mucosal heparin. The heparin proteoglycan fragments obtained by incubation with Fenton's reagent for 60 min had 12 units of anticoagulant activity/mg, which is approx. 26% of the activity of the intact rat proteoglycan heparin.

Degradation of heparin proteoglycan by the xanthine oxidase system

The xanthine oxidase system generates $\text{O}_2^{\cdot-}$, H_2O_2 and $\text{OH}^\cdot$ and has been employed as a cell-free model of the oxidant-generating system of phagocytes [43,44]. Incubation of heparin proteoglycan with xanthine oxidase, acetaldehyde and either $50\text{ }\mu\text{M}$ - FeSO_4 (Fig. 6b) or $10\text{ }\mu\text{M}$ - FeSO_4 (Fig. 6d) resulted in the formation of low- M_r degradation products similar in size to those resulting from exposure of the proteoglycan to Fenton's reagent (Fig. 3d). Like the degradation of proteoglycan heparin by Fenton's reagent, degradation by the xanthine oxidase system in the presence of the higher Fe^{2+} concentration ($50\text{ }\mu\text{M}$) was not inhibited by SOD (Fig. 6c). However, SOD was inhibitory when the lower Fe^{2+} concentration ($10\text{ }\mu\text{M}$) was used. Proteoglycan degradation by the low- Fe^{2+} -supplemented acetaldehyde/xanthine oxidase system was also inhibited by the addition of

Fig. 5. H.p.l.c. elution profiles of Fenton's-reagent-induced heparin-degradation products in comparison with that of commercial heparin

^{35}S -labelled RPMC heparin was incubated with 0.1 mM- H_2O_2 + $50\text{ }\mu\text{M}$ - FeSO_4 for either 5 min (●—●) or 60 min (○—○) at 37°C . These preparations and commercial heparin (—) were then separately chromatographed on a Spherogel TSK 3000 SW column as described in the Materials and methods section. H.p.l.c. was performed with 0.1 M-Tris/sulphate buffer, pH 7.5, at a flow rate of 0.5 ml/min; 1 ml fractions were collected. V_0 and V_t were determined with Blue Dextran and [^{35}S]sulphate respectively.

either catalase ($60\text{ }\mu\text{g/ml}$) or mannitol (0.1 M) and was unaffected by heated SOD (results not shown).

DISCUSSION

We report here that the heparin in rat MCG is susceptible to oxidative degradation by intact normal neutrophils and by cell-free oxygen-radical-generating systems. Phagocytosis of ^{35}S -labelled MCG by human neutrophils resulted in degradation of the ^{35}S -labelled material to lower- M_r products, some of which were released extracellularly with the remainder retained in the neutrophil pellets. Degradation of MCG-associated heparin was markedly diminished when CGD neutrophils were substituted for normal neutrophils, suggesting a role for reactive oxygen species in the mediation of heparin degradation. Reactive oxygen species most probably facilitate heparin breakdown by directly acting on the heparin proteoglycan to generate lower- M_r products that serve as substrates for additional processing by intracellular glycosidase/sulphatases. An alternative possibility is that the leucocyte-derived oxidants act on these enzymes to enhance their heparin-degrading activity.

Our results demonstrate that heparin proteoglycan is degraded by cell-free $\text{OH}^\cdot$ -generating systems, specifically Fenton's reagent (Fe^{2+} plus H_2O_2) and the xanthine oxidase system supplemented by either high ($50\text{ }\mu\text{M}$) or low ($10\text{ }\mu\text{M}$) Fe^{2+} concentrations. Degradation by H_2O_2 and Fe^{2+} was essentially complete in 5 min. In each cell-free system the cleavage of heparin was prevented by catalase, indicating a requirement for H_2O_2 , and was partially inhibited by mannitol, an $\text{OH}^\cdot$ scavenger. SOD had no effect on the degradation of heparin by H_2O_2 plus Fe^{2+} , or by the xanthine oxidase system at high Fe^{2+} concentration, whereas at low Fe^{2+} concentration SOD did inhibit degradation by the xanthine oxidase system. These data suggest that at high Fe^{2+}

Fig. 6. Heparin degradation by the acetaldehyde/xanthine oxidase system: effects of Fe^{2+} concentration and inhibitors

^{35}S -labelled RPMC heparin was incubated at 37°C for 30 min in 0.2 M-sodium acetate buffer, pH 5.5, alone (a) or containing 4 mM-acetaldehyde + xanthine oxidase ($20\ \mu\text{g}/\text{ml}$) + $50\ \mu\text{M}$ - FeSO_4 (b), 4 mM-acetaldehyde + xanthine oxidase ($20\ \mu\text{g}/\text{ml}$) + $50\ \mu\text{M}$ - FeSO_4 + SOD ($50\ \mu\text{g}/\text{ml}$) (c), 4 mM-acetaldehyde + xanthine oxidase ($20\ \mu\text{g}/\text{ml}$) + $10\ \mu\text{M}$ - FeSO_4 (d) or 4 mM-acetaldehyde + xanthine oxidase ($20\ \mu\text{g}/\text{ml}$) + $10\ \mu\text{M}$ - FeSO_4 + SOD ($50\ \mu\text{g}/\text{ml}$) (e). Products were examined by Sepharose 4B chromatography.

concentration the xanthine oxidase system generates $\text{OH}^\cdot$ through the interaction of the H_2O_2 formed with stoichiometric amounts of Fe^{2+} (Fenton's reagent), whereas $\text{O}_2^{\cdot-}$ is needed for the re-utilization of iron ions when low concentrations are employed (iron-ion-catalysed Haber-Weiss reaction).

This oxidative degradation of rat heparin proteoglycan is consistent in most respects with prior reports on the susceptibility of hyaluronic acid and proteoglycans to oxygen radicals. Hyaluronic acid is degraded in the presence of O_2 , Fe^{2+} (or Cu^+) ions and reducing agents such as ascorbic acid [19,20,23], or by O_2 -derived free radicals generated enzymically by xanthine oxidase and hypoxanthine [21,22]. This depolymerization of hyaluronic acid is inhibited by $\text{OH}^\cdot$ scavengers, implicating $\text{OH}^\cdot$ as the active oxygen species. Similarly, heparin proteoglycan from rat skin is degraded by ascorbic acid [25]. Of interest is that, in the degradation of heparin proteoglycan by either H_2O_2 plus Fe^{2+} or the H_2O_2 generated by the xanthine oxidase/acetaldehyde system shown here, the heparin fragments that result are smaller in size than those derived from the exposure of heparin proteoglycan to ascorbate [25].

The products of the oxidative degradation of heparin proteoglycan by cell-free $\text{OH}^\cdot$ -generating systems have an M_r distribution smaller than that of rat heparin glycosaminoglycans (estimated average M_r 80 000) [45] (Fig. 4) and similar to that of commercial pig heparin (estimated M_r range 5000–26 000 [41] (Fig. 5). These observations suggest that each rat heparin glycosaminoglycan side chain is cleaved at as few as two and as many as 15 locations, resulting in oligosaccharide residues heterogeneous in M_r . The heparin-cleavage products produced by normal neutrophils were, in general, smaller than those generated by the cell-free $\text{OH}^\cdot$ -generating systems. The most likely explanation for the difference in size of the products is that the oxidatively cleaved heparin fragments serve as substrates for exoglycosidases, which further cleave the mast-cell heparin. An analogous process has been suggested for the degradation of heparin and heparin sulphate, in which endoglucuronidase-generated oligosaccharides may be substrates for exoenzymes that further degrade the polysaccharide chain [13,14,17].

The demonstration that oxygen-radical-generating systems cleave heparin proteoglycan may be of biological importance. IgE-mediated degranulation of mast cells in allergic reactions leads to the extracellular release of their cytoplasmic granules. These extracellular granules retain such mediators as heparin, chymotrypsin [46] and SOD [47], which may express biological activities in the extracellular space. For example, chymotrypsin retains proteolytic activity [46], and heparin can inhibit both coagulation [48] and formation of the complement-amplification-pathway convertase [6] when these mediators remain granule-associated. The phagocytosis of MCG by neutrophils [49,50], eosinophils [49,51] and macrophages [9,49,50] has been demonstrated, as has the degradation of the ingested [^{35}S]heparin by macrophages [9]. Such phagocytosis of MCG may help limit the biological activities of the granule-associated mediators. For example, the products resulting from the exposure of rat proteoglycans to Fenton's reagent retained only a portion (approx. 26%) of the anticoagulant activity exhibited by the parent molecule. Phagocytes when stimulated by phagocytosis generate $\text{O}_2^{\cdot-}$ and H_2O_2 , which, in the presence of iron, would be expected to form $\text{OH}^\cdot$. It has been proposed that the low free iron concentration in phagocytes limits the formation of $\text{OH}^\cdot$ by phagocytes [52,53], although this finding remains controversial [54–56]. Our findings raise the possibility that the heparin of MCG ingested by phagocytes will be rapidly cleaved by reactive oxygen species to lower- M_r fragments. Since the oxygen-radical-generating system of phagocytes is plasma-membrane-associated, partial degradation of heparin proteoglycan could also occur extracellularly. The fragments formed by the oxygen-radical-cleaved proteoglycan heparin retained anticoagulant activity (although the activity was less than that of the parent compound), and thus have the potential to exert biological activity in tissues. Further, the resulting fragments may provide more suitable substrates for enzymes that can complete the degradation process.

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