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Original Contribution

Efficiencies of fragmentation of glycosaminoglycan chloramides of the extracellular matrix by oxidizing and reducing radicals: potential site-specific targets in inflammation?

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ABSTRACT

Hypochlorous acid and its conjugate base, hypochlorite ions, produced under inflammatory conditions, may produce chloramides of glycosaminoglycans, these being significant components of the extracellular matrix (ECM). This may occur through the binding of myeloperoxidase directly to the glycosaminoglycans. The N–Cl group in the chloramides is a potential selective target for both reducing and oxidizing radicals, leading possibly to more efficient and damaging fragmentation of these biopolymers relative to the parent glycosaminoglycans. To investigate the effect of the N–Cl group, we used ionizing radiation to produce quantifiable concentrations of the reducing radicals, hydrated electron and superoxide radical, and also of the oxidizing radicals, hydroxyl, carbonate, and nitrogen dioxide, all of which were reacted with hyaluronan and heparin and their chloramides in this study. PAGE gels calibrated for molecular weight allowed the consequent fragmentation efficiencies of these radicals to be calculated. Hydrated electrons were shown to produce fragmentation efficiencies of 100 and 25% for hyaluronan chloramide (HACl) and heparin chloramide (HepCl), respectively. The role of the sulfate group in heparin in the reduction of fragmentation can be rationalized using mechanisms proposed by M.D. Rees et al. (*J. Am. Chem. Soc.* **125**:13719–13733; 2003), in which the initial formation of an amidyl radical leads rapidly to a C-2 radical on the glucosamine moiety. This is 100% efficient at causing glycosidic bond breakage in HACl but only 25% efficient in HepCl, the role of the sulfate group being to favor the nonfragmentary routes for the C-2 radical. The weaker reducing agent, the superoxide radical, did not cause fragmentation of either HACl or HepCl although kinetic reactivity had been demonstrated in earlier studies. Experiments using the oxidizing radicals, hydroxyl and carbonate, both potential in vivo species, showed significant increases in fragmentation efficiencies for both HACl and HepCl, relative to the parent molecules. The carbonate radical was shown to be involved in site-specific reactions at the N–Cl groups, reacting via abstraction of Cl, to produce the same amidyl radical produced by one-electron reductants such as the hydrated electron. As for the hydrated electrons, the data support fragmentation efficiencies of 100 and 29% for reaction of carbonate radicals at N–Cl for HACl and HepCl, respectively. For the weaker oxidant, nitrogen dioxide, no fragmentation was observed, probably because of a low kinetic reactivity and low reduction potential. It seems likely therefore that the N–Cl group can direct damage to extracellular matrix glycosaminoglycan chloramides, which may be produced under inflammatory conditions. The in vivo species, the carbonate radical, is also much more likely to be site-specific in its reactions with such components of the ECM than the hydroxyl radical.

The extracellular matrix (ECM) is made up of huge multimolecular complexes with arrays of link proteins and aggrecan molecules along a central hyaluronan backbone. Hyaluronan (HA) is bound by a number of ECM and cell surface proteins [1,2].

With this central structural function, HA is a particularly important component of the ECM [3,4], as demonstrated by the fact that a hyaluronan synthase-2 knockout is embryonically lethal in mice [5]. HA also provides a hydrated environment [6] for growing, moving, and renewing cells and tissues [7]; activates signaling events in cells; and is involved in moderating many cellular processes, including proliferation, migration, adhesion, and apoptosis [8–11]. HA appears to have a range of significant

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biological functions dependent upon its molecular mass. Large-molecular-mass fragments are involved in space-filling and immunosuppressive roles, whereas smaller HA fragments have been shown to be proinflammatory and angiogenic; oligosaccharides may be involved in cell signaling (reviewed in [12]).

Oxidative damage of the extracellular matrix components by either enzymatic or nonenzymatic pathways may have implications for the initiation and progression of a range of human diseases. These include arthritis, kidney disease, cardiovascular disease, lung disease, periodontal disease, and chronic inflammation. Oxidative damage to hyaluronan by reactive oxidative species, and in particular by free radicals, has received much attention, largely through the ease of monitoring its fragmentation using viscometric techniques, which is reviewed in [13]. The potential mechanism of oxidative damage to the ECM and its role in human pathologies have also been discussed in a recent review [14].

Several reactive species may be formed at sites of inflammation, including superoxide ($O_2^{\bullet -}$), hydrogen peroxide, hypochlorite ($HOCl/OCl^-$), and peroxynitrite ($ONOO^-/ONOOH$). The last species may be formed in vivo by the diffusion-controlled reaction between superoxide and nitric oxide ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$) [15], where nitric oxide is generated by macrophage-inducible nitric oxide synthase and endothelial nitric oxide synthase [16,17].

Our previous studies on HA have measured HA fragmentation yields as a proportion of quantifiable fluxes of free radicals produced by ionizing radiation. For this purpose, both viscosity changes and a combination of gel-permeation chromatography with multiangle laser light scattering were used to measure changes in molecular weight of the polydispersed hyaluronan. In this way, the efficiencies of fragmentation of HA by a range of free radicals and reactive oxidative species including hydroxyl radicals, carbonate radicals, dibromide and dichloride radical anions, and peroxynitrite were determined [18,19].

The fragmentation of hyaluronan and other glycosaminoglycans has also been investigated intensively by Davies and co-workers using both electron paramagnetic resonance (EPR) spectroscopy and sensitive polyacrylamide gel electrophoresis (PAGE) techniques. The use of the latter technique showed the novel and potentially biologically significant result that peroxynitrous acid and carbonate and hydroxyl radicals react largely in a site-specific process to produce an array of HA fragments, in a "ladder-type" display, each separated from its neighbor by the molecular mass of the repeating disaccharide unit in HA, thus mimicking to a significant extent the action of the enzyme hyaluronidase [20,21]. Similar site-selective fragmentation was also observed when glycosaminoglycan chloramides (formed through reaction with hypochlorite) were reduced by copper(I) ions and superoxide anion radicals [22,23].

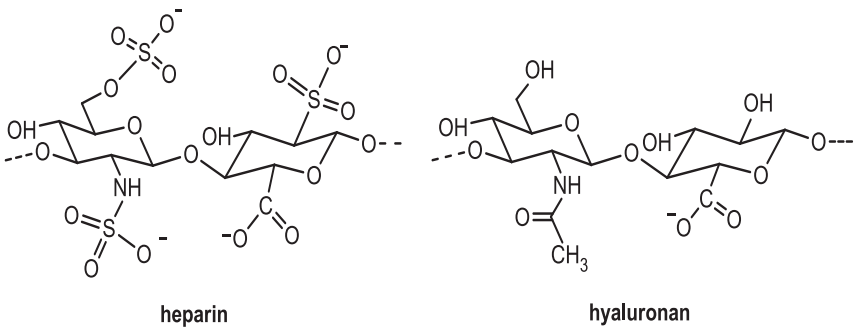
The formation of chloramides and chloramines from the reaction of hypochlorite with amides and amines, respectively, was demonstrated in an early study [24] and is suggested to be a key process in inflammation, in which hypochlorite (from myeloperoxidase) may produce glycosaminoglycan chloramides. In vitro studies of the reactions of hypochlorite with glycosaminoglycans have indeed demonstrated that chloramides are produced in yields and rates of reaction that are dependent upon both pH and ratio of hypochlorite to glycosaminoglycan concentrations [25,26] and have demonstrated that such derivatives may accelerate the fragmentation of glycosaminoglycans within the ECM. Chloramides are weak oxidizing agents and are therefore potential biological targets for reducing radicals and other reducing agents through reaction at the N–Cl group. Indeed, it has been shown that both superoxide radicals and transition metal ions cause the fragmentation of HA through reaction with its chloramide derivative [22]. In the case of the heavily sulfated heparan sulfate, both Cu (I) and Fe(II) were also found to produce fragmentation of its chloramide derivative. An estimate of the efficiency of the fragmentation process was made in a PAGE experiment using a standard octasaccharide for calibration of molecular weight in a completely decomposed chloramide: a yield of 50% was estimated [27].

In this study, ionizing radiation was used to produce selected oxidizing and reducing free radicals whose concentrations can be determined with both significant accuracy and precision and can be controlled. In this way, free radicals can be reacted with the chloramide derivatives of hyaluronan and heparin, the latter being the most heavily charged glycosaminoglycan. The oxidizing radicals were hydroxyl ($^{\bullet}OH$), carbonate ($CO_3^{\bullet -}$), and nitrogen dioxide ($NO_2^{\bullet}$), all potential in vivo species produced via peroxynitrite and other reaction pathways [28,29]. The reducing radicals selected in this study were the hydrated electron (e_{aq}^-) and $O_2^{\bullet -}$. The hydrated electron e_{aq}^- is strongly reducing and may be expected to be highly, perhaps 100%, selective in its attack. It acted as a model in this study for less strongly reducing agents such as glutathione disulfide anion radicals. Superoxide, a much weaker reducing species, was also investigated in this study. The main aim was to measure, for the first time, the efficiencies of fragmentation of hyaluronan and heparin chloramides by all these free radicals and thereby to deduce, by comparison with experiments carried out under the same conditions with the parent glycosaminoglycans, whether the N–Cl group confers selectivity of attack to enhance fragmentation. Any enhanced fragmentation efficiency seen with chloramide derivatives relative to parent molecules is of clear relevance to inflammation within the extracellular matrix where these derivatives are likely to be formed and may therefore present site-specific targets for free radicals and other reactive species.

Materials and methods

Materials

Sodium formate, hypochlorous acid, *tert*-butanol, and sodium bicarbonate were all analytical grade (Sigma–Aldrich). Hyaluronan (80 kDa) was a gift from Novozymes; heparin sodium salt (Alfa



Scheme 1. Structures of hyaluronan and heparin.