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Original Contribution

Chain scission of hyaluronan by carbonate and dichloride radical anions: Potential reactive oxidative species in inflammation?

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Abstract

The reactions of the carbonate and dichloride radical anions, $CO_3^{\bullet -}$ and $Cl_2^{\bullet -}$, with the extracellular matrix glycosaminoglycan hyaluronan (HA) have been studied using the kinetic technique of pulse radiolysis and also by steady-state irradiation combined with gel permeation chromatography/multiangle laser light scattering(gpc/MALLS) to measure the rates of reaction with HA and the yield of HA chain scission, respectively. For comparison, the same measurements were made for the reactions of the free radicals $^{\bullet}OH$, $Br_2^{\bullet -}$, and $N_3^{\bullet}$. The carbonate and dichloride radical anions were found to react relatively quickly with HA (7.0×10^5 and 6.9×10^6 dm^3 mol^{-1} s^{-1}, respectively) although they are much less reactive than the hydroxyl radical, $^{\bullet}OH$. Significant yields (20 and 38%, respectively) of chain scission of HA by these radical anions were also determined from the gpc/MALLS experiments, providing some support for their potential participation in the depolymerization of HA in vivo. These results are compared with data obtained for the other free radicals (hydroxyl, azide radicals, and dibromide radical anions) investigated in this study in order to gain an insight into their mechanism of reaction with HA. Earlier chain scission yields of HA by hydroxyl radicals determined by the authors have also been revised using the gpc/MALLS technique employed in the current study. The yields of 52% (absence of air) and 44% (in air) are much lower than the previous values. In the current study, the effect of oxygen on the yields of HA chain breaks is discussed in terms of the reactivity of HA peroxyl radicals in the presence of superoxide radical anions. The relevance of the results of this study to mechanisms of inflammation is discussed.

Keywords: Hyaluronan; Carbonate radical anion; Dichloride radical anion; Reactive oxidative species; Peroxynitrite; Nitric oxide; Superoxide; Free radical

One of the main secretory components of synovial fluid is hyaluronan (HA). In rheumatoid synovial fluid, the weight average molecular weight is only about 4.8 MDa compared to about 7 MDa in normal fluids [1]. As well as providing a physical structure within the extracellular matrix, HA is known to modulate a range of cellular and tissue functions [2]. HA fragments are known, for example, to modify macrophage expression of chemokines and growth factors as well as cell surface markers and to accumulate at sites of inflammation (see, for example, [3,4]). It remains unclear how such fragments are formed although the production of free radicals and other reactive oxidative species as a result of phagocytosis suggests a role for such species in the fragmentation of HA.

There is thus now a significant amount of evidence to support the involvement of highly reactive species such as $O_2^{\bullet -}$, H_2O_2, $HOCl/OCl^-$, chloramines, and the hydroxyl free radical, $^{\bullet}OH$, in inflammatory diseases(for a review, see [5]). Although both $O_2^{\bullet -}$ and H_2O_2 are unlikely themselves to degrade hyaluronan, both species can participate in reactions with transition metal complexes of copper(I/II) and iron(II/III) to produce the highly reactive and damaging species, the hydroxyl free radical. The probability of these reactions occurring in inflammatory diseases such as rheumatoid synovial fluid is increased because it has been established, for example, that both

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catalase and superoxide dismutase, whose functions are to scavenge H_2O_2 and $O_2^{\bullet -}$, respectively, are barely detectable in such fluids [6].

There have been a number of studies of the reaction of the hydroxyl radical and similar reactive species with HA (for example, [7–9]. A wide range of techniques has been used to follow the degradation of HA, including viscosity changes, end-group analysis, oxygen consumption, peroxide formation, and carbon dioxide release. Electron paramagnetic resonance measurements have also been performed to gain an insight into the molecular mechanism of chain scission by hydroxyl and other reactive species [10,11]. The main thrust of our work has been to measure HA chain scission yields as a proportion of quantifiable hydroxyl radical fluxes produced by ionizing radiation [12–15]. For this purpose, both viscosity changes and more recently a combination of multiangle laser light scattering and gel permeation chromatography (gpc/MALLS) have been used to measure changes in molecular weight. The changes in molecular weight have thus been used to determine the chain break concentration.

Although the hydroxyl radical has long been regarded as the major reactive oxidative species in inflammatory disease, other potential candidates, such as peroxynitrite ($ONOO^-$), carbonate radical anions ($CO_3^{\bullet -}$), and hypochlorite may also have significant roles in, for example, the fragmentation of HA.

Peroxynitrite, a reactive nitrogen species, is produced in the diffusion-controlled reaction between $O_2^{\bullet -}$ and nitric oxide [16]. Given the high concentrations of superoxide radicals produced in the respiratory burst and the likely simultaneous presence of nitric oxide during phagocytosis, it seems highly probable that peroxynitrite may also be produced during phagocytosis. The hypothesis is supported, for example, by the fact that significantly high nitrite concentrations have been found in the serum of patients with rheumatoid arthritis [17]. Peroxynitrite may therefore be a potential oxidant in rheumatic diseases. Recent studies have demonstrated that HA is indeed degraded by peroxynitrite [18–20]. The study in our laboratories demonstrated that peroxynitrite in its protonated form, ONOOH, can cause fragmentation of HA in significant yields [19]. That work also showed that, extrapolated to zero hyaluronan concentration, hydroxyl radicals (formed from a rearrangement of ONOOH) were produced in a 5% yield (as a proportion of the peroxynitrite concentration).

More biologically relevant work is now ongoing in our laboratories to investigate the effect of carbon dioxide on the reactivity of peroxynitrite with hyaluronan.. In other studies, it has been proposed that an adduct (which may also be an oxidant in its own right) is formed between peroxynitrite and carbon dioxide. This adduct has been shown to produce significant yields (up to 33%) of $CO_3^{\bullet -}$ [21–23]. In the work presented here, the experiments with carbonate radicals are seen as a key component in the understanding of the biologically relevant reactions of peroxynitrite with HA in the presence of carbon dioxide.

The fragmentation of hyaluronan by hypochlorite has been studied in considerable detail using EPR and other techniques [10,24,25]. Hypochlorite is produced in chronic inflammatory conditions after the release of myeloperoxidase through the activation of leukocytes [26] and has been shown from EPR measurements to produce HA-derived free radicals in an attack on the *N*-acetyl groups of HA via the production of long-lived chloramides. Decomposition, through one electron reduction, of HA chloramides by transition metal ions leads to the formation of nitrogen- and carbon-centered radicals which led ultimately to HA chain scission [10,24,25]. It was further shown that superoxide radicals could also initiate the reduction of these chloramides [25].

The simultaneous production of superoxide radical anions and HOCl during phagocytosis also allows the possibility of interaction between these same species. It is already known that the reaction between superoxide radical anion and HOCl occurs relatively quickly, 7.5×10^6 dm^3 mol^{-1} s^{-1}, to produce hydroxyl and/or chlorine-derived radicals [27,28] and could compete effectively with the much slower reaction of HOCl with HA (0.01 dm^3 mol^{-1} s^{-1} [29]. The extent of the competition would depend upon the relative concentrations of HA and superoxide radical anions and, given the 9 orders of magnitude difference in rate constants, it seems likely that superoxide radicals would compete effectively to produce hydroxyl and/or chlorine-derived radicals. In the presence of chloride ion in acidic solutions, hydroxyl radicals are known to react at diffusion controlled rates to produce the highly oxidizing radical anion, $Cl_2^{\bullet -}$ [30]. The yield of $Cl_2^{\bullet -}$ is much lower in neutral solutions, although at the relatively high extracellular fluid chloride concentration of about 0.15 mol dm^{-3}, there is evidence that $Cl_2^{\bullet -}$ and related species are produced in significant yields [30]. $Cl_2^{\bullet -}$ or related species may also be formed in biological systems as a result of the reduction of hypochlorite by transition metal complexes or by superoxide radicals [27]. Its reaction with HA may be as selective as found for hypochlorite [10] and is included in the current study. For comparison, the reaction of the related species $Br_2^{\bullet -}$ with HA is also studied here.

Azide radicals may be produced in model studies involving peroxynitrite in which the ozonation of azide is used to prepare peroxynitrite. Any hydroxyl radicals produced by peroxynitrite could react proportionately with any residual azide to produce azide radicals, which in turn may produce unwanted reactions with HA.

The purpose of this study therefore is to establish whether carbonate and dichloride radical anions can cause the fragmentation of hyaluronan and so be considered as possible reactive oxidative species in inflammation processes.

In view of the potential significance of hydroxyl radicals in the depolymerization of HA in vivo, an additional feature of this study is to present revised values of the HA chain scission yields induced by hydroxyl radicals.

Materials and methods

Hyaluronan solution was donated by Biomatrix, Inc. (USA). The sample was dialyzed against water for 5 days and then autoclaved at 128°C for 30 min to produce samples