Oxygen-centred free radicals can efficiently degrade the polypeptide of proteoglycans in whole cartilage

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(Received 11 November 1984)

Bovine nasal cartilage slices, biosynthetically labelled in their proteoglycan with $^{35}$SO$_4^-$, were used as substrate for the attack of free radicals generated on exposure to a Co$^{60}$ source (which allows study of single radical species), and by chemical and enzymatic means. Systems generating hydroxyl (OH') and superoxide (O$_2^-$) radicals degraded the proteoglycan efficiently, while the hydroperoxy radical (HO$_2^-$) was less efficient; addition of appropriate radical scavengers inhibited degradation. The radioactive products were heterogeneous in molecular size, but with doses up to 3600 Gy were the same size range as intact chondroitin sulphate. They contained free amino groups, and more were liberated by aminopeptidase M digestion, implying that at least a small peptide was present. Thus a major site of radical attack may be the polypeptide chain. We suggest that free-radical fragmentation of polypeptides may be important both in extracellular catabolism and in intracellular proteolysis.

The role of oxygen-derived free radicals in degradation of macromolecules and lysis of organisms is gaining increasing attention (1,2). Since the key cells in inflammatory tissue breakdown, such as the macrophage, can produce active oxygen species when stimulated (initially O$_2^-$, which can lead to formation of OH' and H$_2$O$_2$) (3), the possibility exists that these species are involved in the degradation of connective tissue which accompanies chronic inflammation. We present here the first direct evidence, using defined radiation chemical and enzymatic systems, that systems generating both hydroxyl and superoxide radicals have potent degradative actions on whole cartilage in vitro. Cleavage of peptide bonds in the polypeptide of proteoglycan seems to be involved, and the major product is virtually complete chondroitin sulphate chains.

Materials and Methods

Bovine nasal cartilage was biosynthetically labelled in its proteoglycan using $^{35}$SO$_4^-$ (4), and slices were then used as substrate for oxygen radical attack. The generation of free-radical species in
phosphate-buffered saline (PBS), pH 7.2, was performed using a Co$^{60}$ source of 2000 Curie ('cave' type). In such a system OH$, e(aq)$, and H$^+$ are the main primary species formed. Manipulation of the irradiation system allows specific radical formation (5). In order to generate predominantly OH$, saturation with N$_2$O efficiently converts e(aq) into OH$^-$ according to:

$$H_2O + e(aq) + N_2O \rightarrow OH^- + OH^+ + N_2$$

$k(1) = 9.1 \times 10^9$ M$^{-1}$s$^{-1}$.

In such a system a small (roughly 10%) contribution will be due to H$. Alternatively, superoxide radical (O$_2^-$) can be generated by scavenging of OH$^-$ and H$^+$ with sodium formate (10 mM) and irradiating in the presence of N$_2$O/O$_2$ (80:20) according to:

$$HCOO^- + OH^- + COO^- + N_2O$$

$k(2) = 2.9 \times 10^9$ M$^{-1}$s$^{-1}$.

$$HCOO^- + H^+ + COO^- + H_2$$

$k(3) = 2 \times 10^8$ M$^{-1}$s$^{-1}$.

$$COO^- + O_2 + O_2^- + CO_2$$

$k(4) = 2.4 \times 10^8$ M$^{-1}$s$^{-1}$.

In systems containing O$_2$, the superoxide radical may be generated by:

$$e(aq) + O_2 + O_2^-$$

$k(5) = 2 \times 10^{10}$ M$^{-1}$s$^{-1}$.

At low pH, the hydroperoxy radical is formed:

$$HO_2^- \rightleftharpoons O_2^- + H^+ \quad pK_a = 4.8$$

Thus in a PBS air-saturated system the predominant species formed would be OH$^-$ and O$_2^-$. 

**Results and Discussion**

In preliminary experiments, degradation products were found in the medium surrounding the cartilage immediately after irradiation with 1200 Gy, though much more diffused out of the discs during a further 24-h incubation at 4°C, by which time more than 90% of the maximum release of product had occurred. Control discs released no significant radioactivity during parallel incubations at 4°C. Thus a 24-h period of diffusion after the irradiation was chosen as standard. The hydroxyl- and superoxide-radical-generating systems using the cobalt source were each potent in degrading the cartilage (Fig. 1). This was true of living cartilage, of freeze-thawed cartilage (in which the chondrocytes are killed), and of freeze-thawed and heat-denatured cartilage (in which endogenous hydrolases are largely inactivated, so that autolysis is reduced to a low level, permitting the most satisfactory measurement of degradation due to exogenous agents). Data are shown for the latter condition, in which most experiments were conducted, since this was the only one which permitted design of irradiation experiments with enough comparable replicates and
Fig. 1. Dose-response curves for degradation of cartilage proteoglycan by oxygen-derived radicals. 1-mm-thick slices of bovine nasal cartilage were labelled in their proteoglycan by incubation in $^{35}$SO$_4$ as described (4). They were then freeze-thawed, and immersed in a boiling water bath for 8 min, to kill chondrocytes and inactivate endogenous hydrolases. The autolytic release of sulphate-labelled material was reduced to a very low level by this procedure, but as noted before (4), the susceptibility of the proteoglycan to exogenous proteinases was not significantly changed by this procedure. Remaining proteinases were further inhibited by coating the slices with heat-inactivated swine serum (30 min, 37°C), and washing with PBS. Discs were stored at -20°C. For irradiation, single discs were suspended in 1.5 ml of PBS (made using twice-distilled and deionized water), and pregassed as appropriate (●, N$_2$O only; ▲, air only; △, N$_2$O/O$_2$/10-mM formate, pH 7; ○, N$_2$O/O$_2$/formate, pH 4) for 10 min. Irradiation was performed in clean, oven-glowed glass tubes at 17.9 Gy/min, after which discs were stored at 4°C for 24 h. Degradation was then measured as release of $^{35}$SO$_4$ into the fluid phase, expressed as % of the total radioactivity in the system. Radioactivity in the slices was determined after their complete dissolution in HCOOH (98%; 1 ml) at 55°C overnight. Results are means ± S.D. (where this exceeds the size of the symbol), for n= 3, and are representative of several experiments. The difference between degradation in these experiments and that in Table 1 shows the extreme of variation between batches of cartilage discs which we observed.
conditions to permit detailed examination of the degradation. In contrast to the hydroxyl and superoxide conditions, the hydroperoxy-radical system was relatively inactive. This latter observation showed that direct radiation damage (as opposed to radical attack) could not be responsible for much of the degradation, which was confirmed by detailed experiments where discs were heat-dried, and then irradiated with or without rehydration: while the rehydrated discs were degraded to the normal extent, the dehydrated discs suffered less degradation (36-46% of corresponding controls after 1200 Gy, varying slightly between the radical systems). These experiments thus showed unambiguously that systems in which oxygen radicals are generated can degrade the proteoglycan of whole cartilage.

Experiments with selective radical scavengers were undertaken. At the concentrations commonly used (10 mM) the selective scavengers mannitol, ferricyanide, and ascorbate had significant but small inhibitory effects consistent with both hydroxyl and superoxide radicals contributing directly or indirectly to degradation. When mannitol was used at a much increased concentration of 1 M, greater inhibition of degradation was achieved (Table 1). These experiments confirmed the degradative activity of the two systems, but emphasized that the interior of the cartilage discs contains a huge concentration of fixed saccharide units in the proteoglycan, and thus unusually large scavenger concentrations may be necessary to compete effectively. Superoxide dismutase could significantly inhibit the superoxide-mediated degradation, though even when discs were preincubated for 24 h with the enzyme, inhibition was incomplete (Table 1), presumably due to limitations on permeation of large molecules within the cartilage.

Two biochemical systems were then studied to extend the relevance of the above observations. Firstly, in the presence of Fe²⁺, H₂O₂ at pH 5.6 may catalytically generate hydroxyl radicals, whereas at pH 7 the same system produces rather little of any radical (6). An advantage of this system is that all the components are small molecules which can be expected to enter the cartilage freely. In agreement with the radiolysis data, at pH 5.6 this system was very effective in degrading cartilage (Table 2). This could be inhibited by desferrioxamine, which seems at all concentrations to make iron unavailable for generation of hydroxyl radicals from hydrogen peroxide (7). It is notable that in assays of the iron content of the cartilage discs used in these studies, we found that each disc (about 100 mg wet wt.) contained less than 30 ng of iron in total, probably due to the extensive washing procedures after the biosynthetic labelling. The data thus confirmed the effectiveness of systems generating hydroxyl radicals in degrading cartilage. Secondly, enzymatic generation of superoxide was performed using xanthine oxidase and hypoxanthine (8). Unfortunately xanthine oxidase was rather effective in degrading cartilage even without addition of its substrate hypoxanthine (36.5 ± 2.3% [mean ± S.D., for triplicates] in 24-h experiments with 0.6 U/ml xanthine oxidase contributing 4.2 mg/ml protein). This degradation was reduced to about 3% by three agents which inhibit proteolytic activity (9): serum, PMSF (phenyl-methyl-sulfonyl-fluoride), or leupeptin. Proteinases are known to contaminate the commercial xanthine oxidase samples, and so in further experiments with the enzyme in the presence of PMSF to inhibit the proteinases, slight
Table 1. Inhibition of oxygen-radical degradation of cartilage by mannitol and superoxide dismutase

(a) Using the \( \cdot OH^- \)-generating system defined in the text, cartilage discs were irradiated (1200 Gy) with the specified additions of mannitol to scavenge hydroxyl radicals. Because 1-M mannitol becomes solid at 4°C, the diffusion after irradiation was into fresh medium which was in all cases PBS alone. Mannitol and ascorbate had slightly inhibitory effects on the release of degradation product during the first 3 h of the diffusion period, which may be due to action during this period of longer-lived radicals within the cartilage, or of hydrogen peroxide.

(b) Discs were irradiated (1200 Gy) in the superoxide-radical-generating conditions defined in the text, and with the specified additions of superoxide dismutase (SOD; 42 units/mg protein, Sigma). For the preincubation experiment, discs were incubated with SOD for 18 h at 4°C, and the irradiation commenced. Increasing the HCOOH concentration in the superoxide-generating system to 1 M made no difference to the overall rate of degradation, or to its susceptibility to inhibition by SOD.

<table>
<thead>
<tr>
<th>Condition during irradiation</th>
<th>Degradation (%)</th>
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<tbody>
<tr>
<td>(a) Hydroxyl-radical attack</td>
<td></td>
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<tr>
<td>Control</td>
<td>5.20 ± 0.42</td>
</tr>
<tr>
<td>+ 10-mM mannitol</td>
<td>4.58 ± 0.22</td>
</tr>
<tr>
<td>+ 1-M mannitol</td>
<td>1.71 ± 0.33</td>
</tr>
<tr>
<td>Blank (no irradiation)</td>
<td>0.35 ± 0.12</td>
</tr>
<tr>
<td>(b) Superoxide-radical attack</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.39 ± 0.80</td>
</tr>
<tr>
<td>+ 12.5 units/ml SOD</td>
<td>7.27 ± 0.73</td>
</tr>
<tr>
<td>+ 62.5 units/ml SOD</td>
<td>6.86 ± 0.66</td>
</tr>
<tr>
<td>+ 125 units/ml SOD</td>
<td>5.72 ± 0.48</td>
</tr>
<tr>
<td>+ 125 units/ml SOD, preincubated with SOD</td>
<td>5.20 ± 0.38</td>
</tr>
<tr>
<td>Blank (no irradiation)</td>
<td>0.29 ± 0.14</td>
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breakdown was produced in hypoxanthine-dependent fashion (1% in 24 h), and this could be inhibited by superoxide dismutase. Spectrophotometric measurements showed that more than 80% of the hypoxanthine was converted to uric acid during the first 3 h of incubation, in the presence of the cartilage. Whereas the products from irradiation attack (in which radicals are produced throughout the interior of the cartilage) take many hours to diffuse out of the cartilage, the products of xanthine oxidase attack were largely released within 3 h (data not shown). In view of the relatively slight degradation, and the large size of the enzyme (238 kDa), it seems that the superoxide radicals generated by the enzyme are largely on the external surface of the cartilage. The contaminating proteinases probably have lower molecular weights like many proteinases (9), and hence may act to some degree within the cartilage, producing greater degradation.
Table 2. Degradation of cartilage by hydroxyl radicals generated by Fe/H$_2$O$_2$ at pH 5.6

Discs were incubated at 37 for 24 h, with 100-mM acetate buffer pH 5.6, 10 I.U./ml penicillin, and 10 g/ml streptomycin, and the stated additions. H$_2$O$_2$ was used at 300 1 of 30 vol. stock in the final incubation vol. of 1.5 ml, while Fe$^{2+}$ was used as the sulphate, at a final concentration of 1 mM. The discs were then immediately processed as described in Fig. 1. The complete system at pH 7.2 gave only 2.54 0.83% degradation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Degradation (%)</th>
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</thead>
<tbody>
<tr>
<td>H$_2$O$_2$ alone (control)</td>
<td>1.82 0.19</td>
</tr>
<tr>
<td>+ Fe$^{2+}$ (complete system)</td>
<td>17.04 2.5</td>
</tr>
<tr>
<td>+ Fe$^{2+}$/desferrioxamine (0.11 mM)</td>
<td>13.49 1.17</td>
</tr>
<tr>
<td>+ Fe$^{2+}$/desferrioxamine (1.1 mM)</td>
<td>3.35 0.29</td>
</tr>
<tr>
<td>Blank (buffer alone)</td>
<td>0.41 0.12</td>
</tr>
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</table>

The nature of the products of degradation was studied extensively using gel chromatography on Sepharose 2B and 6B (Fig. 2). During attack by the hydroxyl- or superoxide-radical-generating systems, a single main product was detected, which chromatographed in a single symmetrical peak on 2B (Fig. 2). This peak was distinctly subfractionated on Sepharose 6B filtration, and thus was heterogeneous. The product was similar to that of complete dissolution of the discs with papain (Fig. 2), which contains intact single chondroitin sulphate chains (10), and was notably devoid of free sulphate or of other material of very small molecular size. The possibility that the chains generated by radical attack contained small peptides adjacent to the xylose-serine linkage of the glycan to the protein (11) was investigated by reacting the products with fluorescamine, which labels primary amino groups with a fluorophor (12). A positive reaction was obtained; reactive material co-chromatographed with the major radioactive peak. Aminopeptidase M digestion liberated further fluorescamine-reactive material, implying that at least a small peptide was present on these glycosaminoglycan chains, and thus that some primary chain cleavage effected by the radical-generating systems is on the polypeptide moiety. In the more complex irradiation conditions of PBS alone, some substantially larger products were also released (Fig. 2), and this was also observed with the Fe$^{2+}$/H$_2$O$_2$ system.

The conclusion that polypeptide chain scission is responsible for at least some of the proteoglycan breakdown by the radical-generating systems is consistent with previous observations that the second-order rate constants for the reactions of both hydroxyl and superoxide radicals are some orders of magnitude greater with proteins than with polysaccharides (13). Furthermore, we have very recently shown directly that extensive polypeptide scission can occur during attack of hydroxyl radicals on many purified proteins (including some cartilage
Fig. 2a. Gel filtration of degradation products from oxygen-radical attack on cartilage. 1.5-ml portions of supernatants from 1200 Gy irradiation in the OH⁻ (●), O₂⁻ (△), and PBS alone (▲) systems, obtained after the 24-h diffusion period, were chromatographed on Sepharose 2B (K15/90 column, flow rate 6 ml/h, eluant PBS). 1.5-ml fractions were collected. The elution volume of products of OH⁻ attack were the same after 100 Gy as after 1200 Gy. V₀ and Vₜ indicate the void and total volumes of the column, and Vₜhs, the single peak of the elution profile of papain-solubilized radioactivity (95% of the radioactivity was solubilized). The fourth profile shown is that of the products from further irradiation (48 000 Gy) in PBS alone of the material from OH⁻ attack (1200 Gy).
components), and on protein mixtures (14). Chondroitin sulphate, apparently the major product in our system, can be degraded by free radicals though it seems that 1200 Gy (our maximum dose) cleaves less than 1% of the linkages even in chondroitin sulphate in free solution (15). Although the linkages containing galactose and xylose at the polypeptide end of the glycan are exceptionally susceptible to periodate cleavage, this does not imply that they are also more susceptible to radical attack. To support this interpretation we showed that much larger doses of irradiation could degrade the radioactive degradation product peak from gel chromatography into radioactive fragments which were much more retarded on the gel (and thus were smaller: Fig. 2). It has been noted several times before that hyaluronic acid can be depolymerized by oxygen radicals (16). The present work shows that depolymerization (in this case partly of the protein core of the proteoglycan) can occur even within whole large extracellular matrices, which of course present an environment highly distinct from that of the free-fluid phase. The data indicate the likely importance of radical-mediated polypeptide breakage not only in turnover of extracellular materials but also possibly in the breakdown of intracellular proteins (17). In the case of connective-tissue macromolecules, the leukocytes with their specialized surface oxidative systems are likely participants in radical-mediated breakdown:

Fig. 2b. Gel filtration of degradation products from oxygen-radical attack on cartilage. Chromatography on Sepharose 6B (K15/90 column, flow rate 6 ml/h, eluant PBS). 1.5-ml fractions were collected. 1.5-ml portions of the supernatant from 1200 Gy irradiation in PBS alone (▲), or of the papain digest (×), were chromatographed.
in our irradiation system the cartilage is exposed to a maximum of about one micromole of radicals, and this is roughly the radical production of $10^7$ stimulated macrophages in 30 minutes (3). In the case of intracellular proteins, the radical-generating electron-transport systems of the endoplasmic reticulum, and other cellular enzymes and components, may well have the corresponding role.

It should be emphasized that in our complex multiphasic system of cartilage discs, it cannot be safely assumed that the damaging radicals are necessarily those generated initially in the various conditions. Thus while it is not surprising that hydroxyl radicals might fragment proteoglycans, since they can fragment most macromolecules, it would be rather more unexpected if superoxide radicals can directly achieve the same fragmentation, since little precedent for this exists. Indeed, it is very likely that the superoxide radicals supplied to the cartilage discs become converted into many other radical species, perhaps including hydroxyl radicals, which may be directly responsible for fragmentation. Because of the chemical complexity of the cartilage matrix it is not readily possible to decide the nature of the active radicals; but in future studies with isolated proteoglycans in solution we will address this question.

Acknowledgements

We acknowledge with thanks support from the Arthritis and Rheumatism Council (R.T.D.), the SERC and Glaxo Group Research (CRR), and the National Foundation for Cancer Research (LGF). We thank Prof. Robin Willson for discussions, and the MRC for the cobalt source.

References