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Degradation of human proteoglycan aggregate induced by hydrogen peroxide

Protein fragmentation, amino acid modification and hyaluronic acid cleavage

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We have previously shown that treatment of neonatal human articular-cartilage proteoglycan aggregates with H_2O_2 results in loss of the ability of the proteoglycan subunits to interact with hyaluronic acid and in fragmentation of the link proteins [Roberts, Mort & Roughley (1987) Biochem. J. **247**, 349–357]. We now show the following. (1) Hyaluronic acid in proteoglycan aggregates is also fragmented by treatment with H_2O_2. (2) Although H_2O_2 treatment results in loss of the ability of the proteoglycan subunits to interact with hyaluronic acid, the loss of this function is not attributable to substantial cleavage of the hyaluronic acid-binding region of the proteoglycan subunits. (3) In contrast, link proteins retain the ability to bind to hyaluronic acid following treatment with H_2O_2. (4) The interaction between the proteoglycan subunit and link protein is, however, abolished. (5) *N*-Terminal sequence analysis of the first eight residues of the major product of link protein resulting from H_2O_2 treatment revealed that cleavage occurred between residues 13 and 14, so that the new *N*-terminal amino acid is alanine. (6) In addition, a histidine (residue 16) is converted into alanine and an asparagine (residue 21) is converted into aspartate by the action of H_2O_2. (7) Rat link protein showed no cleavage or modifications in similar positions under identical conditions. (8) This species variation may be related to the different availability of histidine residues required for the co-ordination of the transition metal ion involved in hydroxyl-radical generation from H_2O_2. (9) Changes in function of these structural macromolecules as a result of the action of H_2O_2 may be consequences of both fragmentation and chemical modification.

INTRODUCTION

The proteoglycan aggregates of human articular cartilage are composed of proteoglycan subunits in non-covalent associations with hyaluronic acid. These associations are stabilized further by the link proteins, which bind to both the proteoglycan subunit and hyaluronic acid. The mechanical functioning of cartilage is thought to depend on the retention of proteoglycans in the tissue, and this in turn is believed to depend upon the ability of these molecules to form aggregates of high M_r. The proteoglycan content of an aggregate is a function of the length of its hyaluronic acid filament and of the continued ability of proteoglycan subunits and link proteins to interact with each other and with hyaluronic acid.

There is considerable interest in the mechanisms by which proteoglycan aggregates may be modified, particularly concerning the action of proteinases upon the aggregates (reviewed by Muir, 1980). More recently, it has been proposed that reactive oxygen metabolites may also be involved in the degradation of connective tissue that occurs under inflammatory arthritic conditions and in disorders involving excessive deposition of transition metal ions in the joint, such as haemophilia and Wilson's disease (reviewed by Halliwell & Gutteridge, 1984, 1985). The production of partially reduced metabolites of oxygen is a consequence of normal aerobic metabolism (Chance *et al.*, 1979). In addition, phagocytic cells are capable of producing larger amounts of reactive oxygen metabolites at their surfaces during the oxidative burst. These metabolites have been shown to be able to activate (Weiss *et al.*, 1985) or inactivate proteinases and to inactivate proteinase inhibitors (Vissers & Winterbourn, 1987) under appropriate experimental conditions. A number of studies have demonstrated the ability of reactive oxygen metabolites to degrade proteoglycans directly (Bartold *et al.*, 1984; Bates *et al.*, 1984; Chung *et al.*, 1984; Dean *et al.*, 1984), and we have described in detail some structural modifications that occur in neonatal human articular-cartilage proteoglycan aggregates as a result of treatment with H_2O_2 (Roberts *et al.*, 1987). These modifications include fragmentation of the link proteins and loss of the ability of proteoglycan monomers to associate with hyaluronic acid, despite minimal decrease in subunit size. We have suggested that the effects of H_2O_2 are consistent with the hypothesis that some of the changes which occur with increasing age in human cartilage may result through the action of reactive oxygen metabolites.

We here extend our previous studies on the effect of H_2O_2 upon proteoglycan aggregates by describing the

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site of cleavage and two amino acid modifications that occur in the *N*-terminal region of the link protein, the relative lack of fragmentation within the hyaluronic acid-binding region of the proteoglycan subunits and the degradation of hyaluronic acid within the aggregate.

MATERIALS AND METHODS

Materials

Guanidinium chloride and trypsin (tosylphenylalanylchloromethane-treated) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. CsCl was from Accurate Chemical and Scientific Co., Westbury, NY, U.S.A. Materials for electrophoresis were from Bio-Rad Laboratories, Toronto, Ont., Canada. Poly(vinylidene difluoride) transfer membranes were from Millipore Corp. Bedford, MA, U.S.A. H_2O_2 was AnalaR grade (30%, v/v) from BDH Chemicals, Toronto, Ont., Canada. Radiochemicals were from Amersham International, Mississauga, Ont., Canada. Rabbit anti-(mouse IgG1) antibody was from SciCan Diagnostics, Edmonton, Alberta, Canada, and the reagents for hyaluronic acid assay (HA Test 50) were kindly provided as a kit by Pharmacia Diagnostics A.B., Uppsala, Sweden. All solutions were made up freshly in water of resistance greater than 10 MΩ/cm, purified by using a Millipore MilliQ water-purification system.

Methods

Preparation of proteoglycan aggregate. Neonatal human articular cartilage was obtained at autopsy within 18 h of death and extracted in 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 6.0, containing 1 mM each of EDTA, phenylmethanesulphonyl fluoride and iodoacetic acid and 1 μg of pepstatin/ml (Roughley & White, 1980). The extracts were dialysed to associative conditions (Roughley *et al.*, 1982), adjusted to a starting density of 1.5 g/ml by the addition of CsCl and centrifuged at 100000 $g_{av.}$ for 48 h at 10 °C in a fixed-angle rotor. The proteoglycan aggregates were isolated from the lower part of the gradient (density greater than 1.55 g/ml), dialysed twice against water, once against 0.1 M-potassium acetate and exhaustively against water and then freeze-dried. On analysis by Sepharose CL-2B chromatography, 70% of the proteoglycan was shown to be in aggregate form.

Rat chondrosarcoma proteoglycan aggregate was prepared by an identical procedure after harvesting the Swarm chondrosarcoma from Sprague–Dawley rats that had been injected subcutaneously with a chondrosarcoma-cell suspension (Oegema *et al.*, 1975).

Reaction of proteoglycan aggregates with H_2O_2. Proteoglycan aggregates were dissolved at 3 mg/ml in 25 mM-sodium acetate buffer, pH 5.6, containing 80 mM-NaCl, and 200 μl portions of this solution were routinely used for experiments. Experimental additions of appropriate dilutions of H_2O_2 were made in a volume of 10 μl to produce the desired final concentrations, which were in the range 0–240 mM-H_2O_2. Reactions were allowed to proceed for 24 h at 37 °C, and the samples then dialysed against 0.125 M-Tris/HCl buffer, pH 6.8.

Treatment of proteoglycan aggregates with trypsin. Proteoglycan aggregates were treated with trypsin, following treatment with H_2O_2, in the following manner. The samples were dialysed against 0.1 M-Tris/HCl buffer, pH 7.5, and trypsin was added to a final concentration of 10 μg/mg of proteoglycan aggregate. The samples were incubated at 37 °C for 4 h, then phenylmethanesulphonyl fluoride was added to a final concentration of 1 mM, and the samples were dialysed against 0.1 M-Tris/HCl buffer, pH 6.8, before electrophoresis.

Analytical CsCl-density-gradient centrifugation. Analysis of the ability of components of the proteoglycans to interact was performed by CsCl-density-gradient centrifugation under associative conditions with

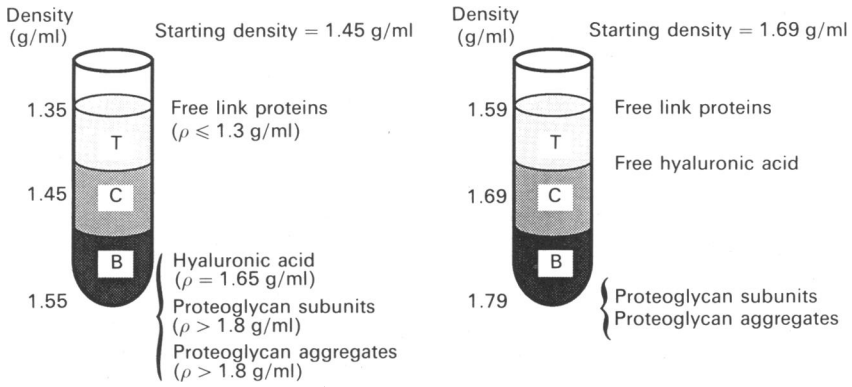

Fig. 1. Effect of starting density on the equilibrium sedimentation of proteoglycan aggregate and its components in CsCl density gradients

Sedimentation positions were analysed by using starting densities of 1.45 and 1.69 g/ml. With a starting density of 1.45 g/ml intact proteoglycan aggregate, free proteoglycan subunit and free hyaluronic acid all sediment to the bottom of the gradient, whereas free link protein sediments to the top. With a starting density of 1.69 g/ml intact proteoglycan aggregate and free proteoglycan subunit sediment to the bottom of the gradient, free hyaluronic acid sediments near the centre and free link protein sediments at the top. Link protein–hyaluronic acid complexes sediment to the bottom of the gradient with a starting density of 1.45 g/ml, but to the top with a starting density of 1.69 g/ml, whereas link protein–proteoglycan subunit complexes sediment to the bottom under both starting conditions. For analysis gradients were divided into three equal fractions representing the bottom (B), central (C) and top (T) thirds. The Figure depicts the average sedimentation position of the various proteoglycan components and also shows the density range obtained under the various starting conditions.

starting densities of 1.45 or 1.69 g/ml, after determining that these conditions allow the differentiation of link protein interaction in the form of proteoglycan aggregate from link protein interacting with hyaluronic acid alone (Fig. 1). CsCl was added to samples of treated proteoglycan solution to produce the desired final density, and 240 μl portions of these solutions were centrifuged at 150000 $g_{max.}$ for 24 h in a Beckman Airfuge (Rostand *et al.*, 1982). It was determined that the interactions between the components of the proteoglycan aggregate were stable in these procedures. At the end of the centrifugation, tube contents were fractionated into three 80 μl fractions, 10 μl of each fraction was assayed for uronic acid by the carbazole method of Bitter & Muir (1962), and the remainder was dialysed against 0.1 M-Tris/HCl, pH 6.8, before electrophoresis.

Electrophoresis and Western blotting. SDS/polyacrylamide-gel electrophoresis was performed in 10% slab gels under the conditions described by Laemmli (1970). Sample preparation, electrophoresis and Western-blot analysis were essentially as previously described (Roberts *et al.*, 1987). All samples were heated in the presence of 5% (v/v) 2-mercaptoethanol before being loaded on to the gels. Proteins were electroblotted on to nitrocellulose membranes in 20% (v/v) methanol/15.6 mM-Tris/120 mM-glycine buffer, pH 8.3. The membranes were blocked in 1% (w/v) bovine haemoglobin in 0.145 M-NaCl/10 mM-sodium/potassium phosphate buffer, pH 7.8, containing 0.05% NaN_3 before incubation with ascitic fluids, which contained monoclonal antibodies as required. These were 9/30/8-A-4 (Caterson *et al.*, 1985*a*,*b*), which recognizes two nearly identical epitopes in intact link protein (Neame *et al.*, 1986), and 12/20/2-A-5, which recognizes an epitope close to the hyaluronic acid-binding region of the core protein of the proteoglycan (Caterson *et al.*, 1985*a*). Both of these antibodies were kindly supplied by Dr. Bruce Caterson, Department of Biochemistry, University of West Virginia, Morgantown, WV, U.S.A. The monoclonal antibody 12/20/2-A-5, which is of the IgG1 subclass, was localized by the use of a second-step rabbit anti-(mouse IgG1) antibody. The nitrocellulose membranes were incubated with monoclonal antibodies as required, and regions of antibody binding were located by the use of ^{125}I protein A as previously described (Roberts *et al.*, 1987).

Sepharose CL-2B chromatography. Before chromatography samples were reduced and alkylated. Guanidinium chloride was added to each sample to a final concentration of 4 M, dithiothreitol was added to a final concentration of 5 mM, and the samples were then incubated at 40 °C for 5 h. Iodoacetamide was added to a final concentration of 20 mM, and the samples were incubated at 40 °C for 1 h and then overnight at 4 °C. All samples were then extensively dialysed against water before being chromatographed on a 110 cm × 1 cm column of Sepharose CL-2B at a flow rate of 6 ml/h in 50 mM-Tris/HCl buffer, pH 7.3, containing 4 M-guanidinium chloride. Each sample contained 1.5 mg of proteoglycan in 1 ml. Fractions (1 ml) were monitored for uronic acid by the carbazole method of Bitter & Muir (1962), or for glycosaminoglycans by the Dimethylmethylene Blue dye-binding assay (Farndale *et al.*, 1986), which was standardized with chondroitin sulphate.

Hyaluronic acid assay. Column fractions were assayed for hyaluronic acid by the radiometric method of Tengblad (1980). The principle of this assay is that hyaluronic acid in the test sample binds to added ^{125}I-labelled hyaluronic acid-binding protein, and unbound ^{125}I-labelled hyaluronic-acid-binding protein is quantified by its binding to hyaluronic acid that is covalently linked to Sepharose beads. The assay was performed under the conditions suggested by Pharmacia, and was standardized with reduced and alkylated neonatal human cartilage proteoglycan and with standard solutions of rooster comb hyaluronic acid that were furnished by Pharmacia.

Purification and sequencing analysis of link proteins. Proteoglycan aggregates were treated with 60 mM-H_2O_2 as above, or untreated. They were then dialysed extensively, first against 0.1 M-Tris/HCl buffer, pH 6.8, and then against 0.2 M-sodium acetate buffer, pH 5.5. Guanidinium chloride was then added to a final concentration of 4 M, and CsCl to a final density of 1.5 g/ml. The link proteins were then isolated from the proteoglycans by CsCl-density-gradient centrifugation at 100000 *g* in a fixed-angle rotor at 10 °C, dialysed extensively against 0.1 M-Tris/HCl buffer, pH 6.8, and concentrated by ultrafiltration on an Amicon YM5 membrane. The link proteins were then separated by electrophoresis, electroblotted on to poly(vinylidene difluoride) membranes and stained with Coomassie

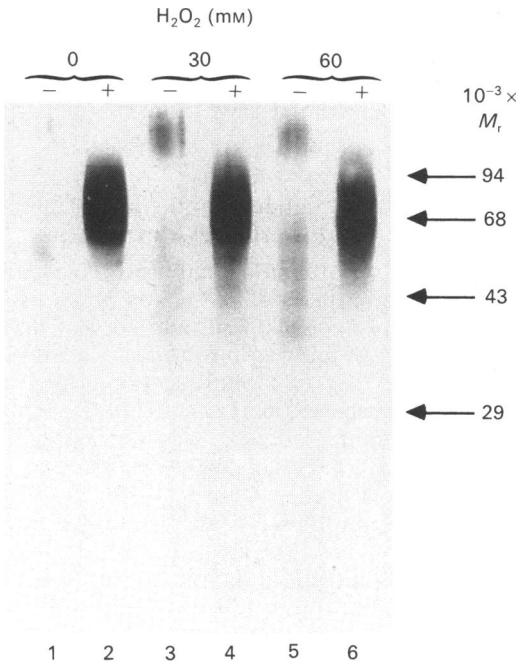

Fig. 2. Effect of H_2O_2 treatment upon the hyaluronic acid-binding region of the proteoglycan subunit

Proteoglycan aggregates were treated in the absence of H_2O_2 (lanes 1 and 2), with 30 mM-H_2O_2 (lanes 3 and 4) or with 60 mM-H_2O_2 (lanes 5 and 6), and then with 10 μg of trypsin/mg of proteoglycan aggregate (lanes 2, 4 and 6) or in the absence of trypsin (lanes 1, 3 and 5). The samples were then electrophoresed and electroblotted, followed by localization of protein components reactive with the monoclonal antibody to the hyaluronic acid-binding region of the proteoglycan subunits.

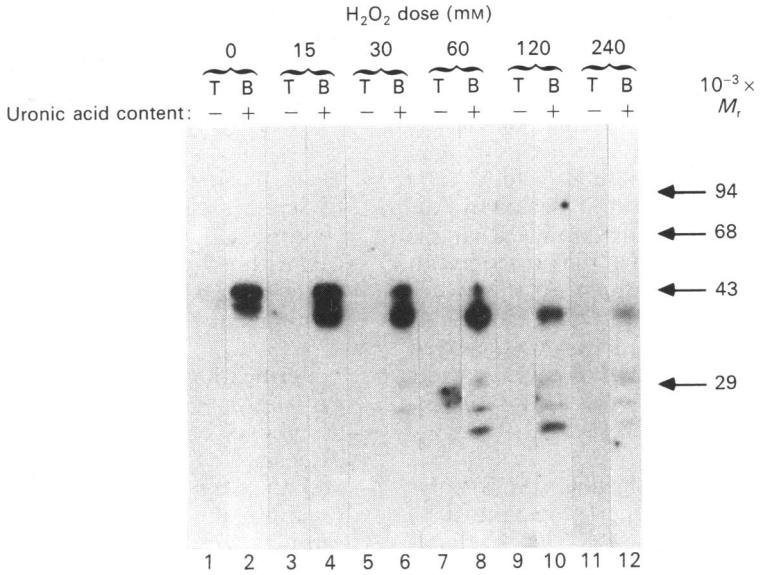

Fig. 3. Effect of H_2O_2 treatment upon the association of link protein with other components of the proteoglycan aggregate by CsCl-density-gradient centrifugation at a starting density of 1.45 g/ml

Proteoglycan aggregate preparations were treated in the absence of H_2O_2 (lanes 1 and 2) or in the presence of H_2O_2 to final concentrations of 15 mM (lanes 3 and 4), 30 mM (lanes 5 and 6), 60 mM (lanes 7 and 8), 120 mM (lanes 9 and 10) or 240 mM (lanes 11 and 12). The samples were ultracentrifuged, divided into three equal fractions, assayed for uronic acid, then electrophoresed and analysed by Western blotting with the monoclonal antibody to link protein. 'B' indicates the bottom of the gradient, and 'T' indicates the top of the gradient. '+' indicates that this fraction contained uronic acid, and '−' indicates absence from the fraction of uronic acid. All of the material that contained uronic acid was present in the bottom fraction in each of these experiments. None of the central fractions contained immunoreactive material.

Brilliant Blue R250 (Matsudaira, 1987). Protein bands were excised and sequenced with an Applied Biosystems 470A gas-phase Sequenator.

RESULTS

Effects of H_2O_2 treatment on the hyaluronic acid-binding region of the proteoglycan subunit

Treatment of human neonatal proteoglycan aggregate with increasing doses of H_2O_2 was previously shown to modify the proteoglycan subunit so that it is no longer able to interact stably with hyaluronic acid. However, no detectable change in subunit size or glycosaminoglycan content was seen (Roberts *et al.*, 1987), indicating that only minimal degradation was occurring under these conditions. The presence and integrity of the hyaluronic acid-binding region of the proteoglycan subunits in these preparations was therefore studied to determine whether free radicals were causing its cleavage in a manner analogous to the initial action of proteinases.

Following treatment of human neonatal proteoglycan aggregate with increasing concentrations of H_2O_2, hyaluronic acid-binding region was localized on immunoblots of SDS/polyacrylamide gels by using a specific monoclonal antibody (Fig. 2). Negligible amounts of material were detected, indicating that radical treatment did not mediate removal of the hyaluronic acid-binding region as an intact unit, even at H_2O_2 concentrations up to 240 mM. To verify whether the hyaluronic acid-binding regions were indeed still present, the radical-treated proteoglycans were incubated with trypsin. On treatment of the proteoglycan aggregate with proteinases such as trypsin, the hyaluronic acid-binding region is liberated as a 65000–67000-M_r component (Perkins *et al.*, 1981). When aggregate samples treated with H_2O_2 were trypsin-digested and analysed as above, hyaluronic acid-binding region was present at all H_2O_2 concentrations used, indicating its continued presence on the proteoglycan subunits even though they no longer can interact with hyaluronic acid.

Analysis of interactions between components of the proteoglycan aggregate by CsCl-density-gradient centrifugation

Treatment of proteoglycan aggregates with up to 240 mM-H_2O_2 resulted in fragmentation of link proteins, but both treated and untreated link proteins sedimented in the lower third of an associative density gradient of starting density 1.45 g/ml, demonstrating that the treatment did not release them from their interaction with both of the other components of the aggregate (Fig. 3). Furthermore, when the treated aggregate preparations were dissociated by the addition of guanidinium chloride to 4 M and the guanidinium chloride was dialysed away, the fragmented link proteins were found to have retained their capacity to reassociate with the other aggregate components. This suggests that H_2O_2 treatment does not render the link proteins susceptible to irreversible denaturation in guanidinium chloride, as they are still able to interact with either the hyaluronic acid or the hyaluronic acid-binding region of the proteoglycan subunits, despite the proteolysis that is observed.

The centrifugation of treated aggregates on associative CsCl density gradients of starting density 1.69 g/ml demonstrated that the fragmented link proteins were not associated with the uronic acid-containing proteoglycan monomers, but that they sedimented with a component of intermediate density between that of isolated link

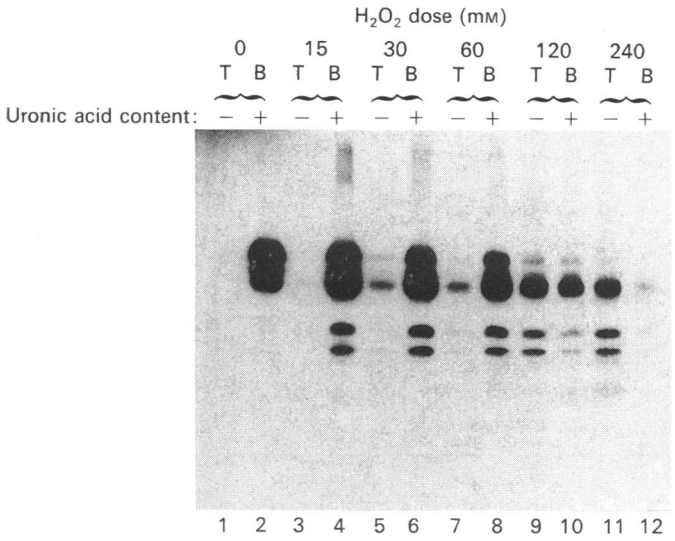

Fig. 4. Effect of H_2O_2 treatment upon the association of link protein with other components of the proteoglycan aggregate by CsCl-density-gradient centrifugation at a starting density of 1.69 g/ml

Proteoglycan aggregate preparations were treated in the absence of H_2O_2 (lanes 1 and 2) or in the presence of H_2O_2 to final concentrations of 15 mM (lanes 3 and 4), 30 mM (lanes 5 and 6), 60 mM (lanes 7 and 8), 120 mM (lanes 9 and 10) or 240 mM (lanes 11 and 12). The samples were ultracentrifuged, divided into three equal fractions, assayed for uronic acid, then electrophoresed and analysed by Western blotting with the monoclonal antibody to link protein. 'B' indicates the bottom of the gradient, and 'T' indicates the top of the gradient. '+' indicates that this fraction contained uronic acid, and '−' indicates absence from the fraction of uronic acid.

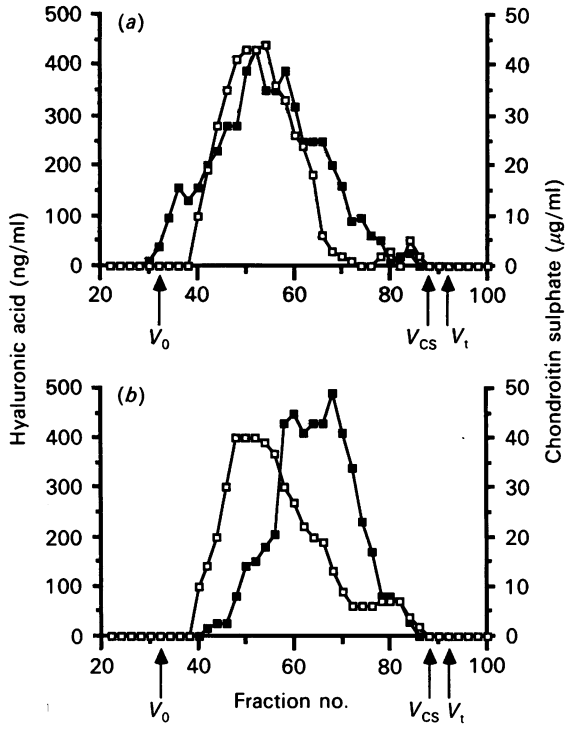

Fig. 5. Effect of H_2O_2 treatment on the size of hyaluronic acid within proteoglycan aggregates

Proteoglycan aggregates were treated in the absence of H_2O_2 (*a*) or in the presence of 120 mM-H_2O_2 (*b*), and the preparations were then reduced and alkylated. Subsequently they were chromatographed on Sepharose CL-2B and the resulting eluent was analysed for glycosaminoglycan (□) and hyaluronic acid (■) content. The void volume (V_0) and total volume (V_t) of the column, and the elution position of chondroitin sulphate (V_{CS}) are indicated.

proteins and proteoglycans (Fig. 4), which can only be hyaluronic acid (see Fig. 1). Thus, not only is the interaction between the proteoglycan subunit and hyaluronic acid impaired by H_2O_2 treatment, but also that between the proteoglycan subunit and link protein. In contrast, the interaction between link protein and hyaluronic acid remains functional.

Analysis of the effects of H_2O_2 treatment upon hyaluronic acid

Proteoglycans were incubated in the absence or in the presence of 120 mM-H_2O_2, then reduced and alkylated under dissociative conditions and chromatographed on Sepharose CL-2B (Fig. 5). Treatment with H_2O_2 resulted in decrease in size of the hyaluronic acid with little change in the size of the uronic acid-containing proteoglycans. It is of interest that only limited cleavage of hyaluronic acid occurred. The smallest hyaluronic acid molecule that it is possible to resolve by the technique employed would have an M_r of about 20000, and would be eluted close to the position indicated for isolated chondroitin sulphate chains (V_{CS}). No degradation products of this size were observed.

Effects of H_2O_2 treatment upon the primary structure of link protein

The first observable change in neonatal human link protein on treatment with H_2O_2 is the complete conversion of the 48000- and 44000-M_r components into a band migrating to a similar position on SDS/polyacrylamide gels to the minor 41000-M_r component. The sequence of the first eight amino acid residues of this H_2O_2-generated component was determined (Fig. 6). When compared with the sequences of the naturally occurring 48000-, 44000- and 41000-M_r molecules (Nguyen *et al.*, 1989), which were determined on link proteins from the same individual, the H_2O_2-generated link protein was observed to start at residue 14 of the 48000- or 44000-M_r molecules and was distinct from the 41000-M_r molecule. In addition, the conversion of two of the first eight amino acid residues to other residues was observed in the H_2O_2-generated link protein: alanine for histidine at residue 3 and aspartate for asparagine at residue 8. These results indicate that both peptide-bond cleavage and amino acid modification may occur as a consequence of free-radical attack. The sequence of rat chondrosarcoma link protein was also determined after treatment of the rat chondrosarcoma proteoglycan aggregate with H_2O_2 under conditions identical with those used for the human. This sequence was not altered by the treatment, at least for the first ten residues, in contrast with treatment of the human proteoglycan aggregate.

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          1         5         10        15        20
(a)                                   Ala-Ile-Ala-Ile-Gln-Ala-Glu-Asp
(b)  Asp-His-Leu-Ser-Asp-Asn-Tyr-Thr-Leu-Asp-His-Asp-Arg-Ala-Ile-His-Ile-Gln-Ala-Glu-Asn
(c)                                                 Ile-Gln-Ala-Glu-Asn
(d)  Asp-His-Leu-Ser-Asp-Ser-Tyr-Thr-Pro-Asp-Gln-Asp-Arg-Val-Ile-His-Ile-Gln-Ala-Glu-Asn
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Fig. 6. *N*-terminal sequence analysis of link protein after treatment of proteoglycan aggregate with 60 mM-H_2O_2

(*a*) Human link protein following treatment with H_2O_2. (*b*) Human link protein, M_r 48000 or 44000 (Nguyen *et al.*, 1989). (*c*) Human link protein, M_r 41000 (Nguyen *et al.*, 1989). (*d*) Rat chondrosarcoma link protein (Neame *et al.*, 1986). Amino acid residues in **boldface** type represent differences due to H_2O_2 treatment, and amino acid residues underlined represent differences between the human and rat sequences.

DISCUSSION

In our previous study (Roberts *et al.*, 1987) it was demonstrated that treatment of neonatal human articular cartilage with low concentrations of H_2O_2 resulted in limited cleavage of link protein and modification of the proteoglycan subunit so that it no longer forms aggregates with hyaluronic acid. Little diminution in size of the proteoglycan subunit was seen. In contrast, treatment with high concentrations of H_2O_2, or in the presence of exogenous metal ions, leads to extensive degradation of both the link protein and the proteoglycan subunit with loss of macromolecular material. Under these latter conditions it is thought that bulk-phase generation of hydroxyl radicals, mediated by the metal ion, is responsible for random degradation of the aggregate components to small fragments. At lower concentrations of H_2O_2, however, a specific set of modifications appears to be occurring, presumably as a result of site-selective generation of the radicals.

The present results indicate that the inability of the radical-treated subunits to aggregate with hyaluronic acid is not due to loss of the hyaluronic acid-binding region. The experimental evidence for this statement is, at first sight, paradoxical, since the presence of hyaluronic acid-binding region was demonstrated by trypsin digestion of the H_2O_2-treated proteoglycan aggregate and it would be expected that this region should be degraded by trypsin treatment if it is not participating in aggregate formation, as is the case for untreated proteoglycan subunits. The amino acid modification seen on sequencing of radical-treated link protein may hold the clue to this paradox, and by analogy, leads us to believe that amino acid modifications may have also occurred in the hyaluronic acid-binding region, which, in addition to causing a decrease in the ability to interact with hyaluronic acid, also lead to a lower susceptibility of this part of the proteoglycan subunit to trypsin treatment, possibly as a result of effects on the conformation of the region and accessibility to the enzyme.

In addition to the loss of ability to interact with hyaluronic acid, it was also found that the interaction of proteoglycan subunit with link protein was abolished by H_2O_2 treatment, although link protein itself was still able to bind to hyaluronic acid. This distinction between the binding of link protein to hyaluronic acid, proteoglycan subunit binding to hyaluronic acid and link protein binding to proteoglycan subunit is consistent with the separate functional domains associated with these interactions on the link proteins (Périn *et al.*, 1987) and the proteoglycan subunits (Neame *et al.*, 1987).

The current study also demonstrates that treatment of proteoglycan aggregates with H_2O_2 results in cleavage of the hyaluronic acid molecule. Cleavage of hyaluronic acid preparations by reactive oxygen metabolites has been studied extensively (Scott *et al.*, 1972; Wong *et al.*, 1981; Greenwald & Moak, 1986), but there are no reports of the effects of reactive oxygen metabolites upon the size of hyaluronic acid in proteoglycan aggregates. Our results show that, even in the presence of 120 mM-H_2O_2, hyaluronic acid was susceptible to only limited cleavage.

Cleavage within the *N*-terminal region of the link protein occurs between residues 13 and 14. Interestingly, histidine occurs at residues 11 and 16, and this amino acid has been implicated in the chelation of the transition metal ions necessary to participate in the conversion of H_2O_2 into the hydroxyl radical. Localization of protein modifications to the vicinity of histidine residues may be a result of chelation of transition-metal ions by this amino acid pair and the generation of a more reactive metabolite of oxygen occurring in a reaction between H_2O_2 and the transition-metal ion. This reactant is postulated to react very closely to its site of generation, and may be a complexed form of the hydroxyl radical (Bors *et al.*, 1979; Samuni *et al.*, 1981; Sutton *et al.*, 1987). In support of this hypothesis is the observation that similar treatment does not result in cleavage of the rat chondrosarcoma link protein, which contains glutamine rather than histidine at residue 11, and therefore might not chelate transition metal ions tightly in this region.

Earlier studies (Creeth *et al.*, 1983; Cooper *et al.*, 1985), using amino acid analysis, showed loss of histidine residues following H_2O_2 treatment of ovarian-cyst mucus glycoproteins and cleavage of a synthetic polyhistidine molecule. These studies led those authors to suggest that cleavage in this glycoprotein occurred at the histidyl bond. In contrast, in the human link protein used in the present study cleavage did not occur adjacent to a histidine residue, though modification of a histidine residue did occur with a conversion into alanine. Such a modification would also be consistent with the amino acid composition data presented for the mucus glycoprotein. Thus, though we would agree that a histidine residue can be modified by radical treatment, our data suggest that this need not necessarily involve concomitant cleavage of the adjacent peptide bond.

Our results suggest that the effects of H_2O_2 upon

connective-tissue macromolecules may include both peptide-bond cleavage and non-proteolytic modification of the protein components, plus glycosyl-bond cleavage of carbohydrates. Such changes, if they occur *in vivo*, would be expected to have a deleterious effect on tissue function.

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