Treatment of cartilage proteoglycan aggregate with hydrogen peroxide

Relationship between observed degradation products and those that occur naturally during aging

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The effects of treatment of purified neonatal human articular-cartilage proteoglycan aggregate with H_2O_2 were studied. (1) Exposure of proteoglycan aggregate to H_2O_2 resulted in depolymerization of the aggregate and modification of the core protein of both the proteoglycan subunits and the link proteins. (2) Treatment of the proteoglycan aggregate with H_2O_2 rendered the proteoglycan subunits unable to interact with hyaluronic acid, with minimal change in their hydrodynamic size. (3) Specific cleavages of the neonatal link proteins occurred. The order in which the major products were generated and their electrophoretic mobilities resembled the pattern observed during human aging. (4) The proteolytic changes in the link proteins were inhibited in the presence of transition-metal-ion chelators, thiourea or tetramethylurea, suggesting that generation of hydroxyl radicals from H_2O_2 by trace transition-metal ions via a site-specific Fenton reaction may be responsible for the selective cleavages observed. (5) Cleavage of the link proteins in proteoglycan aggregates by H_2O_2 was shown to have a limited effect on the susceptibility of these proteins to cleavage by trypsin. (6) The relationship between these changes and those observed in cartilage during human aging suggests that some of the age-related changes in the structure of human cartilage proteoglycan aggregate may be the result of radical-mediated damage.

INTRODUCTION

The proteoglycan monomers of articular cartilage have the ability to interact with hyaluronic acid to form aggregates, which are stabilized by further interaction with glycoproteins termed link proteins. The aggregates are thought to be responsible for the resistance of cartilage towards compression. Human articular-cartilage proteoglycan aggregate exhibits a number of age-related changes that are indicative of proteolytic modification, and that may be associated with altered functional properties. These changes include a decrease in the size of the proteoglycan subunit, ultimately to fragments of M_r approx. 60 000 that bind hyaluronic acid but contain little uronic acid (Roughley *et al.*, 1985), and a number of changes in the structure of the link proteins.

Neonatal and adult human articular-cartilage link proteins have been shown to exist as three major components of M_r 40 000–50 000, of which the largest two appear to differ only in type or degree of glycosylation and give rise to the smallest link protein *in vitro* after treatment with proteinases (Mort *et al.*, 1985). The largest of these three components is most abundant in the neonatal human, but the smallest becomes more predominant with increasing age. Moreover, a number of additional cleavages in the link proteins occur with age, to yield a series of fragments of M_r 26 000–30 000, which are only evident under reducing conditions (Mort *et al.*, 1983).

Proteinase treatment of neonatal cartilage proteoglycan aggregate *in vitro* can reproduce some of the above age-related changes, i.e. decrease in size of the proteoglycan subunits, generation of free hyaluronic acid-binding regions derived from the proteoglycan subunits (Campbell *et al.*, 1986) and cleavage of the larger link proteins to produce the smallest of the three native proteins (Mort *et al.*, 1985). However, the production of fragmented link-protein molecules, which are held together in a pseudo-native conformation by disulphide bonds, has not yet been reproduced enzymically *in vitro*.

In order to investigate the possibility that some of the age-related proteolytic changes observed in cartilage proteoglycan *in vivo* may be generated by a non-enzymic mechanism, such as via the action of reactive oxygen metabolites, we have investigated the effects of H_2O_2 on human neonatal articular-cartilage proteoglycan. H_2O_2 is a product of the metabolism of many cells (Chance *et al.*, 1979), including mononuclear phagocytes and polymorphonuclear leucocytes, which depend in part upon this molecule for their capacity to kill micro-organisms (Nathan, 1983; Murray *et al.*, 1985). H_2O_2 can be produced directly, e.g. by the action of a number of oxidative enzymes, or by the dismutation of superoxide radicals, and the highly reactive hydroxyl radical may be generated from H_2O_2 as a result of excitation with ionizing radiation or via the Fenton reaction with transition-metal ions (Halliwell & Gutteridge, 1984).

Abbreviation used: DTPA, diethylenetriaminepenta-acetic acid.

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Reactive oxygen metabolites have been implicated as active agents in many age-related changes (Harman, 1981), which include the oxidation, fragmentation or cross-linking of biologically important molecules such as DNA, lipids (Pryor, 1978) and a number of proteins, including collagen (La Bella & Paul, 1965) and elastin (La Bella *et al.*, 1966). Degradation of hyaluronic acid in the presence of radical-generating systems has been described (Scott *et al.*, 1972; Greenwald *et al.*, 1976; Greenwald & Moy, 1980), and the presence of transition-metal ions has been shown to be necessary for this effect (Wong *et al.*, 1981). A similar mechanism is implicated in the degradation of ovarian-cyst mucus glycoproteins by H_2O_2 (Creeth *et al.*, 1983; Cooper *et al.*, 1985). Chung *et al.* (1984), Bartold *et al.* (1984) and Bates *et al.* (1984) have shown decreases in the size of pig articular-cartilage proteoglycan, pig gingival proteoglycan and bovine nasal-cartilage proteoglycan respectively after treatment with reactive oxygen metabolites, and Dean *et al.* (1984, 1985) have demonstrated that hydroxyl radicals and superoxide radicals generated by a variety of different radical-generating systems are able to degrade the protein core of proteoglycan in intact bovine nasal cartilage. The results of the present study extend these observations and suggest that a number of age-related changes in human articular-cartilage proteoglycan may be duplicated *in vitro* by the action of reactive oxygen metabolites.

MATERIALS AND METHODS

Materials

Guanidinium chloride, catalase, hyaluronic acid and trypsin [tosylphenylalanyl-chloromethane-('TPCK'-)treated] were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and CsCl was from Accurate Chemical and Scientific Co. (Westbury, NY, U.S.A.). Materials for electrophoresis were from Bio-Rad Laboratories, Toronto, Ont., Canada, and 30% (v/v) H_2O_2 (AnalaR grade) was from BDH Chemicals, Toronto, Ont., Canada. Spectrophor 1 dialysis membrane (6000–8000 M_r cut-off) was from Fisher Scientific, Montreal, Que., Canada. Radiochemicals ($[^{14}C]$ methylated protein standards, $Na^{125}I$ and ^{125}I -Protein A) were from Amersham International, Mississauga, Ont., Canada. All other chemicals were of analytical grade or best commercially available grade. All solutions were made up freshly in water of resistance greater than 10 $M\Omega/cm$ obtained from a Millipore Milli Q water-purification system (Millipore Corp., Bedford, MA, U.S.A.).

Preparation of neonatal human cartilage 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 cartilage 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 100 000 $g_{av.}$ 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.

Analysis of link proteins in adult human cartilage

Human articular cartilage was obtained at autopsy within 18 h of death and extracted as described above. The extract was then dialysed against 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.1% SDS and analysed by SDS/polyacrylamide-gel electrophoresis, electroblotting and immunolocalization.

Reaction of proteoglycan aggregate with H_2O_2

Proteoglycan aggregate was dissolved at 3 mg/ml in 25 mM-sodium acetate/acetic acid buffer, pH 5.6, containing 80 mM-NaCl, and 200 μl portions of this solution were routinely used for experiments. An appropriate dilution of 30% H_2O_2 stock solution in water was added in a volume of 10 μl to the proteoglycan solution. Reactions were carried out at 37 °C for 24 h unless otherwise stated. In an experiment in which the time course of the reaction was investigated, the reactions were terminated by the addition of 20 μl of 250 mM-DTPA in 25 mM-acetate buffer, pH 5.6, stock solution at selected time points. In all other experiments, the reactions were terminated at 24 h by dialysis of the reaction mixture against 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.1% SDS with the use of a Spectrophor 1 dialysis membrane in a multi-well microdialysis apparatus (BRL, Bethesda, MD, U.S.A.) through which fresh dialysis buffer was perfused at a rate of 2 ml/min for at least 12 h. In addition, the effect of addition of various metal-ion chelators and molecules that react with hydroxyl radicals was investigated. DTPA and EDTA solutions were made up as 200 mM stock solutions in 25 mM-acetate buffer, pH 5.6, and tetramethylurea, thiourea, sodium formate and urea were made up as 5 M stock solutions with the pH re-adjusted to pH 5.6 with HCl or NaOH as required. A 10 μl portion of an appropriate dilution of this stock solution was added to the reaction mixture when required.

Isolation of proteoglycan subunits from H_2O_2 -treated proteoglycan aggregate

After treatment with H_2O_2 proteoglycan aggregate solutions were supplemented with guanidinium chloride and CsCl to adjust the density to 1.50 g/ml and the guanidinium chloride concentration to 4 M (to dissociate proteoglycan subunits from hyaluronic acid and link proteins). Each preparation was then centrifuged at 100 000 $g_{av.}$ for 48 h at 10 °C, and the proteoglycan subunits were subsequently collected from the bottom of the gradients (density greater than 1.55 g/ml). Each preparation was then dialysed against 10 litres of 0.2 M-sodium acetate buffer, pH 5.5, and chromatographed through Sepharose CL-2B.

Sepharose CL-2B chromatography

Samples (1 ml containing approx. 2 mg of proteoglycan) were chromatographed on columns (110 cm $\times$ 1 cm) of Sepharose CL-2B at a flow rate of 6 ml/h in 0.2 M-sodium acetate buffer, pH 5.5. The eluate was collected in 1 ml fractions and monitored for uronic acid by the carbazole method of Bitter & Muir (1962). For some experiments the capacity of the isolated proteoglycan subunits to aggregate with hyaluronic acid was investigated by chromatographing preparations in the presence and in the absence of added hyaluronic acid. Hyaluronic acid was added as 20 μl of a 2 mg/ml

solution in water, 30 min before the sample was loaded on to the column.

Analysis of glycosaminoglycan size

The effect of H_2O_2 upon the glycosaminoglycan side chains was investigated as follows. Proteoglycan aggregates at 3 mg/ml were treated with final concentrations of 0, 70 mM- or 140 mM- H_2O_2 or with 70 mM- H_2O_2 + 1 mM- CuSO_4 for 24 h at 37 °C as described above. The samples were next extensively dialysed against 0.125 M-Tris/HCl buffer, pH 6.8, and then against deionized water. Glycosaminoglycans were liberated from the proteoglycans by cleavage in 0.05 M-NaOH containing 1 M- NaBH_4 at 45 °C for 48 h (Carlson, 1968) and neutralized with concentrated acetic acid, and 1 ml portions were analysed by gel-permeation chromatography on a 110 cm $\times$ 1 cm column of Sephacryl S200 in 0.5 M-sodium acetate/acetic acid buffer, pH 5.5, at a flow rate of 6 ml/h. Fractions (1 ml) of the eluate were analysed for uronic acid by the method of Bitter & Muir (1962).

Electrophoresis, electroblotting and immunolocalization

SDS/polyacrylamide-gel electrophoresis was performed in 10 % slab gels under the conditions of Laemmli (1970). Samples were dialysed against 0.1 % SDS/0.125 M-Tris/HCl buffer, pH 6.8, and boiled in the presence of 2 % (w/v) SDS/1 % (v/v) glycerol/0.001 % Bromophenol Blue/5 % (v/v) 2-mercaptoethanol for 3 min before being loaded on to the gels. Proteins were electroblotted on to nitrocellulose membrane in 15.6 mM-Tris/120 mM-glycine/40 % (v/v) methanol, pH 8.3. The membrane was blocked for 16 h in 1 % (w/v) bovine haemoglobin in PBS/azide (10 mM-sodium potassium phosphate buffer, pH 7.2, containing 0.145 M-NaCl and 0.05 % NaN_3).

Link proteins were immunolocalized on transfer membranes by the use of ascitic fluid that contained the monoclonal antibody 9/30/8-A-4, and the hyaluronic acid-binding region of the proteoglycan core protein was immunolocalized by the use of ascitic fluid that contained the monoclonal antibody 12/20/2-A-5. Both of these ascitic fluids were kindly supplied by Dr. Bruce Caterson, Department of Biochemistry, University of West Virginia, Morgantown, WV, U.S.A. The mouse monoclonal antibody 9/30/8-A-4 was produced by immunization with Swarm rat chondrosarcoma link protein and is of the IgG2b subclass (Caterson *et al.*, 1985a,b). The antibody recognizes two nearly identical epitopes in the C-terminal half of the molecule (Neame *et al.*, 1986), and immunologically similar epitopes are present in all link proteins isolated from human, bovine, rat and chicken hyaline cartilage. The mouse monoclonal antibody 12/20/2-A-5 recognizes an epitope that is present in the hyaluronic acid-binding region of the proteoglycan core protein and is of the IgG1 subclass (Caterson *et al.*, 1985a).

Immunolocalization of link proteins was done as follows. The nitrocellulose membrane was incubated at room temperature for 4 h in 1 % bovine haemoglobin in PBS/azide to which was added the monoclonal antibody 9/30/8-A-4 (dilution 1 in 2000). The membrane was then washed with several changes of PBS/azide and incubated for 1 h in PBS/azide containing 1 % haemoglobin and 100 000 d.p.m. of ^{125}I -Protein A/ml. For some experiments ascitic fluid was labelled with ^{125}I by using the chloramine-T method (Greenwood *et al.*, 1963)

and used in place of unlabelled ascitic fluid, which eliminated the need for a ^{125}I -Protein A step. The membrane was washed thoroughly in PBS/azide and dried, and link proteins were located by radioautography with Kodak XAR film.

Immunolocalization of the hyaluronic acid-binding region of the proteoglycan core protein was done as follows. The nitrocellulose membrane was incubated at room temperature for 4 h in 1 % bovine haemoglobin in PBS/azide to which was added the monoclonal antibody 12/20/2-A-5 (dilution 1 in 500), washed with PBS/azide, incubated for 4 h at room temperature in 1 % bovine haemoglobin in PBS/azide containing a rabbit anti-mouse IgG1 antibody (dilution 1 in 1000) (Sci-Can Diagnostics, Edmonton, Alberta, Canada), washed again, incubated for 1 h in PBS/azide containing 1 % bovine haemoglobin and 100 000 d.p.m. of ^{125}I -Protein A/ml, then treated as above.

The [^{14}C]methylated proteins used as M_r standards and their approximate M_r values as indicated by the supplier were as follows: myosin, 200 000; phosphorylase b, 93 000; bovine serum albumin, 69 000; ovalbumin, 46 000; carbonic anhydrase, 30 000; lysozyme, 14 000.

Agarose/polyacrylamide-gel electrophoresis

Agarose/polyacrylamide-gel electrophoresis was done in composite agarose/polyacrylamide gels by the method of McDevitt & Muir (1971). Gels contained 0.60 % agarose, 1.14 % acrylamide and 0.06 % methylenebisacrylamide. The electrophoresis buffer was 10 mM-Tris/HCl buffer, pH 7.5. Samples for electrophoresis were dissolved at 2 mg/ml in water and diluted 1:1 with 50 % (w/v) sucrose containing 0.05 % Bromophenol Blue before analysis. After electrophoresis, the gels were stained with 0.02 % Toluidine Blue in 0.1 M-acetic acid at 25 °C and destained with 3 % acetic acid at 35 °C. The mobility of the stained bands was calculated relative to that of Bromophenol Blue (R_{BB}).

Degradation of link proteins in proteoglycan aggregate, with trypsin

Neonatal proteoglycan aggregate was prepared as described above, and dissolved at 3 mg/ml in 25 mM-acetate buffer, pH 5.6, containing 80 mM-NaCl. To 1 ml portions of this solution were added water or H_2O_2 in 10 μl portions, to give initial concentrations of 0, 6 mM-, 15 mM-, 30 mM- or 60 mM- H_2O_2 . After 24 h treatment at 37 °C, the samples were dialysed exhaustively against 0.1 M-Tris/HCl buffer, pH 7.5, and treated with 10 μg of trypsin/mg of proteoglycan aggregate (or with no trypsin addition) for 4 h at 37 °C. At the end of this incubation period, phenylmethanesulphonyl fluoride was added to all samples to a final concentration of 1 mM, and the samples were dialysed into 0.1 M-Tris/HCl buffer, pH 6.8, containing 0.1 % SDS and analysed by SDS/polyacrylamide-gel electrophoresis followed by electroblotting and immunolocalization of link proteins.

RESULTS

Effect of H_2O_2 on neonatal cartilage proteoglycan aggregate

When neonatal human articular-cartilage proteoglycan aggregate was treated with H_2O_2 , there was a dose-dependent reduction in viscosity, indicating degradation of the aggregate (measured with a Cannon-Manning

semi-micro viscometer at 25 °C; results not shown). The extent of degradation was examined by Sepharose CL-2B chromatography (Fig. 1). Most of the uronic acid-containing material in this preparation was eluted at the column void volume (V_0) before treatment, but after treatment with H_2O_2 less proteoglycan was eluted at V_0 and more was included in the column volume at a position similar to that of intact proteoglycan subunits, indicating that depolymerization of the aggregate had occurred. The extent of this decrease in aggregation was dependent on the H_2O_2 concentration, though at the concentrations used no substantial decrease in hydrodynamic size of the proteoglycan subunits themselves was evident. However, with 1 mM added $CuSO_4$, most of the uronic acid-containing material was eluted near the total column volume, indicating that there is extensive degradation of the proteoglycan subunit in the presence of exogenous Cu^{2+} ions, which would catalyse the generation of high fluxes of hydroxyl radicals.

Agarose/polyacrylamide-gel electrophoresis of these proteoglycan aggregates (Fig. 2) also showed that the major product (mean R_{BB} 0.76) was of similar size to intact proteoglycan subunits (R_{BB} 0.75) in the absence of exogenous $CuSO_4$. The addition of Cu^{2+} ions resulted in a major product of R_{BB} 1.10, which is therefore smaller than the proteoglycan subunit, and a product of R_{BB} 1.49, which is smaller than single chondroitin sulphate chains (R_{BB} 1.30), suggesting that, under conditions that might be expected to generate high fluxes of hydroxyl radicals, both core protein and glycosaminoglycan chains are degraded.

Effect of H_2O_2 on the proteoglycan subunits in the aggregate preparations

In order to investigate the hyaluronic acid-binding status of the proteoglycan subunits after treatment of the

aggregate with H_2O_2 , the subunits were purified from the incubation mixtures by $CsCl$ -density-gradient centrifugation under dissociative conditions. These isolated subunits were then chromatographed through Sepharose CL-2B columns in the presence and in the absence of added hyaluronic acid (Fig. 3). In the absence of added hyaluronic acid, the untreated subunits and H_2O_2 -treated (up to 134 mM) subunits chromatographed in similar positions, indicating that little degradation of the protein core had taken place with H_2O_2 treatment alone. In the presence of added hyaluronic acid, the untreated subunits largely aggregated and were eluted at the V_0 of the column, but this ability of the subunits to associate with hyaluronic acid was progressively lost on treatment with H_2O_2 , in a dose-dependent manner.

Effects of H_2O_2 on glycosaminoglycan and protein components of proteoglycan aggregate

Gel-permeation chromatography on Sephacryl S200 of glycosaminoglycan chains liberated by alkaline borohydride treatment of the proteoglycans showed that treatment with concentrations of up to 140 mM- H_2O_2 caused no change in the size of the glycosaminoglycan chains. Elution profiles were very similar; K_{av} values of 0.14 were obtained for untreated material and samples treated with 70 mM- and 140 mM- H_2O_2 . Though the glycosaminoglycan chains were apparently not cleaved under these conditions, SDS/polyacrylamide-gel electrophoresis followed by electroblotting and immunolocalization revealed cleavage of the link proteins in portions of these samples removed before alkaline borohydride treatment. Immunolocalization of a protein epitope present in the hyaluronic acid-binding region of the core protein, with the monoclonal antibody 12/20/2-A-5 (Caterson *et al.*, 1985a), revealed that H_2O_2 treatment resulted in the release of small amounts of fragments of

Fig. 1. Degradation of purified human neonatal articular-cartilage proteoglycan aggregate by H_2O_2

Proteoglycan aggregates in solution were treated in the absence of H_2O_2 (■) or with initial concentrations of 27 mM- H_2O_2 (□), 134 mM- H_2O_2 (▲) or 27 mM- H_2O_2 + 1 mM- $CuSO_4$ (◇) for 24 h at 37 °C and chromatographed on a Sepharose CL-2B column. V_0 and V_t represent column void volume and total volume respectively.

Fig. 2. Agarose/polyacrylamide-gel electrophoresis of proteoglycan aggregates after H_2O_2 treatment

Proteoglycan aggregates in solution were treated in the absence of H_2O_2 (lane 1) or with initial concentrations of 0.3 mM- H_2O_2 (lane 2), 27 mM- H_2O_2 (lane 3), 134 mM- H_2O_2 (lane 4) or 27 mM- H_2O_2 + 1 mM- $CuSO_4$ (lane 5) for 24 h at 37 °C and analysed by composite agarose/polyacrylamide-tube-gel electrophoresis.

Fig. 3. Ability of repurified proteoglycan subunits to interact with hyaluronic acid after treatment of aggregate with H_2O_2

Proteoglycan aggregates were treated for 24 h at 37 °C (a) in the absence of H_2O_2 , (b) with 27 mM- H_2O_2 , or (c) with 134 mM- H_2O_2 , and then the subunits were purified from these incubation mixtures by CsCl-density-gradient centrifugation under dissociative conditions. The purified subunits were then chromatographed through Sepharose CL-2B in the presence (■) or in the absence (□) of added hyaluronic acid. V_0 and V_t represent column void volume and total column volume respectively.

M_r between 20000 and 90000 that bore this epitope. However, most of the proteoglycan subunits appeared to remain intact, and trypsin treatment of untreated or H_2O_2 -treated proteoglycan aggregates generated a similar major fragment of M_r approx. 68000 containing the 12/20/2-A-5 epitope (results not shown).

It was, however, possible to fragment the glycosaminoglycan chains under these conditions by the addition of transition-metal-ions; treatment of proteoglycan aggregates with 70 mM- H_2O_2 in the presence of 1 mM added $CuSO_4$ yielded glycosaminoglycans of K_{av} 0.74, when analysed as described above. This treatment rendered both hyaluronic acid-binding region and link protein epitopes undetectable by the techniques employed.

It is therefore evident that H_2O_2 treatment of proteoglycan aggregate can result in damage to the hyaluronic acid-binding region of the protein core of the proteoglycan subunits such that they lose the ability to aggregate with hyaluronic acid. Furthermore, in the absence of exogenous Cu^{2+} ions, little or no cleavage occurs in the glycosaminoglycan chains, or in the glycosaminoglycan attachment region of the proteoglycan subunits.

Effect of H_2O_2 on neonatal human articular cartilage link protein

The link proteins in human neonatal articular-cartilage proteoglycan aggregate treated with H_2O_2 were analysed by SDS/polyacrylamide-gel electrophoresis followed by immunoblotting. H_2O_2 treatment generated a major product of similar size to that of the smallest native link protein (M_r approx. 43000) and caused further fragmentation in a dose-dependent manner (Fig. 4), producing fragments of M_r approx. 33000, 32000, 29000, 28000 and 27000, of which the 29000- M_r fragment appears to be most predominant. A time course of the reaction illustrates that generation of the 43000- M_r link protein and the 29000- M_r fragment occur as early events in the fragmentation and that prolonged exposure to H_2O_2 causes more extensive destruction of the molecule to fragments that are no longer detectable by this technique (Fig. 5).

The fragmentation pattern caused by the presence of H_2O_2 resembles the typical distribution found for link protein from post-mortem articular cartilage of mature individuals. Compared with neonatal material, the tissue from a 59-year-old is enriched in a component of M_r

Fig. 4. Degradation of link protein in proteoglycan aggregates by H_2O_2

Proteoglycan aggregates were treated for 24 h at 37 °C in the absence of H_2O_2 (lane 1) or with 13 mM- H_2O_2 (lane 2), 27 mM- H_2O_2 (lane 3), 54 mM- H_2O_2 (lane 4) or 134 mM- H_2O_2 (lane 5). The products were then subjected to SDS/10%-polyacrylamide-gel electrophoresis under reducing conditions, followed by electroblotting and immunolocalization of the link proteins.

Fig. 5. Time course of degradation of link protein in proteoglycan aggregates by H_2O_2 .

Proteoglycan aggregates were treated in the absence of H_2O_2 (lane 11) or with 140 mM- H_2O_2 (lanes 1–10) at 37 °C. The reactions were stopped by the addition of DTPA to a final concentration of 20 mM at the indicated time after H_2O_2 addition: lane 1, 0 h; lane 2, 1 h; lane 3, 3 h; lane 4, 6 h; lane 5, 9 h; lane 6, 12 h; lane 7, 18 h; lane 8, 21 h; lane 9, 24 h; lane 10, 48 h; lane 11, 48 h. The samples were then dialysed against 0.1 M-Tris/HCl buffer, pH 6.8, containing 0.1% SDS, and subjected to electrophoresis, electroblotting and immunolocalization.

43000 and contains major fragments of M_r 32000 and 29000 and minor fragments of M_r between 32000 and 29000 and between 27000 and 25000 (Fig. 6), observations that are consistent with an earlier, more extensive, study of the age-related changes in human link proteins (Mort *et al.*, 1985). The H_2O_2 -generated components of M_r 43000, 32000, 29000 and 27000 appear to be of similar size to those found in mature cartilage. In addition, fragments of M_r less than 40000 are not observed in either case if the samples are electrophoresed under non-reducing conditions (results not shown), suggesting that the H_2O_2 -generated fragments are held together by disulphide bonds in a pseudo-native conformation within the link protein, as is the case for the fragments generated *in vivo* with increasing age (Mort *et al.*, 1983).

Effects of metal-ion chelators and hydroxyl-radical scavengers

The degradation of the link protein in the presence of H_2O_2 was completely inhibited by the presence of the transition-metal-ion chelator DTPA at concentrations of 100 μM or more and was diminished at 10 μM (Fig. 7). The reaction was also inhibited by the presence of 10 mM-EDTA (results not shown), suggesting that the degradative effect of H_2O_2 on link protein is not direct, but requires transition-metal ions, possibly involving the generation of hydroxyl radicals by the Fenton reaction. H_2O_2 may oxidize or reduce transition-metal ions to

generate hydroxyl radicals or superoxide radicals respectively. A mixture of H_2O_2 and trace levels of transition-metal ions will therefore undergo a series of reactions in which hydroxyl radicals and superoxide radicals are generated, as long as the transition-metal ion is able to undergo a reduction-oxidation cycle. Chelation of transition-metal ions with DTPA probably inhibits their reduction with H_2O_2 (Buettner *et al.*, 1978; Cohen & Sinet, 1982), as does chelation of Cu^{2+} ions with EDTA, but not of Fe^{3+} ions with EDTA (Halliwell & Gutteridge, 1985).

In order to investigate the possibility that this reaction was mediated by the action of hydroxyl radicals generated from H_2O_2 by a Fenton reaction, we attempted to inhibit the reaction by the addition of molecules that have been shown to react with hydroxyl radicals with high rates of reaction in aqueous solution. This was done by adding putative scavengers to final concentrations of 200 mM to proteoglycan aggregates that were then treated with 60 mM- H_2O_2 at 37 °C for 24 h. Under these conditions, thiourea proved to be a potent inhibitor of link protein degradation, and tetramethylurea retarded the process, whereas formate and urea were ineffective (results not shown). These observations are consistent with the hypothesis that hydroxyl radicals may be involved in the degradative mechanism of H_2O_2 in this system; thiourea has the highest rate of reaction with hydroxyl radicals in solution, of the three of these molecules for which second-order rate constants have been measured (thiourea, formate and urea) (Dorfman & Adams, 1973; Halliwell & Gutteridge, 1985).

Fig. 6. Comparison of link-protein fragmentation by H_2O_2 with that observed with age

Lane 1, link proteins from a specimen of cartilage obtained at autopsy from a 59-year-old male; lane 2, human neonatal proteoglycan aggregate treated with 60 mM- H_2O_2 for 24 h at 37 °C; lane 3, as lane 2, but without H_2O_2 addition. Electrophoresis, electroblotting and immunolocalization were then performed.

Fig. 7. Inhibition of H_2O_2 -mediated degradation of link protein by the addition of DTPA

Proteoglycan aggregates were treated with 60 mM- H_2O_2 (lanes 2–5) or without H_2O_2 addition (lane 1) for 24 h at 37 °C after the addition of DTPA to a final concentration of 10 mM (lane 2), 100 μM (lane 3), 10 μM (lane 4) or 1 μM (lane 5). Preincubation with DTPA was for 30 min. Electrophoresis, electroblotting and immunolocalization were then performed.

Effect of H_2O_2 on the susceptibility of the link proteins to enzymic proteolysis

Though isolated link proteins are completely degraded by trypsin (Périn *et al.*, 1978), link proteins in proteoglycan aggregates are protected from proteolysis (Heinegård & Hascall, 1974). In order to investigate the possibility that radical-mediated damage might render the link proteins in proteoglycan aggregate more susceptible to degradation by proteinases, human neonatal articular cartilage proteoglycan aggregate was treated with a range of H_2O_2 concentrations for 24 h at 37 °C, then the H_2O_2 was dialysed away and the aggregates were treated with trypsin. The fragmentation pattern of link proteins was then analysed as before.

The link proteins in aggregate that had not been treated with H_2O_2 were cleaved by trypsin to produce a link protein of M_r 41 000–43 000, which is of similar size to the smallest native link protein (Fig. 8, lane 6). As has been reported previously, for treatment of link protein in proteoglycan aggregate with clostripain (Mort *et al.*, 1985) or with neutral metalloproteinase purified from human articular cartilage (Campbell *et al.*, 1986), this

Fig. 8. Effect of H_2O_2 treatment upon susceptibility of link protein in proteoglycan aggregates to proteolysis by trypsin

Neonatal proteoglycan aggregates were treated for 24 h at 37 °C with no addition (lanes 1 and 6) or additions of H_2O_2 to initial concentrations of 6 mM (lanes 2 and 7), 15 mM (lanes 3 and 8), 30 mM (lanes 4 and 9) or 60 mM (lanes 5 and 10). The samples were then dialysed versus 0.1 M-Tris/HCl buffer, pH 7.5, and treated with 10 μg of trypsin/mg of proteoglycan aggregate (lanes 6–10) or without addition of trypsin (lanes 1–5) for 4 h at 37 °C. Then 1 mM-phenylmethanesulphonyl fluoride was added to all samples and the samples were dialysed into 0.1 M-Tris/HCl buffer, pH 6.8, containing 0.1% SDS and subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions, electroblotted and the link proteins immunolocalized.

proteinase treatment did not completely degrade the link protein, and in this case produced few or no fragments of M_r less than 41 000. Trypsin treatment of the H_2O_2 -degraded link proteins resulted in degradation of the larger components, but no general change in the pattern of fragments of M_r less than 41 000 beyond that observed after H_2O_2 treatment only, except that the major 29 000- M_r H_2O_2 -generated fragment was cleaved in such a position as to decrease its apparent M_r by approx. 1000. Free-radical-mediated damage to link proteins in proteoglycan aggregates does not therefore appear to increase the susceptibility of these proteins to complete degradation by trypsin in this system, but can result in an increased susceptibility to limited modification.

DISCUSSION

Exposure of neonatal human articular-cartilage proteoglycan aggregate to H_2O_2 results in depolymerization of the aggregate, including degradation of protein components, under conditions that cause little or no cleavage in the glycosaminoglycan chains. This degradation appears to be site-selective, initially generating proteoglycan subunits that lack the ability to bind to hyaluronic acid but show little or no decrease in size. Though this treatment releases a small number of fragments of core protein containing an epitope that is associated with the hyaluronic acid-binding region of the proteoglycan subunit, most of the subunits appear to be intact. We are unable to distinguish between the possibilities that modification of important amino acid residues or that limited proteolysis is responsible for the loss of function that we observed in the hyaluronic acid-binding region after H_2O_2 treatment. Concomitantly, with loss of hyaluronic acid-binding ability, generation of the smallest native link protein occurs, followed by internal fragmentation within disulphide-bonded loops. These effects are inhibited by the metal-ion chelators EDTA and DTPA, implicating transition-metal ions in the degradative mechanism, and by molecules that react with hydroxyl radicals, which is consistent with the hypothesis that hydroxyl radicals are involved. The local generation of hydroxyl radicals from H_2O_2 by a Fenton reaction in a site-specific manner is probably responsible, and such a mechanism has been proposed for the H_2O_2 -induced degradation of ovarian-cyst glycoproteins (Creeth *et al.*, 1983; Cooper *et al.*, 1985). In contrast, when Cu^{2+} ions are added, much more extensive degradation occurs.

The nature of the responsible metal ion in this system *in vitro* is unknown at present, though iron ions are probably not involved, since iron-EDTA is known to be an effective generator of hydroxyl radicals from H_2O_2 , and EDTA inhibits the degradative effects of H_2O_2 on link protein. Other transition-metal ions, such as Cu^{2+} , present as counterions in the proteoglycan aggregates are probably responsible for the effect. It was noted that, though the pattern of link-protein fragmentation produced by H_2O_2 treatment was consistent between proteoglycan preparations from different donors, the extent of degradation that resulted from a given H_2O_2 dose varied between preparations. This may reflect different amounts of chelated transition-metal ions in different preparations.

It has been proposed (Samuni *et al.*, 1981; Creeth *et al.*, 1983; Cooper *et al.*, 1985) that the site of degrada-

tion of proteins by H_2O_2 is dependent on the protein primary structure, with the position of amino acid residues that chelate transition-metal ions determining the site of generation of the hydroxyl radical, which then reacts preferentially very close to its site of generation. The time course of degradation of link protein is consistent with this hypothesis, since the initial cleavages are at specific sites. The more extensive degradation seen at later stages may reflect the release of metal ions from their chelation sites as the result of destruction of those sites.

The pattern of link-protein degradation that results from H_2O_2 treatment is similar to that observed during the course of human aging, in that the initial result is the increased abundance of the component of M_r 43 000 followed by the accumulation of smaller fragments. Though treatment with a neutral proteinase from cartilage apparently reproduces a part of the age-related change in link proteins (i.e. the production of the 43 000- M_r component) (Campbell *et al.*, 1986), the fragmentation of link-protein molecules to smaller components that are held together in a pseudo-native conformation by disulphide bonds, as occurs with increasing age, has not previously been reported *in vitro*, by the use of proteinases that are likely to be present within the joint *in vivo*, or by other means. The pattern of 26 000–32 000- M_r link-protein fragments produced from isolated neonatal human articular-cartilage proteoglycan aggregate by H_2O_2 treatment is not identical with that observed in adult human articular cartilage; however, some of the radical-generated fragments do have their counterparts in the situation *in vivo*. The possibility remains that the additional fragments of lower M_r value that are observed *in vivo* may be the products of subsequent proteinase action after initial free-radical cleavage. Such a pathway has been reported for other proteins: the susceptibility of haemoglobin and fibronectin to proteolysis by a number of purified enzymes has been shown to be increased by pretreatment of these proteins with H_2O_2 (Fligel *et al.*, 1984), and Wolff & Dean (1986) have demonstrated that hydroxyl-radical attack on bovine serum albumin increases its susceptibility to digestion by trypsin. The oxidative inactivation of glutamine synthetase from *Escherichia coli* has been shown to sensitize this enzyme to proteolysis by a purified metalloproteinase from *E. coli* and several purified eukaryotic enzymes (Rivett *et al.*, 1985). Changes in the tertiary structure of the proteins may be responsible for these effects. We have observed only a very slightly increased susceptibility to proteolysis in the case of link protein in proteoglycan aggregate after H_2O_2 treatment. The limited nature of the effect may be due to the fragmented link protein not losing its functional properties after this modification, but remaining bound to the proteoglycan aggregate, in a site that may sterically prevent access of trypsin to link protein.

A postulated mechanism by which site-specific generation of hydroxyl radicals from H_2O_2 in articular cartilage might cause age-related changes in the proteoglycans' structure would require the progressive accumulation of these transition-metal ions in joints with age. This has not been investigated, but it is known that the concentrations of iron as ferritin in the synovial membrane and synovial fluid are considerably elevated in patients with rheumatoid arthritis (Senator *et al.*, 1968), as is the concentration of copper in synovial fluid

from patients with rheumatoid arthritis or osteoarthritis (Niedermeyer & Griggs, 1971; Scudder *et al.*, 1978). Gutteridge *et al.* (1981, 1982) have shown that non-protein-bound iron may be present in human rheumatoid synovial fluid in the 2–4 μ M range. It is possible that, even in individuals who exhibit no gross pathological changes, low concentrations of transition-metal ions may become available in the joint for chelation by components of proteoglycan aggregates and therefore be able to participate in site-specific Fenton reactions with H₂O₂. Low concentrations of H₂O₂ may be released by phagocytic cells present in the synovial fluid during periods of joint inflammation throughout life. It is probable that adult cartilage is poorly protected against this process; Blake *et al.* (1981) have shown rheumatoid synovial fluid to contain extremely low or undetectable activities of superoxide dismutase or catalase.

We suggest that cleavage by H₂O₂-driven mechanisms may contribute to the fragmentation pattern of macromolecules observed with increasing age in human articular cartilage.

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