Delayed aggregation of proteoglycans in adult human articular cartilage

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The biosynthesis and macromolecular organization of proteoglycans was studied in explants of adult human articular cartilage. In a series of pulse-chase experiments, labelling with (³⁵S) sulphate, it was shown that the proteoglycan monomer is synthesized as a precursor that has a low affinity for hyaluronic acid. These findings suggest a possible mechanism by which the rate of incorporation of proteoglycans into the extracellular matrix may be controlled.

Articular cartilage contains few cells surrounded by a large amount of extracellular matrix, consisting mainly of collagen and proteoglycan. The proteoglycans are largely responsible for the high elasticity and resilience of the tissue (Kempson et al., 1971). The proteoglycan monomer consists of a central protein core to which a large number of chondroitin sulphate and keratan sulphate chains are covalently bound. At one end of the protein core a globular region, the hyaluronic-acid-binding region, is capable of interacting specifically with hyaluronic acid (Hascall & Heinegård, 1974; Heinegård & Hascall, 1974). The aggregate complex is further stabilized by link proteins (Hardingham, 1979). One property of aggregation may be to prevent diffusion of the proteoglycans out of the tissue since solutions such as 4-M guanidinium chloride, that dissociate the aggregate, enhance the extraction of proteoglycans (Sajdera & Hascall, 1969).

The basic process by which proteoglycan aggregates are synthesized and assembled has been extensively studied in cultures of Swarm rat chondrosarcoma chondrocytes (Kimura et al., 1979). In this system aggregation was shown to be an extracellular event, occurring soon after secretion, and at no stage during synthesis was a non-aggregating form of monomer identified (Kimura et al., 1980). In agreement with these findings Bayliss et al. (1983a) showed that proteoglycans synthesized by human chondrosarcoma in organ culture were highly aggregated. However, those synthesized by adult human articular cartilage are partially aggregated and were only converted to an aggregating form over a relatively long period in culture.

As a result of the above observations, we have re-examined this delay in aggregation and the factors that influence it.
Materials and Methods

Human articular cartilage was obtained from femoral heads, either at operation for subcapital fracture of the neck of femur or hind-quarter amputation. Full-depth pieces of tissue were incubated in Eagles's Basal Medium (Gibco, Europe), containing 0.05-mM MgSO₄, 20-mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), 10-mM glutamine, and carrier-free (³⁵S)sulphate (1 mCi/g cartilage) (Amersham International). The cartilage was pre-equilibrated with the isotope for 1 h at 4°C to ensure full penetration of (³⁵S)sulphate (Maroudas & Evans, 1974) before incubating for 4 h at 37°C (pulse). Incorporation of isotope was then stopped by freezing the tissue at -20°C or the cartilage sample was incubated for a further 18 h in fresh medium, without isotope containing 0.8-mM MgSO₄ (chase). The cartilage samples were sectioned at 20 µm on a cryostat and the proteoglycans extracted with 4-M guanidinium chloride, pH 7.0, containing proteinase inhibitors, at 4°C for 24 h, as described previously (Bayliss et al., 1983b). Extracts were dialysed to remove the guanidinium chloride and their density was adjusted to 1.5 g/ml with CsCl. After centrifuging at 100 000 g at 4°C for 48 h, the gradients were fractionated into three equal-sized fractions (A₁, A₂, and A₃ in the terminology of Heinegård, 1977) and proteoglycan aggregates were recovered from the high-density A₁ fraction. Proteoglycan monomers were prepared from the A₁ fraction by dissociative density-gradient centrifugation in the presence of 4-M guanidinium chloride. Monomers were recovered from the high-density fraction (A₁D₁D₁ in the terminology of Heinegård, 1977). The rate of sulphate incorporation by cartilage samples was determined as described previously (Bayliss et al., 1983a).

The size distribution of proteoglycan monomers and the proportion of aggregated proteoglycans in a preparation was determined by chromatography on a column (150 cm x 0.75 cm) of Sepharose 2B (Bayliss et al., 1983b). Column fractions were assayed for uronic acid, by an automated modification of the Bitter and Muir (1962) method, and for hexose and protein by automated methods described by Bayliss et al. (1983b). Glycosaminoglycan chains were prepared by alkaline borohydride digestion of proteoglycan aggregates. Chondroitin sulphate and keratan sulphate chains were isolated from this digest by the method of Sweet et al. (1979) and their sizes determined by gel chromatography on Sepharose S-300 and Biogel P-10 respectively. The molar ratio of chondroitin-4-sulphate and chondroitin-6-sulphate was measured using the method of Siato et al. (1968).

Results and Discussion

In a preliminary series of experiments the structures of the newly synthesized proteoglycans were compared with those already incorporated in the extracellular matrix. The (³⁵S)proteoglycan monomers, obtained by reduction and alkylation of an A₁ fraction (Heinegård, 1977), had a higher molecular weight than the large non-labelled proteoglycan monomers rich in chondroitin sulphate (pool I) (Fig. 1). It is notable that much less radioactivity was associated with the smaller proteoglycan monomer species rich in keratan sulphate (pool II) that is characteristic of adult human articular cartilage (Bayliss et
al., 1983b), suggesting that this proteoglycan population may arise from slow extracellular proteolysis, rather than being a separate gene product. Alternatively, the proteoglycans in pool II may represent a species that has a very slow rate of turnover.

The chondroitin sulphate and keratan sulphate chains isolated from the \((^{35}S)\)proteoglycan were similar in size to those from non-labelled molecules and the molar ratio of chondroitin-6-sulphate to chondroitin-4-sulphate was approx. 5:1 in both cases. Using the alkaline borohydride/ethanol precipitation procedure described by Sweet et al. (1979), it was also shown that 15\% of the \((^{35}S)\)sulphate was incorporated into keratan sulphate.

Thus, the \((^{35}S)\)proteoglycans, although larger in size, were similar in composition to non-labelled molecules. Nevertheless, there were considerable differences in the aggregating properties of the two populations. Although the majority of non-labelled proteoglycans in the A1 fractions after the 4-h pulse were highly aggregated, most of the \((^{35}S)\)proteoglycans were still in monomeric form (Fig. 2a). During an 18-h chase period, however, the proportion of \((^{35}S)\)proteoglycan aggregate always increased to a value similar to that of the non-labelled molecules (Fig. 2b), although the extent of this increase varied between different individuals. These findings confirm and extend previous work (Bayliss et al., 1983a) which compared the rates of proteoglycan aggregation in articular cartilage and chondrosarcoma from the same amputated limb. Together the results suggest that a precursor form of proteoglycan is first synthesized which subsequently 'matures' over a period of time.

![Gel chromatography on Sepharose CL-2B of non-labelled monomer and \((^{35}S)\)proteoglycan monomer isolated from normal articular cartilage (70-yr-old).]
Fig. 2. Gel chromatography on Sepharose CL-2B of aggregate Al-fractions from normal articular cartilage (65-yr-old). (a) The cartilage sample was incubated in the presence of (35S)sulphate for 4 h before extraction. (b) After labelling for 4 h the cartilage sample was incubated in the absence of isotope for a further 18 h.

The participation of the chondrocyte in the 'maturation' process was shown by freezing and thawing the tissue after the 4-h pulse prior to the 18-h chase incubation. This procedure killed the cells, as they were unable to incorporate (35S)sulphate, but it did not prevent the maturation process from taking place (Fig. 3). On the other hand, the temperature at which the chase was carried out affected the rate of 'maturation'. The process was inhibited at temperatures below 37°C and was augmented somewhat at higher temperatures (Fig. 4). The results suggest that the macromolecular change was the result either of the direct action of an extracellular enzyme (analogous to the conversion of pro-collagen to collagen), or of a slow conformational change possibly involving formation of the disulphide bonds that are present in the hyaluronic-acid-binding region of the molecule (Hardingham et al., 1976). Both processes would be temperature dependent and therefore inhibited at the low temperatures at which extraction and purification were carried out. Evidence in support of the latter mechanism was obtained by incubating the 4-h pulse-Al fraction in the presence of 10-mM N-ethylmaleimide (NEM). Although the rate of 'maturation' was always less with purified proteoglycans in solution than when they were 'chased' in the tissue, NEM completely
Fig. 3. Gel chromatography on Sepharose CL-2B of (35S)proteoglycan isolated from normal cartilage (55-yr-old). (a) Labelled for 4 h; (b) labelled for 4 h and chased for 18 h; or (c) labelled for 4 h and subjected to six cycles of freezing and thawing before the 18-h chase.

inhibited the 'maturation' that did take place during the 18-h chase of two 4-h pulse-Al preparations. It could also be argued that NEM was preventing 'maturation' by inhibiting an enzyme. However, this mechanism would require a close association of the enzyme with proteoglycan for it to be recovered in the high-density regions of the caesium chloride gradient. Furthermore, it is unlikely that a mammalian enzyme would still be active at 60°C, whereas Hardingham (1979) has shown that the isolated aggregate complex is stable at this temperature. Experiments designed to test the first suggested mechanism were unsuccessful, namely to effect the conversion by digesting the 4-h pulse-Al fraction with low concentrations of trypsin. Higher concentrations resulted in degradation of the proteoglycans. Whichever mechanism is active, the effect is very specific and the extent to which 'maturation' occurs is probably sensitive to many factors, e.g. the level of enzyme inhibitor/activator complexes in the tissue or the correct microenvironment for reduction/oxidation. It is not surprising, therefore, that the rates of 'maturation' are very slow in the dilute solutions that result after extraction and purification.

Although these experiments suggested that the precursor did not bind to hyaluronic acid, further studies demonstrated that this was not
Fig. 4. Effect of temperature on (\(^{35}\)S)proteoglycan 'maturation'. Samples of normal articular cartilage (58-yr-old), labelled with (\(^{35}\)S)sulphate for 4 h at 37°C, were chased for 18 h at 4°C, 18°C, 37°C, and 60°C. The extent of aggregation of each preparation was determined by gel chromatography on Sepharose CL-2B.

Fig. 5. Interaction of non-labelled monomer (●), precursor (\(^{35}\)S)proteoglycan monomer (○), and 'mature' (\(^{35}\)S)proteoglycan monomer (▲) with hyaluronic acid. Aliquots of the \(^{32}\)S-labelled monomer preparations (AID1D1), containing 1 mg of non-labelled monomer (A1D1D1) as a carrier, were mixed with various concentrations of human umbilical-cord hyaluronic acid in 100-mM sodium phosphate buffer pH 7.0 for 18 h at 4°C. The extent of aggregation was assessed on Sepharose CL-2B.
the case. Fig. 5 shows that higher concentrations of hyaluronic acid were necessary to aggregate the precursor (³⁵S)proteoglycan monomer than that required to fully aggregate the non-labelled monomer and the 'mature' (³⁵S)proteoglycan monomer.

At present, it is not clear how the hyaluronic-acid-binding region is modified, but such a slow structural change would allow time for the molecule to diffuse away from the pericellular environment before becoming immobilized as aggregates. This may be another mechanism, besides the availability of hyaluronic acid and link protein, that determines the rate of incorporation of monomers into aggregate structures. Elucidation of the factors that control this process should provide better understanding of the pathways of matrix turnover in normal cartilage and in degenerative joint disease.

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References