Age-related decrease in the link-stability of proteoglycan aggregates formed by articular chondrocytes

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Chondrocytes were isolated from the articular cartilage of rabbits aged between 6 and 50 weeks and labelled with [³⁵S]sulphate after 48 h in monolayer culture. The percentage of the total proteoglycan monomers synthesized by each culture that were present as link-stabilized aggregates was shown to be about 83% at 6, 9 and 12 weeks, 73% at 15 weeks, 48% at 30 weeks and 32% at 50 weeks. The proliferative activity of the cells in culture also decreased markedly with the age of the donor. The results suggest that aging of chondrocytes *in vivo* is accompanied by a decrease in their capacity for link-protein synthesis.

When chondrocytes are isolated from rat chondrosarcoma (Kimura *et al.*, 1980), foetal-bovine tracheal cartilage (Bjornsson & Heinegard, 1981), chick embryos (Lohmander *et al.*, 1979) or immature rabbit articular cartilage (Plaas *et al.*, 1983), the cells secrete both aggregating proteoglycan monomers and link protein. The amount of link protein secreted has been shown to be sufficient for the formation of a high percentage of link-stabilized aggregates (Bjornsson & Heinegard, 1981) or even in excess of proteoglycan monomer, as in chondrosarcoma cultures (Kimura *et al.*, 1980).

Immature rabbit chondrocytes produce 75–80% link-stabilized aggregates in culture, but this percentage decreased markedly after the cells had formed a confluent monolayer and ceased to proliferate (Plaas *et al.*, 1983). In the present paper we show that aging of articular chondrocytes *in vivo* is also accompanied by a marked decrease in their proliferative activity and in the percentage of link-stable aggregates formed in monolayer culture.

Experimental

Materials

Materials were obtained as previously described (Plaas *et al.*, 1983). In addition, PD-10 columns were obtained from Pharmacia, and Linbro tissue-culture multiwell plates were from Flow Laborato-

ries (Irvine, Ayrshire, Scotland, U.K.).

Chondrocyte cultures

Chondrocytes were isolated from the articular cartilage of New Zealand White rabbits aged 6, 9, 12, 15, 30 and 50 weeks, as previously described (Plaas *et al.*, 1983) and transferred to multiwell plates (2 cm² approx.) at 5 × 10⁵ cells/ml of Ham's F-12 medium supplemented as previously described (Plaas *et al.*, 1983).

DNA synthesis by chondrocytes was determined as previously described (Plaas *et al.*, 1983).

Pulse-chase radiolabelling and isolation of proteoglycans in chondrocyte cultures

At 48 h after initial plating, [³⁵S]sulphate (25 µCi; 83.3 µCi/µmol) was added in 0.5 ml of fresh medium to adherent cells and incorporation was allowed for 24 h at 37°C. After incubation the medium was removed and the cell layer was washed with 0.5 ml of non-radioactive medium and this washing was added to the pulse medium (pulse medium samples). Fresh non-radioactive medium (0.5 ml) was added and incubation allowed to proceed for a further 24 h before medium collection (chase medium 1); this procedure was repeated for a second 24 h chase period (chase medium 2). The cell layers were then extracted with 0.5 ml of 0.5 M-sodium acetate, pH 6.8 (containing 10 mM-sodium EDTA, 5 mM-benzamidine hydrochloride, 0.1 mM-phenylmethanesulphonyl fluoride and 100 mM-6-aminohexanoic acid) at 4°C for 24 h (associative extract). The inextractable residue was solubilized by treatment with 0.5 ml of 0.5 M-NaOH for 24 h at room temperature. Medium samples and associative extracts were stored at –20°C until analysis.

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Total ^{35}S -labelled macromolecules present in media, associative extracts and alkali digests were determined by Sephadex G-25/PD-10 column chromatography as previously described (Stevens *et al.*, 1981).

Determination of percentage of link-stabilized aggregates

Samples of ^{35}S -labelled proteoglycan in pulse or chase media or associative extracts (50–250 μl) were added to 10 μl of hyaluronate oligosaccharides (1 mg of hexuronate/ml) prepared as previously described (Plaas *et al.*, 1983) and fractionated on columns of Sepharose CL-2B, also as previously described (Plaas *et al.*, 1983). The percentage of total [^{35}S]proteoglycan monomer that was eluted in the void volume of the column (about 1–14 g of eluant) was taken as the percentage of link-stabilized aggregate present (Kimura *et al.*, 1979).

Cartilage explants

Cartilage slices were prepared and labelled with [^{35}S]sulphate as described by Sandy *et al.* (1980). The conditions of culture and the determination of the distribution of ^{35}S -labelled macromolecules were as previously described (Sandy *et al.*, 1978).

Results and discussion

Total proteoglycan synthesis by chondrocytes from rabbits of each age varied between 11.8 and 19.2 nmol/24 h per 10^6 cells (Table 1). Although there was close agreement between the total activity observed in replicate cultures established from any one cell preparation, there was no marked correlation between activity and the age of the donor animal. It seems likely, however, that, in these experiments, proteoglycan synthesis would be more influenced by variation in the preparation and maintenance of the cells *in vitro* than by the age of the rabbits.

On the other hand the distribution of ^{35}S -

labelled monomers between the pulse media, chase media and cell layer showed a marked age-related change (Table 1). Thus, although 49.4% of newly synthesized proteoglycans were released into the medium from the cell layer in cultures of 6-week-old cells, this fraction increased with age and accounted for about 69% in cultures of 30- and 50-week-old cells. This increase was reflected in a markedly higher [^{35}S]proteoglycan content of the associative extract of 6-week-old cells (4.68 nmol/ 10^6 cells; 25.4% of total) than of 50-week-old cells (0.98 nmol/ 10^6 cells; 5.1% of total).

Since this associative extract contains proteoglycans retained by the newly formed extracellular matrix, these results showed that the proteoglycans secreted by 6-week-old cells were much more readily incorporated into this matrix than were those proteoglycans secreted by 50-week-old cells under the same culture conditions.

An explanation for this difference in matrix retention was obtained when the link-stability of aggregates in medium and extracts was determined (Fig. 1). In cultures with high proteoglycan retention in the cell layer (6, 9, 12 weeks), more than 75% of aggregates in pulse medium, chase media and associative extract were in a link-stabilized form. Conversely, the poor retention around mature chondrocytes was associated with low link-stability, which was only 13% in the pulse medium of cells from 50-week-old rabbits. This low link-stability of aggregates in samples from mature cells was due to a deficiency in supply of functional link protein, since addition of exogenous link protein as previously described (Plaas *et al.*, 1983) produced more than 75% link-stability in all samples.

A low- M_r non-aggregating proteoglycan (K_{av} = 0.70; Fig. 1) was synthesized by all cultures and was present only in the pulse media (column 1, Fig. 1), presumably owing to a high rate

Table 1. *Synthesis and distribution of ^{35}S -labelled proteoglycan monomers in chondrocyte cultures*

Chondrocytes isolated from rabbits of each age were labelled with [^{35}S]sulphate for 24 h, and the [^{35}S]proteoglycans were isolated from the pulse medium, two chase media, associative extract and residue as described in the Experimental section. The values given are means $\pm$ S.D. for quadruplicate cultures derived from the pooled tissue from two animals. The values in parentheses are the mean percentages of the total [^{35}S]proteoglycan present in each fraction.

Age of rabbit (weeks)	[^{35}S]Sulphate incorporated (nmol/24 h per 10^6 cells)					
	Total	Pulse medium	Chase medium 1	Chase medium 2	Associative extract	Residue
6	18.4 $\pm$ 0.55	9.07 $\pm$ 0.55 (49.4)	2.90 $\pm$ 0.31 (15.8)	1.03 $\pm$ 0.05 (5.5)	4.68 $\pm$ 0.79 (25.4)	0.71 $\pm$ 0.11 (3.9)
9	13.1 $\pm$ 0.38	7.06 $\pm$ 0.13 (54.0)	1.96 $\pm$ 0.13 (15.0)	1.24 $\pm$ 0.01 (9.5)	2.34 $\pm$ 0.33 (18.0)	0.46 $\pm$ 0.05 (3.5)
12	11.8 $\pm$ 0.43	6.29 $\pm$ 0.18 (53.5)	2.12 $\pm$ 0.12 (18.0)	0.99 $\pm$ 0.03 (8.4)	1.95 $\pm$ 0.16 (16.5)	0.42 $\pm$ 0.06 (3.6)
15	15.0 $\pm$ 0.29	9.64 $\pm$ 0.22 (64.5)	1.84 $\pm$ 0.21 (12.3)	0.85 $\pm$ 0.09 (5.7)	1.79 $\pm$ 0.18 (12.0)	0.82 $\pm$ 0.06 (5.5)
30	18.6 $\pm$ 0.46	12.9 $\pm$ 0.48 (69.0)	1.86 $\pm$ 0.47 (10.0)	1.15 $\pm$ 0.11 (6.2)	1.43 $\pm$ 0.20 (7.7)	1.32 $\pm$ 0.13 (7.1)
50	19.2 $\pm$ 0.36	13.2 $\pm$ 0.61 (68.9)	2.19 $\pm$ 0.10 (11.4)	0.88 $\pm$ 0.10 (11.4)	0.98 $\pm$ 0.04 (5.2)	1.92 $\pm$ 0.09 (10.0)

Fig. 1. Determination of the percentage of link-stabilized aggregates in samples from chondrocyte cultures. Chondrocyte cultures from rabbits of different ages [(a) 6 weeks; (b) 9 weeks; (c) 12 weeks; (d) 15 weeks; (e) 30 weeks; (f) 50 weeks] were labelled with [^{35}S]sulphate, and samples of pulse medium (column 1), chase medium 1 (column 2), chase medium 2 (column 3) and associative extract of cell layers (column 4) were added to hyaluronate oligosaccharides and fractionated on Sepharose CL-2B as described in the Experimental section. The percentage of proteoglycan monomer in link-stabilized aggregate (shaded areas) is given.

of diffusion from the cell layer. It always represented a higher percentage of the total ^{35}S -labelled macromolecules synthesized by cells from 6-week-old rabbits (about 15%) than from 50-week-old rabbits (about 8%).

The percentage of the total proteoglycan mon-

omers synthesized by each culture, which were isolated as link-stabilized aggregates, was calculated from the data in Table 1 and Fig. 1 (also assuming 95% link-stability of aggregates in the residue samples). Chondrocytes isolated from rabbits aged 6, 9, 12, 15, 30 and 50 weeks produced

Table 2. *Synthesis and distribution of ^{35}S -labelled proteoglycan in cartilage explants*

Cartilage explants from 6-week-old and 50-week-old rabbits were labelled with [^{35}S]sulphate for 2h, maintained in non-radioactive medium for 3 days and then digested with papain (see the Experimental section for details). The values given are means $\pm$ 1 s.d. for quadruplicate cultures derived from the pooled tissue from two animals. The values in parentheses are the mean percentages of the total [^{35}S]proteoglycan present in each fraction.

Age of rabbit (weeks)	[^{35}S]Sulphate incorporated (pmol/h per mg wet wt.)					
	Total	Pulse medium	Chase medium 1	Chase medium 2	Chase medium 3	Residue
6	42.9 $\pm$ 2.3	0.64 $\pm$ 0.08 (1.5)	6.52 $\pm$ 0.47 (15.2)	3.18 $\pm$ 0.17 (7.4)	1.72 $\pm$ 0.08 (4.0)	30.9 $\pm$ 0.60 (71.9)
50	7.54 $\pm$ 0.47	0.23 $\pm$ 0.02 (3.1)	2.72 $\pm$ 0.15 (36.1)	1.27 $\pm$ 0.08 (16.9)	0.69 $\pm$ 0.04 (6.1)	2.85 $\pm$ 0.14 (37.8)

aggregates that were 85, 83, 81, 73, 48 and 32% link-stabilized respectively.

Poor matrix retention of newly synthesized proteoglycans around mature cells as compared with immature cells was also observed in labelling of cartilage explants (Table 2). Thus cartilage from mature rabbits released [^{35}S]proteoglycan extensively during maintenance in culture and retained only 37.8% of the total after 3 days. On the other hand, immature cartilage retained 71.9% of the total under the same conditions. The relatively poor retention by mature cartilage did not appear to be due to degradation of newly synthesized proteoglycan monomer in these samples; the elution profiles obtained by dissociative Sepharose CL-2B chromatography of chase media 1 were essentially identical for the two ages, and showed that most of the released proteoglycan was about the size of proteoglycan monomer.

It should be noted that total proteoglycan synthesis (pmol/h per mg wet wt.) by immature cartilage was about 6-fold greater than that by mature cartilage (Table 2). This can be partly explained by the higher cell density (about 2-fold) of immature tissue, but it also indicates that, in freshly prepared explants, immature chondrocytes are significantly more active than mature chondrocytes, a difference not observed in isolated cell preparations (Table 1).

It seems likely that the relatively low retention of [^{35}S]proteoglycans around mature cells in monolayer culture (Table 1) and explant culture (Table 2) is due in both cases to poor link-stabilization of newly formed aggregates. It is of particular interest that, in monolayer, the major decrease in link-stabilization occurred between 15 and 30 weeks of age, which coincides with the major decrease in proliferative activity of the cells (Table 3). This appears to be related to our previous work with immature cells in monolayer culture in which there was a marked decrease in link-protein secretion and link-stabilization of medium aggregates after the cells had reached confluence and ceased to proliferate (Plaas *et al.*, 1983).

The poor link-stability of aggregates isolated from the medium of non-proliferative cultures in

Table 3. *Proliferative activity of chondrocytes in culture*

Chondrocytes isolated from rabbits of each age were labelled with [^3H]thymidine for 24h, the [^3H]DNA was isolated and radioactivity determined (see the Experimental section). Results shown are means $\pm$ 1 s.d. for quadruplicate cultures derived from the pooled tissue from two animals.

Age of rabbit (weeks)	Incorporation of [^3H]thymidine into DNA (d.p.m./24h per cell)
6	11.84 $\pm$ 0.31
9	7.71 $\pm$ 0.11
12	6.03 $\pm$ 0.05
15	5.50 $\pm$ 0.04
30	1.34 $\pm$ 0.12
50	0.45 $\pm$ 0.02

the present work is also apparently due to an insufficient supply of functional link protein, since in all cases high link-stability was obtained after addition of exogenous link. This lack of link protein would arise if, in mature cell cultures, the secreted link was more tightly bound than proteoglycan monomer by pericellular matrix components, such as collagen or hyaluronic acid. Alternatively, mature chondrocytes might synthesize or secrete a lower ratio of link protein to proteoglycan monomer than do highly proliferative cells.

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