Biochem. J. (1990) 267, 803–808 (Printed in Great Britain)

Effects of tissue compression on the hyaluronate-binding properties of newly synthesized proteoglycans in cartilage explants

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The effects of tissue compression on the hyaluronate-binding properties of newly synthesized proteoglycans in calf cartilage explants were examined. Pulse–chase experiments showed that conversion of low-affinity monomers to the high-affinity form (that is, to a form capable of forming aggregates with 1.6% hyaluronate on Sephacryl S-1000) occurred with a $t_{\frac{1}{2}}$ of about 5.7 h in free-swelling discs at pH 7.45. Static compression during chase (in pH 7.45 medium) slowed the conversion, as did incubation in acidic medium (without compression). Both effects were dose-dependent. For example, the $t_{\frac{1}{2}}$ for conversion was increased to about 11 h by either (1) compression from a thickness of 1.25 mm to 0.5 mm or (2) medium acidification from pH 7.45 to 6.99. Oscillatory compression of 2% amplitude at 0.001, 0.01, or 0.1 cycles/s during chase did not, however, affect the conversion. Changes in the hyaluronate-binding affinity of [^{35}S]proteoglycans in these experiments were accompanied by no marked change in the high percentage ($\sim$ 80%) of monomers which could form aggregates with excess hyaluronate and link protein. Since static tissue compression would result in an increased matrix proteoglycan concentration and thereby a lower intra-tissue pH [Gray, Pizzanelli, Grodzinsky & Lee (1988) J. Orthop. Res. 6, 777–792], it seems likely that matrix pH may influence proteoglycan aggregate assembly by an effect on the hyaluronate-binding affinity of proteoglycan monomer. Such a pH mechanism might have a physiological role, promoting proteoglycan deposition in regions of low proteoglycan concentration.

INTRODUCTION

The load-bearing properties of cartilage are largely dependent on the high density of matrix-affixed charge residing on the extracellular matrix proteoglycans [1–3]. The ionized carboxy and sulphate groups of the glycosaminoglycan side chains endow cartilage with a large osmotic swelling pressure which is balanced by the tensile strength of the collagen framework [4]. Most cartilage proteoglycans (60–85%) appear to exist in aggregates, in which monomers are non-covalently bound to hyaluronate in an interaction stabilized by link protein [5–7]. Such aggregates are macromolecular complexes that can consist of $\sim$ 30–100 proteoglycan monomers affixed to a $\sim$ 1.3-μm-long hyaluronate backbone [6,8]. The size of the aggregate effectively immobilizes proteoglycans within the collagenous meshwork, preventing monomers from diffusing out of the tissue [7,9]. In contrast, catabolized monomers, which are unable to aggregate with hyaluronate, are able to diffuse within the matrix and are preferentially released from thin cartilage slices *in vitro* [10,11].

The mechanism by which proteoglycan aggregates are assembled biosynthetically has been extensively studied in chondrocyte culture [12,13]. In studies of cartilage explants, it has been shown that monomers, once secreted from chondrocytes, appear to undergo a maturation process in the extracellular matrix whereby their binding affinity for hyaluronate increases [14]. This conversion process occurs in articular cartilage *in vivo* [15] and in purified proteoglycan solutions [16]. With purified samples, the conversion can be catalysed by mild base [16,17] or by incubation of monomer in aggregate with hyaluronate, or hyaluronate and link protein, but not by incubation with link protein alone [16]. Within explant cultures, the process appears to occur in the absence of continued cellular activity and can be slowed by low temperature [18,19], but is promoted by a low partial pressure of oxygen [20]. This conversion appears to be blocked by *N*-ethylmaleimide [18], consistently with the involvement of free thiol groups in the process.

Both static and dynamic compression of cartilage explants at physiological magnitudes can alter proteoglycan metabolism [21,22]. Static compression of cartilage can inhibit proteoglycan biosynthesis [21–25], possibly through a physicochemical alteration of intra-tissue ion concentrations [24,26]. Compression increases the concentration of negatively charged glycosaminoglycans, leading to a decreased intra-tissue pH (see [24] for analysis). Such an intra-tissue acidification can independently account for the observed inhibition of proteoglycan synthesis [24]. On the other hand, physiological levels of dynamic compression, consisting of oscillatory displacements of $\sim$ 1–5% at frequencies of 0.01–1 cycle/s, can stimulate synthesis [21]. Further, the physical loading protocols in our studies [21] are relevant to articular cartilage *in vivo*, where compression can be considered to be a combination of a time-average static component and a time-varying dynamic compressive component.

In the present study we used pulse–chase methods to examine the effects of static compression and oscillatory compression on the acquisition of hyaluronate-binding affinity by newly synthesized proteoglycans, as well as the role of altered intra-tissue pH in mediating the effects of static compression.

EXPERIMENTAL

Materials

Materials for cartilage explant and culture and for proteoglycan isolation and analysis were obtained as described previously [15,21]. In addition, sterile-filtered 1.0 M-NaOH and 1.0 M-HCl were from Sigma (St Louis, MO, U.S.A.). Tissue-Tek

Abbreviation used: $t_{\frac{1}{2}}$, half-life for the conversion process.

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embedding solution (O.C.T. compound 4583) and Tissue-Tek II disposable specimen moulds were from Miles Diagnostics, Elkhart, IN, U.S.A. Sephacryl S-1000 was from Pharmacia, Piscataway, NJ, U.S.A.

Cartilage explant, culture and radiolabelling

Cartilage discs, 1 mm thick × 3 mm diameter, were explanted from the femoropatellar grooves of 1–2-week-old calves, and cultured in Dulbecco modified Eagle medium with 44 mM-$NaHCO_3$ 10 mM-Hepes, 0.1 mM non-essential amino acids, additional 0.4 mM-L-proline, ascorbate (20 μg/ml) and 10% foetal-bovine serum in a humidified CO_2/air (1:19) incubator at 37 °C, as previously described [21]. Medium (pH 7.45) was changed daily and, on the first day, was supplemented with 100 μg of penicillin/ml and 100 units of streptomycin/ml. All medium was prepared by temperature and CO_2 equilibration in the incubator for 1–2 h just before use. After 2–6 days of culture, discs swelled axially to a thickness of 1.25–1.4 mm and attained relatively steady-state rates of biosynthesis [21]. Discs were then pulse-radiolabelled for 1.5 h in medium including 100 μCi of [^{35}S]sulphate/ml (1 ml/4 discs) before use in the various chase protocols (described below).

Static compression. Pulse-labelled discs were washed three times (1 ml/disc) over a total of 0.5 h with culture medium (pH 7.45), and then placed in individual wells of compression chambers (0.8 ml of medium/disc), where they were subjected to uniaxial, radially unconfined, static compression between two impermeable platens essentially as described previously [21]. Cartilage discs were compressed to a thickness of 1.25, 1.00, 0.75 or 0.50 mm. In such a configuration, stress relaxation (and concomitant fluid flow relative to the solid matrix) is 90% complete within ~ 15 min [21]; thus the period required to reach mechanical equilibrium was short relative to the 3–24 h period of chase incubation in the pulse–chase protocols.

Oscillatory compression. Pulse-labelled discs were washed as described above and then placed in a dynamic compression chamber (25 ml of medium/12 discs) held within a mechanical spectrometer (Dynastat; IMASS, Hingham, MA, U.S.A.), as described previously [21]. This apparatus allowed cartilage discs to be subjected to a time-varying uniaxial, radially-unconfined compression. During the 8.5 h compression and chase period, discs were subjected to a static compression to a thickness of 1.00 mm and a superimposed 2% (0.02 mm peak) oscillatory strain. Oscillations were at frequencies of 0 (static control), 0.001, 0.01, or 0.1 cycles/s. Control studies showed that showed that injection of a CO_2/air (1:g) mixture into the chamber fixed the bicarbonate-buffered medium at pH ~ 7.4.

pH variation. Pulse-labelled discs were washed and then compressed to a static thickness of 1.25 mm as described above, except that pH-adjusted medium was used during wash and chase. Medium with pH of 6.99–7.70 in the CO_2 incubator was prepared from the above medium formulation by adding combinations of 1 M-HCl, water and 1 M-NaOH in a total added volume of 2.24 ml/l of medium such that 18.8, 11.2 or 0 mmol of HCl/l of medium or 22.4 mmol of NaOH/l of medium was added. Since the pH of the medium was not significantly altered during the 24 h chase period (pH change $\leqslant$ 0.02 pH unit), it was routinely measured after chase using medium pooled from four discs with a Ross combination pH-electrode (Orion Research, Boston, MA, U.S.A.).

In all pulse–chase protocols, tissue incubations were either on a rotary shaker (model 3520; Labline Instruments, Melrose Park, IL, U.S.A.) at 60 rev./min (during static compression) or with medium recirculation (during dynamic compression [21]) to minimize stagnant films. In each protocol, discs from adjacent anatomical locations within a single joint were evenly distributed among the different treatment groups, and each treatment group consisted of four to six discs. For a given chase duration in the static-compression protocols, separate chambers were used to impose the different treatment conditions in order to allow samples to be tested simultaneously. In oscillatory-compression protocols, a single dynamic chamber was used in sequential 12 h experiments for the treatment conditions.

After chase, discs were rinsed over 0.5 h with two changes (1 ml/disc) of a phosphate-buffered saline solution (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4,$7H_2O$/1.5 mM-KH_2PO_4) at 4 °C, including proteinase inhibitors (0.1 M-6-aminohexanoic acid, 10 mM-Na_2·EDTA, 10 mM-benzamidine hydrochloride, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl chloride, 1 μM-pepstatin and 1 μM-leupeptin) [27] at pH 7.4 and stored at −20 °C.

Extraction and isolation of proteoglycans from cartilage discs

Groups of four to six discs (~ 48–72 mg wet wt. of cartilage) from each pulse–chase condition were then frozen in Tissue-Tek embedding solution within 10 mm × 10 mm × 5 mm specimen moulds and cryostat-sectioned to 20 μm. Cartilage slices were then extracted for 36–42 h at 4 °C by addition of 4 M-guanidinium chloride, 50 mM-sodium acetate, 10 mM-Hepes and proteinase inhibitors, pH 7.0. The tissue residue was treated with 0.5 ml of 0.5 M-NaOH for 1 day at 22 °C to solubilize essentially all remaining glycosaminoglycans, and then neutralized with an equal volume of 0.5 M-HCl. Control studies where slices were rinsed free of embedding solution before guanidinium chloride extraction indicated that the small volume (~ 0.2 ml) of embedding solution had no effect on subsequent proteoglycan analysis procedures.

Cartilage extracts were filtered over glass wool, adjusted to a density of 1.45 g/ml with solid CsCl, centrifuged at 38000 rev./min in a Beckman 50Ti rotor ($r_{av.}$ = 8.02 cm) for 72 h at 12 °C, and sliced into four equal gradient fractions (D1, 1.56 g/ml; D2, 1.48 g/ml; D3, 1.43 g/ml; D4, 1.38 g/ml). The terms D1, D2, D3, and D4 refer to the fractions of highest to lowest buoyant density obtained by subjecting cartilage extracts to CsCl-density-gradient centrifugation under dissociative conditions and are based on the terminology of Heinegård & Sommarin [27]. D1 fractions were dialysed exhaustively against 0.15 M-sodium acetate, pH 6.8 at 4 °C, and then stored at −20 °C.

Analytical procedures

Samples were assayed for proteoglycan as sulphated glycosaminoglycan by reaction with dimethyl-Methylene Blue [28]. The concentration of hyaluronate in Healon was taken as given by Pharmacia (10 mg/ml). Radioactivity was determined by liquid-scintillation counting after addition of 2 ml of ScintiVerse BioHP; samples with guanidinium chloride were prepared to a volume of 0.5 ml by addition of 2 M-guanidinium chloride/0.5 M-sodium acetate, pH 6.8, whereas samples with Triton X-100 (~ 0.5 ml) were prepared by addition of 1 ml of 10% (w/v) sodium dodecyl sulphate. DNA was determined by reaction with the dye Hoechst 33258 [29].

Proteoglycan aggregation experiments with hyaluronate and link protein

Portions of D1 samples (containing 250 μg of proteoglycan and ~ 75000 c.p.m. of radioactivity in ~ 0.6 ml) were fractionated on Sephacryl S-1000 columns (1 cm × 45 cm), eluted at 9 ml/h (11.5 cm/h) in 0.5 M-sodium acetate/0.1% (v/v) Triton

X-100/0.02% NaN_3, pH 6.8 (essentially the buffer described in [16]); fractions (0.6 ml) were collected and assayed for both proteoglycan and radioactivity. To determine the percentage of tissue proteoglycans and newly synthesized [^{35}S]proteoglycans with a high affinity for hyaluronate (see below for definition), samples were mixed with 1.6% (w/w) Healon, and allowed to aggregate for 2 h at 22 °C before chromatography as described above. To determine maximum aggregatability, samples were mixed with 5% (w/w) Healon and 5% (w/w) link protein, adjusted to 4 M-guanidinium chloride, allowed to dissociate for 2 h at 4 °C, dialysed for 24 h against 0.15 M-sodium acetate, pH 6.8 at 4 °C, and analysed by chromatography as described above. The percentage of proteoglycans in aggregate was computed by considering all the proteoglycans eluted earlier than the local minimum at elution volume (V_e) ~ 16 ml as aggregate, and all eluted between this minimum point and that near V_e ~ 28 ml as monomer. Recovery of proteoglycan and ^{35}S on chromatography was typically 90–100%. In some samples, 4 μg of DNA and 0.04 μCi of ^3H_2O were added as markers for the void (V_0) and total column (V_t) volume respectively.

Definition of high-affinity and low-affinity proteoglycans

Throughout the present paper we define proteoglycan monomers (total tissue or radiolabelled) which form aggregates with 1.6% Healon on Sephacryl S-1000 as high-affinity and those which fail to form aggregates as low-affinity (see [15,16]). Thus the definition of low-affinity and high-affinity monomer given in references [15,16] has been adopted here, with the exceptions that 1.6% (w/w) Healon was used and chromatography was on Sephacryl S-1000.

Analysis of medium

Chase and wash solutions were analysed for ^{35}S-labelled macromolecules by adjusting samples to 2 M-guanidinium chloride/0.5 M-sodium acetate/1 mM-Na_2SO_4, pH 6.8, and fractionating 0.5 ml portions on a PD-10 column of Sephadex G-25 in the same buffer.

RESULTS

Radiolabelling and isolation of proteoglycans

Newly synthesized proteoglycans were pulse-radiolabelled with [^{35}S]sulphate and isolated after a 0–48 h chase. Virtually all of the [^{35}S]proteoglycans remained in the tissue during pulse and subsequent chase protocols; during 1.5 h radiolabelling and 24 h chase, < 1% and < 2% respectively of the ^{35}S-labelled macromolecules were released into the medium. In addition, a high degree of proteoglycan extraction from the cartilage explants was achieved by cryostat-sectioning discs to 20 μm before 4 M-guanidinium chloride extraction; under all compression and pH conditions this procedure yielded a > 93% extraction of both proteoglycans and ^{35}S, consistent with yields from adult human articular cartilage [30].

From these extracts, proteoglycans were purified by CsCl equilibrium-density-gradient centrifugation. Under all compression and pH conditions, the distribution of proteoglycan and ^{35}S in the gradient fractions was similar, with > 90% of the proteoglycan and > 93% of the ^{35}S in the D1 fraction. Consistently with the low rate of ^{35}S loss during chase, the specific radioactivity of D1 proteoglycans (^{35}S c.p.m./μg of proteoglycan) was not significantly altered after 0–48 h of chase (typical S.D. ~ 15% within an experiment). Thus the D1 proteoglycans studied consistently represented a very high percentage of the total monomers, both tissue and radiolabelled, in the system.

When D1 samples (without addition of hyaluronate) were analysed on Sephacryl S-1000 (Fig. 1), more than 92% of both

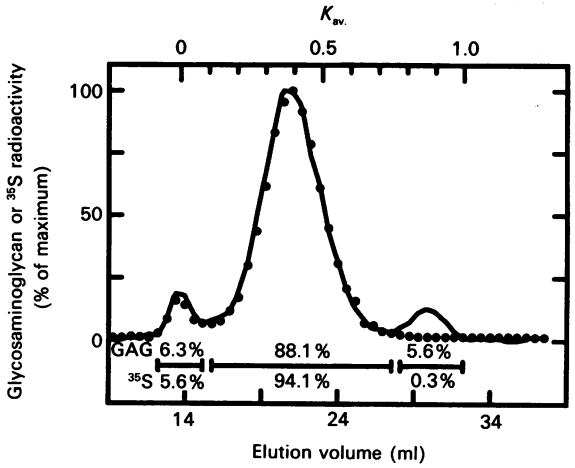

Fig. 1. Size distribution on Sephacryl S-1000 of a D1 proteoglycan sample from cartilage discs labelled with [^{35}S]sulphate

Free-swelling discs were radiolabelled and then held at a swollen thickness of 1.25 mm during 3 h chase in pH 7.45 medium. Proteoglycans were extracted, D1-purified and fractionated on a Sephacryl S-1000 column internally calibrated with calf thymus DNA and ^3H_2O using an elution buffer consisting of 0.5 M-sodium acetate, 0.1% Triton X-100, 0.02% NaN_3, pH 6.8 (see the Experimental section for details); fractions were assayed for glycosaminoglycan (——) and ^{35}S radioactivity (●). The percentage of total glycosaminoglycan (GAG) and ^{35}S radioactivity (^{35}S) are identified above and below (respectively) the indicated regions.

the tissue and [^{35}S]proteoglycans were eluted in the free-monomer position, confirming their isolation from endogenous hyaluronate in these samples. Since these proteoglycan monomers were more included on Sephacryl S-1000 (peak $K_{av.}$ ~ 0.38) than on Sepharose CL-2B (peak $K_{av.}$ ~ 0.28; results not shown), fractionation on Sephacryl S-1000 allowed a more clear resolution of proteoglycan aggregates from monomers; thus all subsequent chromatography was done on Sephacryl S-1000.

The small peak ($K_{av.}$ ~ 0.85) of dimethyl-Methylene Blue-reactive material, which was eluted just ahead of the total column volume, has not been identified, but may be free chondroitin or dermatan sulphate chains, since a component similarly eluted from bovine cartilage D1 preparations has also been observed by uronic acid assay after fractionation on Sepharose CL-2B [20].

Hyaluronate binding properties of proteoglycan monomers

The affinity of monomers for hyaluronate (see definition in the Experimental section) was determined by chromatography on Sephacryl S-1000 after aggregation with 1.6% hyaluronate. A typical set of S-1000 profiles for control discs (pH 7.45, 1.25 mm static compression) is shown in Fig. 2. It is clear that the newly synthesized proteoglycans in the pulse sample contained very little high-affinity monomer, but that there was a conversion from the low-affinity form into the high-affinity form with time. On the other hand, the percentage of total tissue proteoglycan in the high-affinity form (49–59%) was not markedly altered by the chase period.

Since the affinity of total tissue monomers for hyaluronate was essentially the same at all chase times, the conversion of low-affinity ^{35}S-labelled monomers into the high-affinity form could be quantified (see [20]) as the ratio of the percentage of high-affinity ^{35}S-labelled monomers to the percentage of high-affinity tissue monomers. A plot of this ratio against chase period for the control samples (data from Fig. 2) was then used to calculate a half life ($t_{\frac{1}{2}}$) of the conversion process (Fig. 3); $t_{\frac{1}{2}}$ is defined as the time take to bring about a change in ratio which was 50% of the

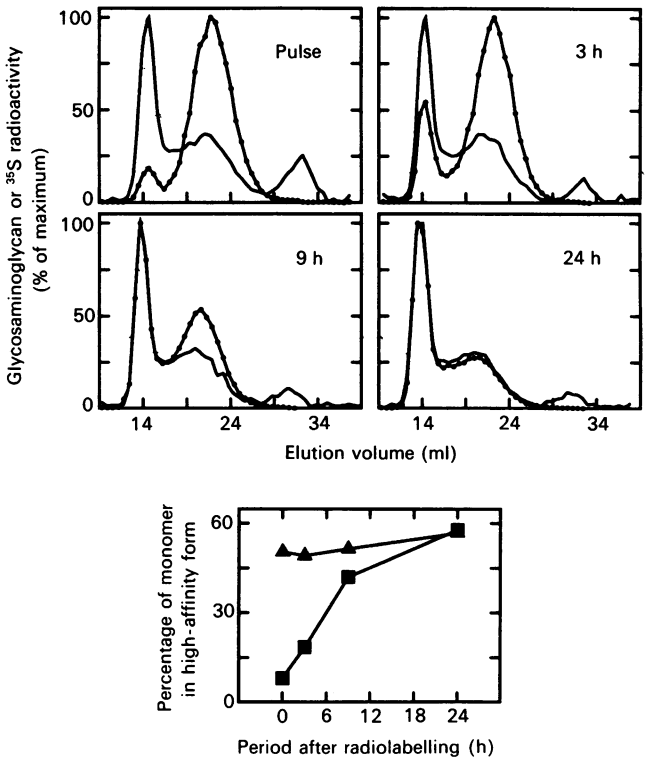

Fig. 2. Effect of chase period on the hyaluronate-binding properties of proteoglycan monomers from cartilage discs after radiolabelling with [^{35}S]sulphate

Portions of proteoglycan monomer samples were allowed to react with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000; fractions were assayed for glycosaminoglycan (——) and ^{35}S radioactivity (●). The elution profiles were from cartilage discs pulse-radiolabelled for 1.5 h and then held at a swollen thickness of 1.25 mm during a chase period of 3 h, 9 h or 24 h, all in pH 7.45 medium. The percentage of (■) radiolabelled monomers and (▲) total issue monomers with a high affinity for hyaluronate (see the Experimental section for definition) after different chase periods are shown in the bottom panel.

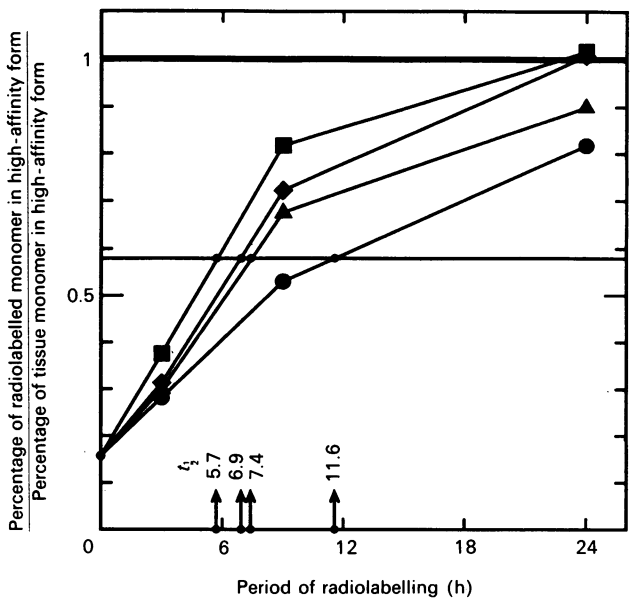

Fig. 3. Effect of static compression on the hyaluronate binding properties of radiolabelled monomers from cartilage discs during various chase periods

Cartilage discs were radiolabelled for 1.5 h and then compressed to a thickness of 1.25 (■), 1.00 (◆), 0.75 (▲) or 0.50 mm (●) during a 0–24 h chase period, all in medium at pH 7.45. Portions of D1 monomer samples were allowed to react with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000, and the percentage of radiolabelled monomers and tissue monomers with a high affinity for hyaluronate were determined (see the Experimental section for details). The ratio of these percentages is plotted, and the time ($t_{\frac{1}{2}}$) for this ratio to reach 0.58 under each compression condition was computed by linear extrapolation.

difference between unity and the ratio in the pulse sample (0.16). Thus, in the control condition shown (Fig. 3, 1.25 mm at pH 7.45), the time taken for the ratio to reach $(0.16+1.00)/2 = 0.58$, was 5.7 h by linear extrapolation.

Effect of increasing degrees of static compression on conversion

Static compression during chase markedly slowed the conversion of the newly synthesized monomers into the high-affinity form (Fig. 3). With increasing compression to 1.00, 0.75 and 0.50 mm, the conversion $t_{\frac{1}{2}}$ was lengthened to 6.9, 7.4 and 11.6 h respectively. Thus this delay in conversion was also dependent on the degree of compression in a dose-dependent fashion, such that a measurable delay was observed even at a minimal compression to 1.00 mm. In addition, virtually identical trends were obtained whether chromatography was done with 0.8% or 1.6% (w/w) hyaluronate and whether samples were run on Sephacryl S-1000 or Sepharose CL-2B (results not shown). Further, since even the maximally compressed samples were fully converted into the high-affinity form by 48 h of chase (results not shown), compression delayed, but did not prevent, conversion.

Effect of altered medium pH on conversion

Since tissue compression would lead to an increase in intra-tissue proteoglycan concentration and thereby a lowering of intra-tissue pH [24], the effect of altered medium pH on monomer affinity conversion was next determined while holding cartilage thickness constant at 1.25 mm (Fig. 4). Medium at acidic pH (6.99 and 7.24, S.D. ~ 0.02, $n = 3$) markedly delayed the conversion relative to medium at normal pH (7.45). Interestingly, medium at basic pH (7.70) accelerated the conversion process. With decreasing medium pH of 7.70, 7.45, 7.24 and 6.99, the conversion $t_{\frac{1}{2}}$ increased to 3.3, 5.7, 8.3 and 10.6 h respectively. Thus the effect on conversion of medium acidification from pH 7.45 to 7.24 and 6.99 (cartilage discs at 1.25 mm) was very similar to the effect of static compression from 1.25 to 0.75 and 0.50 mm respectively. These comparisons were meaningful, since in the two different experiments of Figs. 3 and 4 the conversion half-lives for control discs (1.25 mm at pH 7.45) were essentially identical (5.7 h).

Oscillatory compression

Oscillatory compressions of 2% strain were superimposed on a static offset compression to 1.00 mm during 9 h chase. The ratio of percentage of high-affinity radiolabelled monomers to high-affinity tissue monomers at frequencies of 0 (control), 0.001, 0.01 and 0.1 cycle/s were 0.76 ± 0.04, 0.80 ± 0.02, 0.71 ± 0.02, and 0.80 ± 0.02 (mean ± range for replicate samples) respectively. Thus none of these compression protocols markedly altered the conversion of ^{35}S-labelled monomers compared with the 1.00 mm static control condition where no oscillation was superimposed. Moreover, the degree of affinity conversion at 9 h in the 1.00 mm static controls within this dynamic-compression apparatus as measured by this ratio (0.76) compared well with the conversion in samples treated similarly within the static-compression apparatus (0.72, Fig. 3).

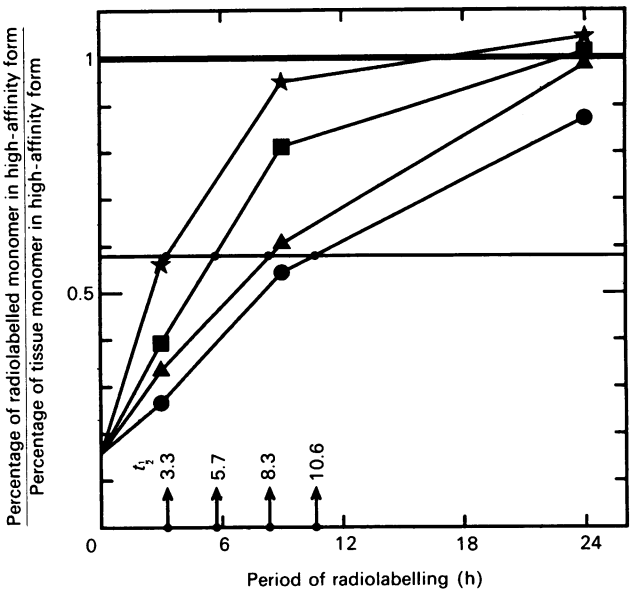

Fig. 4. Effect of medium pH on the hyaluronate-binding properties of radiolabelled momomers from cartilage discs during various chase periods

Cartilage discs were radiolabelled for 1.5 h in medium at pH 7.45 and then bathed in HCl- or NaOH-titrated medium at pH 7.70 (★), 7.45 (■), 6.24 (▲)or 6.99 (●) while held at a compressed thickness of 1.25 mm during a 0–24 h chase period. Portions of D1 monomer samples were allowed to react with 1.6% (w/w) hyaluronate and fractionated on Sephacryl S-1000, and the percentage of radiolabelled monomers and tissue monomers with a high affinity for hyaluronate were determined (see the Experimental section for details). The ratio of these percentages is plotted, and the time ($t_{\frac{1}{2}}$) for this ratio to reach 0.58 under each pH condition was computed by linear extrapolation.

Aggregatability of proteoglycan monomers with excess hyaluronate and link protein

The maximum capacity of D1 proteoglycans to form aggregates was assessed by mixing portions with excess (5%, w/w) hyaluronate and (5%, w/w) link protein before chromatography. The high aggregatability of both tissue and [^{35}S]proteoglycans (range 77–86% in eight samples) was not markedly altered, even after the most extreme chase conditions (medium pH of 6.99 or 7.70 or compression from 1.25 mm to 0.5 mm) and chase durations (either short 3 h chase or long 24 h chase). Further, the ratios of aggregatable ^{35}S-labelled monomers to aggregatable tissue monomers were similar and ranged from 0.95 to 1.00.

DISCUSSION

The results described here clearly demonstrate that the hyaluronate-binding properties of newly synthesized proteoglycans within the extracellular matrix of immature cartilage explants can be altered by mechanical and chemical stimuli. Static compression and acidic medium pH were both shown to slow the normal acquisition of high-binding affinity for hyaluronate by newly secreted proteoglycans in explants. Since the proteoglycans extracted and purified in the D1 fraction represented a consistently high proportion of the radiolabelled and total tissue proteoglycan population, differences in ^{35}S-labelled monomer properties after different chase conditions were attributable to effects on the majority of newly synthesized proteoglycans, and not to a selective extraction or a loss into the medium of [^{35}S]proteoglycans. Further, decreases in the affinity of ^{35}S-labelled monomers for hyaluronate after either tissue compression or low-pH incubation were not due to degradation of hyaluronate-binding sites, since neither compression nor low pH during chase affected the maximum capacity of tissue or ^{35}S-labelled monomers to form aggregates with excess hyaluronate and link protein.

The mechanism by which compression leads to a delay in conversion may be physicochemical. Since cartilage has a highly negatively charged matrix (due to the ionized acidic carboxy and sulphate moieties on proteoglycans), the intra-tissue concentration of cations (e.g. H^+) is increased (and that of anions is decreased) compared with concentrations in the medium [1,2]. Compression of cartilage would increase the density of tissue charge and thereby further decrease intra-tissue pH. The charge density of our calf articular-cartilage discs is ~0.2–0.3 M, as assessed by chemical titration [31], glycosaminoglycan analysis [21] or n.m.r. measurements of interstitial ion concentrations [32]. Thus a similar acidic intra-tissue pH of pH ~6.7 in our cartilage discs would be achieved by either (1) compressing discs to 0.50 mm in normal pH ~7.45 medium, or (2) holding discs at 1.25 mm in pH ~7.0 medium [24]. The similar delay in affinity conversion under these comparable intra-tissue pH conditions and the dose-dependence of delay at intermediate levels of compression or medium acidification (Figs. 3 and 4) are therefore consistent with the hypothesis that static compression delays the extracellular processing of newly synthesized proteoglycans by decreasing the intra-tissue pH.

As part of the pH study, we found that a higher medium pH of 7.70 markedly accelerated the conversion of [^{35}S]proteoglycans. This is consistent with the previous finding that the conversion process in solutions markedly increases with pH from 7.4 to 9.2 [16,17]. Further, if the alkali effect [16,17] is the result of catalysing disulphide-bond formation or rearrangement in the G1 domain of proteoglycan monomer, then the effects of cartilage compression and altered medium pH described here probably also occur by influencing the structure of the G1 domain. Such a direct effect on proteoglycan structure would likely be independent of chondrocyte activity, and thus would be in accord with affinity conversion occurring within the extracellular matrix in the absence of live cells [18,19].

The effects of static compression on the conversion of newly synthesized proteoglycans from low-affinity form to high-affinity form is likely to be of physiological relevance, since the magnitude of compression to elicit a delay was ~20% (from 1.25 mm to 1.00 mm), which is in the physiological range [33]. It is interesting that oscillatory compression protocols of 2% strain at frequencies of 0.001–0.1 cycle/s did not affect the extracellular conversion of [^{35}S]proteoglycans, but were previously observed to stimulate synthesis of proteoglycan [21]. The null effect of oscillatory compression on [^{35}S]proteoglycan conversion is not surprising, since during oscillation cycles the peak amplitude of dynamic compression (0.02 mm) would result in a negligibly small pH change compared with the pH change with the static-offset compression (1.25 mm − 1.00 mm = 0.25 mm). The stimulatory effects of oscillatory compression on proteoglycan synthesis are consistent with the hypothesis that dynamic physical stimuli other than changes in intra-tissue pH (such as hydrostatic pressure, fluid flow, cell deformation or streaming potentials) can alter cell-dependent processes in cartilage [21]. Thus static and dynamic compression may exert differential effects on proteoglycan synthesis and extracellular proteoglycan processing through distinct physical mechanisms.

Mechanical forces *in vivo* are thought to influence the structural adaptation of cartilage, whereas abnormal forces predispose cartilage to degeneration and osteoarthritis [21]. The physico-

chemical modulation of affinity conversion described here could have an important physiological role in the formation and remodelling of a mechanically functional extracellular matrix. Newly synthesized proteoglycans with a low affinity for hyaluronate may be able to diffuse through proteoglycan-rich regions (of relatively low intra-tissue pH) and then become stabilized into aggregates with hyaluronate in proteoglycan-poor regions (of relatively high intra-tissue pH), where they are functionally required. Also, during static compression, low-affinity proteoglycans may have a longer time to diffuse through the compacted and dense matrix meshwork before being converted into the high-affinity form. It remains to be investigated whether the low-affinity proteoglycans are actually more mobile *in situ* within the extracellular matrix than are the high-affinity proteoglycans.

We thank Holly M. Misto, Carl R. Flannery, and J.-Y. Howard Doong for technical assistance, Dr. Christopher J. Handley for suggesting the use of Sephacryl S-1000, and Dr. Martha L. Gray and Dr. James H. Kimura for critical discussions. This project was supported by National Institutes of Health Grant AR33236.

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Received 16 November 1989/5 February 1990; accepted 15 February 1990