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The Mouse Patella Assay

An Easy Method of Quantitating Articular Cartilage Chondrocyte Function in vivo and in vitro

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Summary. An easy method of quantitating articular cartilage chondrocyte function in mice is described, using a defined and anatomically intact cartilage structure, the patella. To avoid damage to the cartilage before incubation, ^{35}S incorporation studies were performed leaving the patellae surrounded by a minimal area of non-cartilaginous connective tissue. The patellae were then punched out so that the ^{35}S activity incorporated into the cartilage could be measured. Incorporation of ^{35}S was almost completely blocked by 0.1 mM cycloheximide. Patellar cartilage from left and right knee joints of individual mice shows identical incorporation of ^{35}S, as indicated by a right/left ratio of 1.01 in vitro and 0.99 in vivo, respectively. ^{35}S incorporation values of patellae of mice of different ages do show considerable variation, but within properly age-matched groups of mice the incorporation values are comparable. A known suppressive effect of acute joint inflammation on proteoglycan synthesis was reliably quantitated in the zymosan-induced arthritis model, as indicated by 39% in vitro and 35% in vivo inhibition of ^{35}S incorporation. The patella assay can be used for quantitative measurement of chondrocyte metabolism in normal and pharmacologically altered cartilage.

Key words: Articular cartilage – Chondrocyte function – Proteoglycan synthesis – Arthritis

Introduction

Chondrocyte proteoglycan synthesis has been studied in articular cartilage of humans and experimental animals using various radioisotopes, in particular ^{35}S. Most of these studies were performed in vitro and used damaged cartilage structures such as slices or explants [1–4]. For comparison of chondrocyte function in various cartilage samples, incorporation of radioisotopes has to be expressed in relation to some tissue parameter, e.g. weight, DNA, or uronic acid content of the cartilage. Moreover, care has to be taken to control the depth of the cartilage samples and to define the localization of the cartilage in the joint. Proteoglycan synthesis is reported to vary with the distance from the articular surface [4] and may also differ considerably in various locations in the joint, e.g. weight-bearing areas versus non-weight-bearing areas [5].

Alterations in chondrocyte function have attracted attention, not only in degenerative joint disease [6–8], but also in inflammatory arthritis [9–13]. In addition, studies are carried out to investigate the modulatory effect of drugs on normal and augmented articular cartilage proteoglycan synthesis [14–17].

This paper describes a mouse cartilage assay that uses a defined structure, in this case the whole patella, and therefore the additional determination of a tissue parameter is not required. The method is fast, easy to reproduce and makes use of an anatomically intact cartilage structure. Suppression of chondrocyte proteoglycan synthesis as observed during acute joint inflammation [13], can be reliably quantitated. Moreover, the development of an articular cartilage assay in small animals such as mice enables not only in vitro but also in vivo incorporation studies with small amounts of radioisotopes, and makes it possible to study the influence of in vivo administered drugs on articular cartilage chondrocyte function in groups of inbred animals.

Materials and Methods

Animals. Male C57Bl mice, in different age groups ranging from 6 to 22 weeks, were used.

In vitro ^{35}S Incorporation in Patellar Cartilage. Groups of five mice of various ages were killed by cervical dislocation. The patellae were removed from the knee joints, leaving the cartilage surrounded by a minimal area of non-cartilaginous connective tissue, and placed in 5 ml incubation medium, consisting of RPMI-Hepes and RPMI-HCO_3 (1:1) (Flow Laboratories, Irvine, Scotland) with added penicillin (100 U/ml) and streptomycin (100 µg/ml). After preincubation at 37 °C in a 5% CO_2 atmosphere for 45 min, the patellae were transferred to Flow plastic incubation vials (2 ml) containing 0.5 ml RPMI and 5 µCi ^{35}S-sulphate (Radiochemical Centre, Amersham, England) and incubation was continued for 3 h, unless stated otherwise. At the end of the incubation period the patellae were washed three times with 10 ml saline, fixed overnight in 0.5% cetylperidiniumchloride in 4% phosphate-buffered formalin, and decalcified in 5% formic acid for 1 day to enable proper dissection of the patella. After

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decalcification the whole patellae could easily be punched out from the surrounding connective tissue with an agar gel punch (diam. 1 mm). After solubilization in 1 ml Lumasolve containing quaternary ammonium hydroxide (Lumac, Basle, Switzerland), the ^{35}S radioactivity of the patellar cartilage was counted in a liquid scintillation spectrometer.

In two experiments normal mouse serum (20%) and/or L-glutamine (1 mM) was added to the incubation medium. The effect of a protein synthesis inhibitor on ^{35}S incorporation was tested by addition of cycloheximide [18] to a concentration of 0.1 mM, both during preincubation and during incubation with ^{35}S.

Autoradiography. To show the localization of ^{35}S incorporation three punched-out patellae were embedded in fresh liver tissue and fixed overnight in 4% phosphate-buffered formalin. The tissues were processed and embedded in paraffin wax and autoradiography was performed on 6-μ sections as previously described for whole joint sections [13].

In vivo ^{35}S Incorporation. Mice were injected intravenously into the tail vein with 2 μCi ^{35}S per gram body weight and killed at various time intervals. The patellae were removed, washed and processed for total ^{35}S radioactivity counting as described above for the in vitro ^{35}S incorporation. Blood samples were obtained from the tail (10 μl) at short intervals after IV injection and lysed in 1 ml water. Total ^{35}S activity was determined by liquid scintillation counting.

Effect of Joint Inflammation on ^{35}S Incorporation. Zymosan (Koch-Light Lab., Colnbrook, Bucks, England) was sterilized by autoclaving and 300 μg in 10 μl saline was injected into the right knee joints of 10 normal C57Bl mice. Previous observations have shown that zymosan induces pronounced joint inflammation 24 h after injection [13, 19]. Left knee joints were injected with 10 μl saline as control. The animals were killed 24 h later. Five mice were injected IV with 2 μCi ^{35}S per gram body weight 2 h before being killed so that ^{35}S incorporation in vivo could be studied, while in another group of five mice ^{35}S incorporation was studied in vitro using isolated patellae. The ^{35}S content of the patellae was determined as described above. The decrease in ^{35}S content of the cartilage from the zymosan-injected knee joint was expressed as a percentage of the ^{35}S content of the contralateral saline-injected control joint of the same animal.

Results

In vitro ^{35}S Incorporation in Patellar Cartilage

Figure 1 shows an autoradiograph of a histological section of a punched-out specimen. The ^{35}S labelling is clearly present in the cartilage and absent in the non-cartilaginous part of the patella. To establish that the ^{35}S labelling of the patellar cartilage reflects incorporation rather than diffuse labelling, an incubation was performed in the presence of the protein synthesis inhibitor cycloheximide. Table 1 shows that the ^{35}S content of the patellae was less than 10% of the normal values in the presence of cycloheximide.

^{35}S incorporation values in cpm of paired patellae of individual normal mice are presented in Table 2. No difference was found between the ^{35}S content of left and right patellae, as indicated by a mean of individual right (R)/left (L) ratios of 1.01 (SD = 0.13). When a mean R/L ratio was calculated from mean values in cpm of left and right patellae instead of from individual R/L ratios a slightly higher standard deviation was found (SD = 0.22).

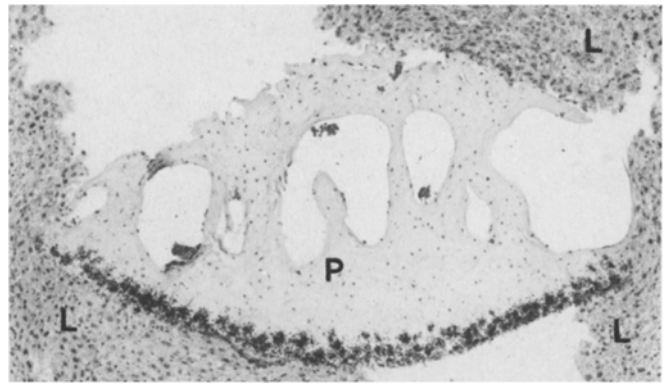

Fig. 1. Autoradiograph of a section of a punched-out patella (*P*), embedded in liver tissue (*L*). The patella was incubated for 3 h in vitro with 10 μCi ^{35}S per ml. Note the distinct labelling of the cartilage and the absence of labelling in the non-cartilaginous part of the patella (H.E., 80×)

Table 1. Effect of cycloheximide on in vitro ^{35}S incorporation in normal patellar cartilage

Mouse	^{35}S content [a]	
	Control [b]	Cycloheximide [c]
1	747	69
2	759	62
3	574	69

[a] Cpm in patellar cartilage after 3 h incubation with 10 μCi/ml ^{35}S

[b] Left patellae were (pre-) incubated in normal medium

[c] Right patellae were incubated in the presence of 0.1 mM cycloheximide both during 30 min preincubation and 3 h ^{35}S incorporation

Table 2. In vitro ^{35}S incorporation in patellar cartilage of left and right knee joints of normal mice

Mouse	^{35}S content [a]		Right/left ratio
	Left patella	Right patella	
1	742	881	1.18
2	708	628	0.89
3	651	695	1.07
4	707	717	1.01
5	971	870	0.90
Mean ± SD	755 ± 125	758 ± 112	1.01 [b] ± 0.13 1.00 [c] ± 0.22

[a] Cpm in patellar cartilage after 3 h incubation with 10 μCi/ml ^{35}S

[b] Mean R/L ratio calculated from individual R/L ratios

[c] Mean R/L ratio calculated from mean values in cpm of right and left patellae

Table 3. In vitro ^{35}S incorporation values in patellar cartilage from normal mice of different age

Age (weeks)	^{35}S content[a]
6 – 8	1 425[b] ± 142
8 – 10	1 266 ± 128
12 – 14	916 ± 138
20 – 22	438 ± 59

[a] Cpm in patellar cartilage after 3 h incubation with 10 µCi/ml ^{35}S

[b] Each value represents the mean ± SD calculated from groups of five mice

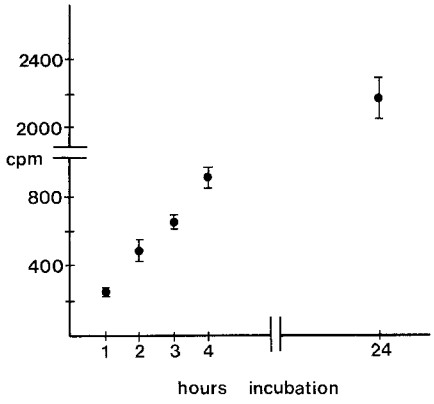

Fig. 2. ^{35}S content of normal patellae (in cpm) after various periods of incubation with 10 µCi ^{35}S per ml. Values represent the mean ± SD calculated from groups of 5 mice

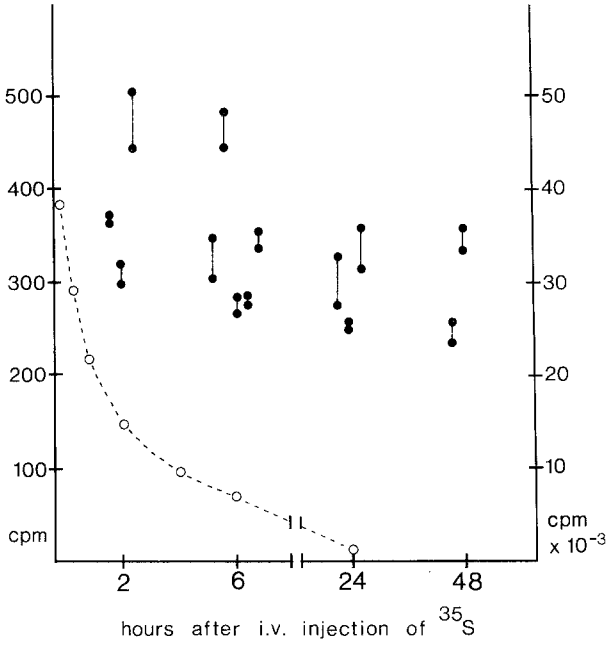

Fig. 3. ^{35}S content of patellar cartilage (*left axis*) and 10 µl blood (*right axis*) at various hours after IV administration of 2 µCi ^{35}S per gram body weight. Groups of mice aged 6–10 weeks were killed after 2, 6, 24 and 48 h. •—• represent ^{35}S values of the left and the right patella of individual mice. ○ - - - ○ represents mean values of the ^{35}S blood content of 3 mice

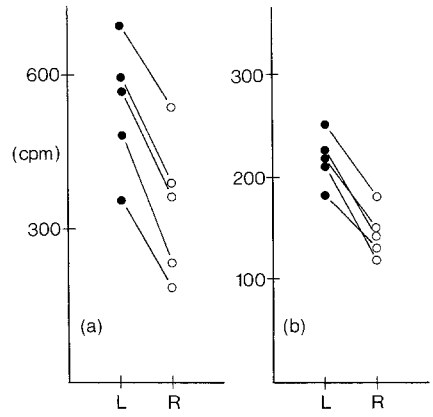

Fig. 4. Effect of arthritis on in vitro (**a**) and in vivo (**b**) ^{35}S incorporation in patellar cartilage. Values represent the ^{35}S content (cpm) of patellar cartilage from saline-injected (•) and zymosan-injected (○) knee joints of individual mice

Table 3 shows the influence of age on the absolute ^{35}S incorporation values of patellar cartilage of normal mice. The ^{35}S content decreases clearly with increasing age of the animals, which shows the need to work with groups of mice of the same age when mean values of groups of mice have to be compared instead of individual R/L ratios. On the other hand, variation in ^{35}S incorporation is small within the age groups, as indicated by the low standard deviations.

Figure 2 shows ^{35}S incorporation values after various periods of incubation. The incorporation proceeds linearly for at least 4 h. Addition of normal mouse serum (up to 20%) to the incubation medium did not alter the incorporation values measured after 3-h incubation. On the other hand, ^{35}S incorporation values were increased by 30% when incubations were performed in the presence of 1 mM L-glutamine, an essential amino acid known to stimulate various synthetic processes.

In vivo ^{35}S Incorporation in Patellar Cartilage

To measure in vivo incorporation ^{35}S was administered IV. Figure 3 shows ^{35}S values of blood samples and ^{35}S values of left and right patellae of normal mice at various times after IV injection. As in the in vitro experiments, no difference was found between the ^{35}S content in the left and right patellae. The mean R/L ratio calculated from individual R/L ratios 6 h after IV injection was 0.99 (SD = 0.14, $n = 5$). The ^{35}S content did not increase between 2 and 6 h after IV injection.

Effect of Joint Inflammation on ^{35}S Incorporation

One day after arthritis induction with zymosan in the right knee ^{35}S incorporation was measured both in vitro and in vivo. Figure 4 shows ^{35}S values of the arthritic cartilage as compared with the paired control cartilage of the same

animal. In all mice ^{35}S incorporation was decreased in the arthritic cartilage. Mean inhibition calculated from inhibition values of individual mice was 39% (SD = 11%) in vitro and 35% (SD = 8%) in vivo, respectively.

Discussion

The present investigation shows that the patella of the mouse is a suitable anatomical entity for easy quantitative measurement of chondrocyte function. The mouse patella assay is fast, enables both in vitro and in vivo studies, and makes use of a defined and anatomically intact cartilage structure.

Chondrocyte proteoglycan synthesis has been widely studied in vitro using either cartilage slices or explants [1–4]. When slices were incubated in culture medium, the rate of proteoglycan synthesis as measured by incorporation of ^{35}S increased markedly with incubation time, probably in reaction to proteoglycan depletion from damaged cartilage under these conditions [20, 21]. In the present study care was taken to avoid damage to the cartilage before incubation. In dissecting the patellae of the mouse, only the surrounding non-cartilaginous connective tissue was damaged leaving the patellar cartilage intact. Upon incubation high ^{35}S incorporation did occur in the damaged connective tissue adjacent to the cut surface (data not shown), but at the end of incubation the patella was punched out from this tissue and only ^{35}S incorporation in intact patellar cartilage was measured.

One advantage of the patella assay is that it is easy and fast. Determination of tissue weight, DNA or uronic acid content is unnecessary, because a defined structure is used, in this case the whole patella. Measurements in the patellae of non-inflamed mouse knee joints revealed no difference in ^{35}S incorporation between left and right patellae (see values in Table 2 and Fig. 3). The standard deviation was only slightly higher when a mean R/L ratio was calculated from mean values of left and right patellae of groups of mice instead of from R/L ratios of individual mice. This higher variation can be caused by interindividual differences, e.g. age of the animals (Table 3) or variation in the endogenous sulphate pools [22].

Changes in chondrocyte function can be reliably quantitated. Joint inflammation is known to have a suppressive effect on chondrocyte proteoglycan synthesis, both from observations in the rabbit [11, 12] and from our autoradiographic studies in arthritic mice [13]. Figure 4 shows that induction of joint inflammation by IA injection of zymosan induces decreased ^{35}S incorporation in patellar cartilage of all mice studied.

Absolute values of in vitro ^{35}S incorporation in patellar cartilage were not influenced by addition of serum to the incubation medium, but the addition of L-glutamine had an enhancing effect, suggesting the latter to be an essential amino acid for murine chondrocytes. Similar effects have been described for chondrocytes of other species. Maroudas [2] showed that addition of serum had no effect on short-term ^{35}S incorporation in human cartilage slices. Recently, Handley et al. [23] showed L-glutamine to be an essential amino acid for avian chondrocytes.

An interesting finding was that in vivo ^{35}S incorporation in patellar cartilage reached a plateau 2 h after IV administration of ^{35}S. Similar observations have been made in mouse costal cartilage [22] and it was suggested that this was caused by rapid disappearance of the circulating ^{35}S. Our experiments also revealed a rapid decrease in the ^{35}S content of the peripheral blood (Fig. 3), but after 2 h the amount of ^{35}S in the blood was still 30% of the value measured shortly (5 min) after IV injection, an amount too large to be ignored. A possible explanation for an early plateau may be that a large part of just-synthesized ^{35}S-labelled proteoglycan present in patellar cartilage at 2 h remains in situ for a long period, but that a small part is released from the cartilage shortly after synthesis. The amount synthesized between 2 and 6 h may then just about counterbalance this short-term loss, resulting in similar values of ^{35}S at 2 and 6 h.

In conclusion, the development of a cartilage assay in small animals such as mice enables comparable in vitro and in vivo studies on articular cartilage chondrocyte function. The influence of drugs on articular cartilage chondrocyte function has so far been studied mainly in vitro. Numerous cartilage samples have to be obtained from single individuals and drugs in variable concentrations have to be introduced into in vitro culture systems [14–17]. In vivo studies with drugs require groups of comparable animals. The development of a cartilage assay in mice makes it possible to work with inbred strains and with large groups of inexpensive animals, which are comparable when properly age-matched.

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