Evaluation of the effects of antiarthritic drugs on the secretion of proteoglycans by lapine chondrocytes using a novel assay procedure

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SUMMARY A new method is described for separating free $^{35}\text{SO}_4^{--}$ from $^{35}\text{SO}_4$ labelled proteoglycans synthesised by rabbit articular chondrocytes cultured in the presence of excess $^{35}\text{SO}_4^{--}$. The procedure uses the low solubility product of barium sulphate to remove, by precipitation, free $^{35}\text{SO}_4^{--}$ from culture medium. Optimum recovery of $^{35}\text{SO}_4$ labelled proteoglycans was achieved after papain digestion to release $^{35}\text{SO}_4$ -glycosaminoglycans, and addition of chondroitin sulphate before the precipitation step. Using this assay, we studied the effect of six drugs—indomethacin, diclofenac, sodium pentosan polysulphate, glycosaminoglycan polysulphate ester, tiaprofenic acid, and ketoprofen—on the secretion into the medium of labelled proteoglycans by lapine chondrocytes. The six drugs were tested at 0.1, 1, 10, 50, and 100 $\mu\text{g/ml}$ over four consecutive 48 hour culture periods. A consistent concentration-response pattern was found for the four non-steroidal anti-inflammatory drugs (NSAIDs) studied. Generally they inhibited proteoglycan secretion at 50 and 100 $\mu\text{g/ml}$ but had no effect at lower concentrations. Inhibition of secretion was strongest with indomethacin and diclofenac at 50 and 100 $\mu\text{g/ml}$. In contrast with the NSAIDs studied, the two sulphated polysaccharides (sodium pentosan polysulphate and glycosaminoglycan polysulphate ester) at low concentrations increased proteoglycan secretion by chondrocytes, with maximal stimulation occurring at 1 $\mu\text{g/ml}$. Sodium pentosan polysulphate, but not glycosaminoglycan polysulphate ester, showed inhibitory activity at 50 and 100 $\mu\text{g/ml}$.

Key words: non-steroidal anti-inflammatory drugs, sodium pentosan polysulphate, glycosaminoglycan polysulphate ester, diclofenac, indomethacin, ketoprofen, tiaprofenic acid.

Non-steroidal anti-inflammatory drugs (NSAIDs) still represent the mainstay of therapeutic management of osteoarthritis. It is now recognised, however, that such drugs, although effective at relieving the symptoms of the disease, may do little to improve the condition, and in some instances may contribute to its progression (see review by Burkhardt and Ghosh¹). In recent years new NSAIDs and other drugs have become commercially available which are reported to be free of deleterious effects on

cartilage metabolism, but it is often difficult to assess the validity of such claims because of the limitations of the methods used. Ideally the influence of a drug on cartilage cells should be monitored at different drug concentrations, over different time periods, and under carefully controlled conditions but in many cases these criteria are not fully observed. The most convenient means of assessing the effects of drugs on chondrocytes is by *in vitro* assays. Although this approach has its shortcomings it can provide a primary screening system, which can be followed up by animal experiments. Measurement of proteoglycan production by cartilage explants or chondrocyte monolayers generally involves pulsing the cultures with $^{35}\text{SO}_4^{--}$ to radiolabel the newly

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synthesised proteoglycans. This method, however, requires the subsequent separation of the resulting $^{35}\text{SO}_4$ labelled proteoglycans from the large amounts of unincorporated $^{35}\text{SO}_4^{--}$. This may be achieved by removal of the free $^{35}\text{SO}_4^{--}$ by gel filtration chromatography of each sample,² exhaustive dialysis of individual samples,³ or papain digestion and cetylpyridium chloride precipitation of the $^{35}\text{SO}_4$ -glycosaminoglycans.⁴ These methods are time consuming and tedious and, in the latter method in particular, the number of steps involved could result in significant cumulative experimental errors.

In the present study we describe a rapid, simple, and reproducible method for separating free $^{35}\text{SO}_4^{--}$ from $^{35}\text{SO}_4$ labelled proteoglycans in chondrocyte or cartilage cultures. The efficiency of this method allows for comprehensive comparative experiments of the effects of drugs on the production of proteoglycans by chondrocytes under a variety of conditions. We used this method to compare the effects of six currently available antiarthritic agents, each at five concentrations, on the total proteoglycan secretion of cultured rabbit articular chondrocytes, measured for four consecutive 48 hour culture intervals.

Materials and methods

ANIMALS

New Zealand white rabbits (6–8 weeks old) of either sex were obtained from the University of Sydney animal house, Castle Hill, NSW, Australia.

PREPARATION OF PRIMARY CHONDROCYTE CULTURES

Rabbits were killed by intracardiac injection of pentobarbitone (May and Baker Australia, Pty Ltd, West Footscray, Victoria, Australia). Both hind legs were removed, and, under aseptic conditions, the knee joints were opened and the articular cartilage was shaved off the femoral condyles, the tibial plateaux, and the patellae. The shavings were transferred to a 15 ml plastic centrifuge tube (Corning Laboratory Science Company, Stone, Staffordshire, UK) containing phosphate buffered saline (PBS). After washing with two 15 ml volumes of PBS the fragments were resuspended in 15 ml of 0.25% trypsin (1:250, Gibco Laboratories, Grand Island, New York, USA) in PBS and incubated at 37°C for one hour. The trypsin solution was aspirated and replaced with 15 ml of a solution of 0.2% collagenase (type 1A, Sigma Chemical Company, St Louis, MO, USA) in Ham's F12 medium (Flow Laboratories, McLean, Va, USA) containing 10% fetal calf serum (FCS) (Commonwealth Serum Laboratories, Parkville, Victoria, Australia),

80 U/ml gentamicin (David Bull Laboratories Pty Ltd, Rydalmere, NSW, Australia), 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; Sigma Chemical Company, St Louis, MO, USA), and incubation was continued at 37°C for three hours with occasional shaking. The digest was washed three times with 15 ml volumes of Ham's F12 medium plus 10% FCS (F12-FCS), the cells were counted with a haemocytometer, and 2×10^6 viable cells in 12 ml F12-FCS were plated into 75 cm² tissue culture flasks (Miles Laboratories, Naperville, IL, USA). A preparation from one rabbit typically yielded approximately 10×10^6 chondrocytes. The medium was changed after three days and subsequently every two to three days. Confluence was reached by the chondrocyte cultures in six to eight days.

PREPARATION OF TEST CULTURES

Six drugs were tested for their effects on proteoglycan secretion by articular chondrocyte cultures. They were glycosaminoglycan polysulphate ester (Luitpold Werk, Munich, FRG), sodium pentosan polysulphate (Benechemie, Munich, FRG), indomethacin (Merck Sharp and Dohme Australia Pty Ltd, Granville, NSW, Australia), diclofenac sodium (Ciba Geigy Australia Ltd, Pendle Hill, NSW, Australia), ketoprofen (May and Baker Australia Pty Ltd, West Footscray, Victoria, Australia), and tiaprofenic acid (Roussel Pharmaceuticals Pty Ltd, Castle Hill, NSW, Australia).

Medium was decanted from confluent chondrocyte cultures, cells were washed with two 5 ml volumes of PBS, and then 3 ml of 0.2% trypsin, 0.1% edetic acid (Ajax Chemical Company, Sydney, Australia) in PBS was added, and cultures were incubated at 37°C for two to three minutes until the cells detached. The cells were aspirated and washed in three 15 ml volumes of F12-FCS, then counted, and the concentration adjusted to 2×10^5 cells/ml. The cell suspension was dispensed into the wells (10^5 cells/well in 0.5 ml) of 24-well plates (Flow Laboratories, McLean, Va, USA). At this plating density the cells had attached to form confluent monolayers after an overnight incubation. The medium was changed and each well received 450 μl of F12-FCS containing 4.3×10^6 disintegrations per minute $^{35}\text{SO}_4^{--}$ (92.5 kBq/well) (New England Nuclear Corp, Bedford, MA, USA). The various drugs were dissolved and appropriately diluted in serum free Ham's F12 medium and delivered to the wells in 50 μl volumes, to give final volumes in the wells of 500 μl and the desired final drug concentrations of 0, 0.1, 1, 10, 50, and 100 $\mu\text{g/ml}$. Four replicate wells were used for each drug concentration. Sufficient F12-FCS plus $^{35}\text{SO}_4^{--}$ was prepared in a single batch

for the experiment to ensure that all wells were exposed to an equal concentration of $^{35}\text{SO}_4^{--}$ at each medium change. Medium was changed and fresh drug solutions were added after two, four, and six days of culture. At these medium changes and on day 8 when the experiment was terminated the exhausted medium was pipetted from each well into tubes and stored for assay.

$^{35}\text{SO}_4$ -PROTEOGLYCAN ASSAY

The level of secretion of labelled proteoglycans by the chondrocytes was measured by separating free (unincorporated) $^{35}\text{SO}_4^{--}$ from $^{35}\text{SO}_4$ labelled proteoglycans, as described below. The principle of the separation method is based on the insolubility of barium sulphate (BaSO_4). The chondrocyte culture supernatants (500 μl), which were collected into 75 $\times$ 10 mm plastic tubes (Edwards Medical Supplies, Narellan, NSW, Australia), were subjected to papain digestion to release glycosaminoglycans. The stock solution used was 2.5 mg/ml papain (crude powder) (Sigma Chemical Co, MO, USA), 7.9 mg/ml cysteine-HCl (Sigma Chemical Co, MO, USA) in PBS preactivated at 60°C for 30 minutes. Papain stock solution (125 μl) was added to each tube, which was capped and incubated at 60°C for two hours. After papain digestion 300 μl of solution containing equal volumes of 0.2 M Na_2SO_4 and 50 mg/ml chondroitin sulphate (crude shark and whale, Sigma Chemical Co, MO, USA) was added to each tube. Tubes were vortexed, 0.4 M BaCl_2 (150 μl) was then added, tubes were again vortexed and centrifuged for 10 minutes at 1500–2000 g. The precipitation step was then repeated on the supernatant. From each tube 500 μl of supernatant was pipetted and transferred to a clean tube, great care being taken not to disturb the precipitate. One hundred microlitres of a 2.5 solution of chondroitin sulphate (50 mg/ml) and BaCl_2 (0.4 M) was added, tubes were vortexed, 100 μl of 0.2 M Na_2SO_4 was added, tubes were vortexed again, and centrifuged as before. Aliquots (100 μl) of this supernatant were carefully removed by pipette (again great care being taken not to disturb the precipitate) and transferred to scintillation vials (Packard Instruments Co Inc, Downers Grove, IL, USA). The radioactivity in the samples was determined by routine β scintillation counting. As only relative proteoglycan synthesis under different culture treatments was of interest the results were expressed as disintegrations per minute of $^{35}\text{SO}_4$ labelled proteoglycan/ 10^5 cells.

PREPARATION OF PURIFIED

$^{35}\text{SO}_4$ -PROTEOGLYCAN

Confluent rabbit chondrocyte cultures in 75 cm^2 flasks containing 12 ml of medium were pulsed with

$^{35}\text{SO}_4^{--}$ (185 kBq/ml) for 48 hours. The medium was removed and concentrated to approximately 5 ml using an Amicon YM10 membrane (Amicon Corp, Danvers, MA, USA). The sample was then applied to a 1.6 $\times$ 90 cm Sepharose CL2B column (Pharmacia South Seas Pty Ltd, Sydney, NSW, Australia) which had been precalibrated with DNA (V_0) and $\text{Na}_2^{35}\text{SO}_4$ (V_t). The column was eluted with 0.5 M sodium acetate (pH 6.8) at a flow of 10 ml/h and fractions (3.0 ml) monitored for radioactivity. The $^{35}\text{SO}_4$ -proteoglycans eluted at V_0 as a single peak of aggregated proteoglycans and free $^{35}\text{SO}_4^{--}$ eluted at V_t . The V_0 fractions were pooled and used as a source of purified $^{35}\text{SO}_4$ -proteoglycans to validate the proteoglycan assay. The specific activity of the $^{35}\text{SO}_4$ -proteoglycan preparation determined after hexuronate analysis⁵ was 15 000 cpm/ μg hexuronic acid.

STATISTICAL ANALYSIS

The data generated on the effects of each drug studied on proteoglycan synthesis were analysed by two factor analysis of variance. Each analysis had six rows (drug concentrations: 0, 0.1, 1, 10, 50, and 100 $\mu\text{g}/\text{ml}$) and four columns (culture periods: day 0–2, 2–4, 4–6, and 6–8) and tested three null hypotheses: (1) that there was no significant difference in the proteoglycan synthesis of cultures exposed to any of the six drug concentrations (difference between rows); (2) that there was no significant difference in the proteoglycan synthesis of cultures during any of the four culture periods (difference between columns); and (3) that the 'dose-reponse' to the drug was not significantly different at any of the four culture periods (interaction). As all the null hypotheses proposed were rejected and the differences were highly significant (see Figs 1–6), the significant differences between all 24 means (6×4) were determined using the Student-Neuman-Keules test and the 5% level of the Q distribution.⁶

Results

PROTEOGLYCAN ASSAY

To separate free $^{35}\text{SO}_4^{--}$ from $^{35}\text{SO}_4$ -proteoglycans in the chondrocyte culture supernatant we used the very low solubility product of BaSO_4 (1.2×10^{-10}) relative to the moderate solubility of the glycosaminoglycan barium salts. Table 1 summarises the development of the method, in which free $^{35}\text{SO}_4^{--}$ and purified $^{35}\text{SO}_4$ -proteoglycan and $^{35}\text{SO}_4$ -glycosaminoglycans were subjected to BaSO_4 precipitation to arrive at conditions for optimum recovery. It can be seen from Table 1 that in an experimental situation with pulsed chondrocyte culture supernatant the addition of excess Ba^{++} to samples would

Table 1 Optimisation of the separation of free $^{35}\text{SO}_4^-$ from $^{35}\text{SO}_4$ -proteoglycans using barium sulphate precipitation

	Disintegrations per minute (dpm) in 100 μl supernatant			
	After 1st precipitation and centrifugation		After 2nd precipitation and centrifugation	
	dpm	Recovery (%)	dpm	Recovery (%)
<i>Free $^{35}\text{SO}_4^-$</i>				
Not precipitated with BaSO_4	543 171	—	387 980	—
BaSO_4 precipitated minus chondroitin sulphate	207	0	34	0
BaSO_4 precipitated plus chondroitin sulphate	1 603	0	126	0
<i>Non-digested $^{35}\text{SO}_4$-proteoglycan</i>				
Not precipitated with BaSO_4	1 407	—	1 005	—
BaSO_4 precipitated minus chondroitin sulphate	50	4	23	2
BaSO_4 precipitated plus chondroitin sulphate	1 227	87	84	84
<i>Papain digested $^{35}\text{SO}_4$-proteoglycan</i>				
Not precipitated with BaSO_4	1 330	—	950	—
BaSO_4 precipitated minus chondroitin sulphate	593	45	152	16
BaSO_4 precipitated plus chondroitin sulphate	1 363	102	1 040	109

Free $^{35}\text{SO}_4^-$ alone, $^{35}\text{SO}_4$ -proteoglycan, and papain digested $^{35}\text{SO}_4$ -proteoglycan were subjected to two successive precipitations with excess Ba^{++} and carrier chondroitin sulphate (see text). In each case precipitations were carried out with and without exogenous chondroitin sulphate. The data demonstrate how the precipitation method removes free $^{35}\text{SO}_4$ from the $^{35}\text{SO}_4$ -proteoglycan but that (i) exogenous chondroitin sulphate is required to prevent precipitation of $^{35}\text{SO}_4$ -proteoglycan; (ii) the presence of this chondroitin sulphate increases free $^{35}\text{SO}_4^-$ background, and so requires a second precipitation to reduce free $^{35}\text{SO}_4^-$ to negligible levels. Even in the presence of chondroitin sulphate there was some precipitation of $^{35}\text{SO}_4$ -proteoglycan unless it was previously digested to $^{35}\text{SO}_4$ -glycosaminoglycans with papain.

not be sufficient, for although $\text{Ba}^{35}\text{SO}_4$ would be precipitated, so too would almost all of the labelled proteoglycans (4% recovery in Table 1). Digestion of the proteoglycan aggregates with papain to yield free $^{35}\text{SO}_4$ -glycosaminoglycan reduced the loss upon precipitation to 55%. Addition of exogenous chondroitin sulphate to the $^{35}\text{SO}_4$ -glycosaminoglycans before precipitation, however, afforded 100% recovery of $^{35}\text{SO}_4$ -glycosaminoglycans in the supernatant. When large amounts of $^{35}\text{SO}_4^-$ were present in supernatants the background radioactive level after a single precipitation step was still relatively high, but after a second precipitation step the total recovery of $^{35}\text{SO}_4$ -glycosaminoglycans was preserved while the $^{35}\text{SO}_4^-$ background was reduced more than 10-fold to an acceptably low level.

EFFECT OF ANTIARTHRITIC DRUGS ON PROTEOGLYCAN BIOSYNTHESIS BY RABBIT CHONDROCYTES

Chondrocyte cultures were exposed to drugs and $^{35}\text{SO}_4^-$ for four consecutive 48 hour culture intervals, and the $^{35}\text{SO}_4$ -proteoglycans which had accumulated in the medium at the end of each culture interval were determined by the papain/double precipitation procedure described above. The six drugs, glycosaminoglycan polysulphate ester, sodium pentosan polysulphate, indomethacin, diclofenac, ketoprofen, and tiaprofenic acid, were tested at concentrations of 0 (control), 0.1, 1, 10, 50, and

100 $\mu\text{g/ml}$. Figures 1–6 show the results obtained for each drug. As the purpose of this study was to compare the effects of drugs at different concentrations relative to a drug free control culture the data presented represent the disintegrations per minute of one fifteenth (dilution due to the assay) of the $^{35}\text{SO}_4$ -glycosaminoglycans produced by 10^5 cells during the indicated 48 hour periods. For all cultures studied a common pattern of chondrocyte secretory activity was observed. During the first 48 hour period proteoglycan secretion was low, increasing to a maximum on days 2–4. A progressive decline in activity then occurred over the next two culture intervals in all experiments (see Figs 1–6).

Glycosaminoglycan polysulphate ester

Figure 1 shows secretion of $^{35}\text{SO}_4$ -proteoglycan by chondrocytes cultured in the absence and presence of increasing concentrations of glycosaminoglycan polysulphate ester over an eight day culture period. For the period days 0–2 glycosaminoglycan polysulphate ester caused no significant effect on proteoglycan secretion up to a concentration of 10 $\mu\text{g/ml}$, but at 50 and 100 $\mu\text{g/ml}$ the drug slightly depressed proteoglycan secretion. During the next two day culture period the pattern of response to increasing concentrations of glycosaminoglycan polysulphate ester had changed substantially and became constant for the duration of the experiment. Inhibition of proteoglycan secretion was no longer observed at

Fig. 1 Effect of glycosaminoglycan polysulphate ester on the secretion of proteoglycans by chondrocytes. Confluent chondrocyte monolayer cultures were grown for eight days, constantly exposed to the indicated drugs plus $^{35}\text{SO}_4^{--}$. The medium, containing fresh drugs and radiolabel, was changed every 48 hours and the exhausted medium from each well was retained for assay of the $^{35}\text{SO}_4$ -proteoglycans which had accumulated during the previous 48 hours of culture. Each plotted curve comprising six data points is a concentration-response curve, the six concentrations being in every case, from left to right, $\bullet$ 0, $\star$ 0.1, $\blacktriangle$ 1.0, $\blacksquare$ 10, $\blacklozenge$ 50, and $\blacktriangledown$ 100 $\mu\text{g/ml}$. All values within each individual figure were compared by a single statistical analysis (see 'Materials and methods'), and the rankings of the magnitudes of the individual values (means of four culture wells) are indicated by the letters assigned to each point. Those means which do not share a letter in common are significantly different at the 5% level. Any two or more means which have a letter in common are not significantly different. For example, a value designated 'IJ' is not different from a value labelled 'HI' (both have the letter 'I') or from a value labelled 'JKL' (both have the letter 'J'), but the value labelled 'HI' is significantly greater than the value labelled 'JKL' (no letter in common).

Fig. 2 Effect of sodium pentosan polysulphate at various concentrations on proteoglycan secretion by lapine chondrocytes. See legend to Fig. 1.

any concentration, even at 50 and 100 $\mu\text{g/ml}$ inhibition by glycosaminoglycan polysulphate ester was statistically indistinguishable from that of the controls. Glycosaminoglycan polysulphate ester at 0.1, 1, and 10 $\mu\text{g/ml}$, however, stimulated proteoglycan secretion significantly (except for days 4–6 where 10 $\mu\text{g/ml}$ was not significantly different from the control). Stimulation of proteoglycan secretion was maximal at 1 $\mu\text{g/ml}$ over the two to four day culture period.

Sodium pentosan polysulphate

The response of chondrocytes to sodium pentosan polysulphate showed similarities with their response to glycosaminoglycan polysulphate ester (Fig. 2). A pattern was established by days 2–4, which persisted for the subsequent culture intervals. At days 0–2 the proteoglycan secretion of the control chondrocytes and those cultured with 0.1, 1, and 10 $\mu\text{g/ml}$ sodium pentosan polysulphate were not significantly different. At 50 and 100 $\mu\text{g/ml}$ of the

Fig. 3 Effect of indomethacin at various concentrations on proteoglycan secretion by lapine chondrocytes. See legend to Fig. 1.

Fig. 4 Effect of diclofenac at various concentrations on proteoglycan secretion by lapine chondrocytes. See legend to Fig. 1.

drug proteoglycan secretion was inhibited. This significant diminution of proteoglycan secretion at high concentrations of sodium pentosan polysulphate, unlike glycosaminoglycan polysulphate ester, persisted throughout the experiment. Like glycosaminoglycan polysulphate ester, sodium pentosan polysulphate at 0.1 and 1 $\mu\text{g/ml}$ stimulated proteoglycan secretion from day 2 onwards, the maximum stimulation being produced by 1 $\mu\text{g/ml}$ at each two day culture interval. The response with time to 10 $\mu\text{g/ml}$ sodium pentosan polysulphate was variable, the effect being stimulatory during days 2–4, inhibitory during days 4–6, and the same as that of the controls on days 0–2 and days 6–8.

Indomethacin

The pattern of response of the chondrocyte cultures to indomethacin was consistent over each culture interval (Fig. 3). Proteoglycan secretion was unaffected by concentrations of this drug of 0.1, 1, and 10 $\mu\text{g/ml}$, but was profoundly inhibited at 50 and 100 $\mu\text{g/ml}$. By days 4–6 $^{35}\text{SO}_4$ -proteoglycan secretion in the presence of 100 $\mu\text{g/ml}$ indomethacin was essentially abolished, being reduced to the level of background disintegrations per minute.

Diclofenac

The pattern of proteoglycan secretory activity of chondrocytes cultured in medium containing diclofenac was found to be almost identical to that of cultures exposed to indomethacin (Fig. 4). Diclofenac at 0.1, 1, and 10 $\mu\text{g/ml}$ did not significantly

influence proteoglycan secretion by chondrocytes. At 50 and 100 $\mu\text{g/ml}$ of the drug, however, a major inhibitory effect on proteoglycan secretion was observed, with proteoglycan levels approaching background in the presence of 100 $\mu\text{g/ml}$ of the drug during days 4–6 and 6–8. A comparison of Figs 3 and 4 suggests that diclofenac at 50 $\mu\text{g/ml}$ was slightly more inhibitory than indomethacin at 50 $\mu\text{g/ml}$.

Ketoprofen

This drug elicited very little effect on proteoglycan secretion by rabbit chondrocytes over the concentration range studied (Fig. 5). During days 0–2 all cultures secreted the same amount of $^{35}\text{SO}_4$ -proteoglycan. The same was observed during days 6–8. On days 4–6 chondrocytes cultured in the presence of 100 $\mu\text{g/ml}$ ketoprofen secreted significantly less proteoglycan than controls, but the magnitude of the reduction was modest. On days 2–4, when secretion levels were highest overall, a statistically significant diminution of proteoglycan secretion relative to control values resulted from both 50 and 100 $\mu\text{g/ml}$ ketoprofen, but the diminished levels of proteoglycan secretion resulting from these two drug concentrations were not distinguishable statistically from each other.

Tiaprofenic acid

The proteoglycan secretion of articular chondrocytes cultured in the presence of tiaprofenic acid was broadly similar to that observed with ketoprofen, but concentration effects were more pronounced

Fig. 5 Effect of ketoprofen at various concentrations on proteoglycan secretion by lapine chondrocytes. See legend to Fig. 1.

Fig. 6 Effect of tiaprofenic acid at various concentrations on proteoglycan secretion by lapine chondrocytes. See legend to Fig. 1.

(Fig. 6). During days 0-2 there was a slight but significant reduction in proteoglycan secretion at concentrations of 10, 50, and 100 µg/ml, but the proteoglycan levels produced at these three drug concentrations were not different from one another. During the two to four day culture period the drug at 0.1, 1, and 10 µg/ml had no significant effect on secretion compared with the controls, whereas 50 µg/ml and 100 µg/ml tiaprofenic acid reduced proteoglycan secretion. This pattern was repeated in the following culture period (days 4-6) and maintained for days 6-8. In this last culture period, however, the magnitude of the reduction in the level of proteoglycan secretion at 50 and 100 µg/ml tiaprofenic acid, though still significant, was only slight.

Discussion

Our objective in the present study was to devise a simple in vitro model which would permit a thorough comparison of drug effects over a range of concentrations and time periods. Of particular importance was the need to maintain reproducibility by the elimination of time consuming steps, such as multi-column chromatographic separations or techniques involving overnight precipitation and subsequent washing steps, which would introduce cumulative losses. As is evident from Table 1 the use of the low solubility of BaSO₄ in conjunction with papain digestion and the introduction of chondroitin sulphate as a carrier permitted a satisfactory separation

of unincorporated ³⁵SO₄⁻ from ³⁵SO₄ labelled glycosaminoglycans released by cells into the culture medium.

From the results it was evident that the six drugs studied fell into three groups of two, which had similar effects on proteoglycan secretion by chondrocytes. The similarity of the response of chondrocytes to indomethacin and diclofenac was striking. Both drugs showed no effect on proteoglycan secretion at concentrations up to and including 10 µg/ml, and both strongly inhibited proteoglycan secretion at 50 and 100 µg/ml. The pattern of response to different concentrations was established in the initial period of exposure to the drug and did not change for the duration of the experiment. Anefeld *et al* reported similar effects for these two drugs using a very different system based on morphometric assessment of chondrocyte ultrastructure.⁷ The morphometric evaluation of rat articular chondrocytes after 12 weeks of twice weekly administration of drugs at doses corresponding to the drugs' ED₅₀ showed similar scores for diclofenac and indomethacin for a number of variables, including endoplasmic reticulum, Golgi bodies, mitochondria, fat vacuoles, glycogen, and mortality. It was concluded that indomethacin and diclofenac both inhibited chondrocyte metabolic activity, whereas piroxicam and tiaprofenic acid studied in the same system had stimulatory effects on the cells. Using a similar ultrastructural study, Fassbender observed that treatment of rats with indomethacin resulted in damage to chondrocyte ultrastructure,

whereas tiaprofenic acid caused only insignificant changes.⁸ In experiments measuring proteoglycan biosynthesis by chondrocytes or cartilage explants various effects of indomethacin have been reported. Proteoglycan synthesis by bovine chondrocytes was inhibited by indomethacin at a concentration of 10^{-5} mol/l in a study undertaken by Mitrovic *et al*³ but was not inhibited by 1.4×10^{-5} M indomethacin according to a report by Klamfeldt.⁹ Palmoski and Brandt found that proteoglycan synthesis in canine cartilage explants was unaffected by indomethacin at concentrations up to 15 µg/ml (4.2×10^{-5} mol/l),¹⁰ and, surprisingly, in a recent paper by Muir *et al*¹¹ proteoglycan synthesis by canine cartilage explants exposed to 5×10^{-7} and 10^{-6} M indomethacin was reported to be significantly lower than that of controls, but at the higher concentrations of 5×10^{-6} and 10^{-5} mol/l this drug had no significant suppressive effect on biosynthesis. Much higher concentrations of indomethacin were used by McKenzie *et al*, who found that cartilage explants from human osteoarthritic femoral heads synthesised significantly less proteoglycans when exposed to 0.5 mM and 1 mM, but not 0.1 mM (36 µg/ml), indomethacin.¹² The response of degenerate cartilage to indomethacin was also studied by Brandt and Palmoski, who reported that at 10^{-6} mol/l this NSAID suppressed by 46% the glycosaminoglycan synthesis of atrophic canine knee cartilage, whereas in normal cartilage proteoglycan synthesis was unaffected.¹³ Relatively few studies have described the in vitro effects of diclofenac on proteoglycan biosynthesis. Mohr and Kirkpatrick measured the incorporation of $^{35}\text{SO}_4^-$ into rabbit chondrocyte cultures and reported that the presence of 5 µg/ml of diclofenac had no effect.¹⁴

In all these studies proteoglycan synthesis was measured at a single timepoint only after a period of exposure to the drug. In addition, the different sources of cartilage, the variable periods of exposure to the drug, the widely varying range of concentrations selected for study, and other aspects of the experimental designs have all contributed to the confused and inconsistent results obtained in the above in vitro assessments of NSAIDs. From the present study we may conclude that although indomethacin and diclofenac showed an inhibitory effect on proteoglycan secretion at 50 and 100 µg/ml, such concentrations would be in excess of those achieved in synovial fluid during normal therapeutic administration of these drugs to arthritic patients.^{15 16}

The effects of tiaprofenic acid and ketoprofen on the secretion of proteoglycan by chondrocytes were essentially similar in our experiments. These two drugs are structurally related in that the benzene ring attached to the 2 position of propionic acid in

ketoprofen is replaced by a thiophene ring in tiaprofenic acid. As the aromatic character of thiophenes is high it is perhaps not unexpected that these two drugs should behave similarly.

Tiaprofenic acid and, especially, ketoprofen had comparatively weak inhibitory effects on proteoglycan secretion. The effect was inconsistent and was not strongly dependent on concentration (see Figs 5 and 6). Inhibition of proteoglycan secretion by high doses of these drugs was most evident during days 2–4, after which the percentage inhibition at 50 and 100 µg/ml diminished with time. Tiaprofenic acid at 10 µg/ml, a concentration which is achieved clinically in serum and synovial fluid,¹⁷ significantly depressed proteoglycan secretion during the initial culture interval, but the effect was very slight and was transient. Studies by other workers have assessed the in vitro effects of tiaprofenic acid on murine patella cartilage,¹⁸ canine articular cartilage explants,¹⁹ and chick²⁰ and rabbit¹⁶ chondrocyte cultures. In none of these studies was a significant inhibition of proteoglycan synthesis reported, but these investigations did not include concentrations of tiaprofenic acid as high as 50 and 100 µg/ml which were found to be inhibitory in the present study. de Vries *et al* reported that tiaprofenic acid at 30 µg/ml did not suppress glycosaminoglycan synthesis by murine patella cartilage in vitro,¹⁸ but the explanted patellae were exposed to the drug for only 30 minutes, so the acute response to the drug measured by these authors could not be compared with our study, which may be considered as equivalent to a chronic effect.

The two sulphated polysaccharides studied, glycosaminoglycan polysulphate ester and sodium pentosan polysulphate, represent a relatively new class of antiarthritic agents, whose pharmacological properties have been recently reviewed.^{1 21} Their effects on chondrocyte proteoglycan secretion were, correspondingly, quite different from those of the NSAIDs. During the initial period of exposure (days 0–2) proteoglycan secretion was significantly suppressed by concentrations of 50 and 100 µg/ml of both drugs. Although this pattern was maintained for sodium pentosan polysulphate, glycosaminoglycan polysulphate ester was not inhibitory at any concentration during the subsequent culture intervals. The significant and substantial stimulation of proteoglycan secretion in the presence of low concentrations of both these sulphated polysaccharides, once established, persisted for the duration of the experiment. This result is in contrast with the effects of the NSAIDs, none of which caused any significant stimulation of proteoglycan secretion over the concentration range examined. Our findings that sulphated polysaccharides could stimulate pro-

teoglycan secretion are consistent with other reports where chondrocyte cultures were employed.^{22 23} The stimulation of proteoglycan secretion by these drugs in this study occurred at concentrations which could be readily achieved in cartilage during human administration of drugs to patients for the treatment of osteoarthritis.²⁴ Our in vitro findings are therefore consistent also with the chondroprotective action of these drugs reported to occur in a number of animal models.^{1 21}

The simple method used in the present study appears to offer distinct advantages over existing procedures. By reducing the tedium and experimental variability associated with other methods it has facilitated the evaluation of the effects of drugs on chondrocyte proteoglycan biosynthesis under a variety of conditions. This method should therefore find application not only in the screening of antiarthritic drugs but also in other areas where knowledge of proteoglycan biosynthesis in cell or organ cultures is an experimental objective.

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