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COMPARISON OF THE EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs) ON PROTEOGLYCAN SYNTHESIS BY ARTICULAR CARTILAGE EXPLANT AND CHONDROCYTE MONOLAYER CULTURES

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Abstract—Experiments were conducted to study proteoglycan biosynthesis by rabbit articular chondrocytes cultured in the presence of NSAIDs and $^{35}SO_4^{2-}$ for up to 8 days. Both articular cartilage explants and confluent chondrocyte monolayer culture models were used. Medium was changed every 2 days and the [$^{35}SO_4$]proteoglycans which had accumulated in the medium and the extracellular matrix during the culture intervals were assayed separately. In long-term experiments, drugs were removed on day 8, and proteoglycan production during a 10–12 day culture interval also was assayed. The drugs studied were diclofenac, indomethacin, ketoprofen, piroxicam and tiaprofenic acid, at concentrations of 0, 0.1, 1, 10, 50 and 100 μg/mL. Whereas proteoglycan production by cell cultures was maximal early in the culture period, explants produced more proteoglycans as time progressed. The highest concentrations of all of the drugs, especially diclofenac and indomethacin, inhibited proteoglycan secretion by both cell and explant cultures. However, after removal of the drugs from the cultures, suppressed proteoglycan production reversed to levels equivalent to, or higher than controls in the cell cultures, but largely persisted in explant cultures. About 70–80% of proteoglycans produced by explants were retained in the matrix, whereas about 80–90% of proteoglycans produced by cell cultures were secreted into the medium. Where drugs inhibited proteoglycan production, the levels were reduced by approximately the same proportions in both extracellular matrix and culture medium fractions. Of the NSAIDs examined only ketoprofen demonstrated a stimulatory effect on PG synthesis in explant cultures at a physiological concentration (0.1 μg/mL).

There continues to be concern about the possible long-term adverse effects on articular cartilage of chronic non-steroidal anti-inflammatory drug (NSAIDs) therapy. While *in vivo* evidence has been presented that some of the older drugs in this class, e.g. aspirin [1] and indomethacin [2] can be detrimental to bone and cartilage metabolism, the majority of studies have utilised *in vitro* methods of assessment. In spite of the fact that the relevance of data obtained *in vitro* to the clinical situation can always be questioned, cell and tissue culture methods offer considerable advantages over animal experiments in terms of efficiency and ethical considerations and are widely used. Many different models have been described, including human [3], dog [4, 5], pig [6] and mouse [7] articular cartilage explants, as well as rabbit [8, 9], bovine [10] and chicken [11] chondrocyte monolayer cultures. However, the variety of species used and the different experimental designs employed make comparison between studies difficult to interpret and often lead to controversy. Further limitations are that, in many studies, proteoglycan biosynthesis was measured on a single occasion only, and after a relatively brief period of exposure of the cells or explants to the drugs [5–11]. In those studies that did include multiple timepoints, relatively short periods of 6 [3] and 24 hr [4] were employed.

Because NSAID therapy in arthritic patients is usually long-term, we thought it appropriate, in this and a previous study [12], to expose chondrocyte monolayer and cartilage explant cultures to the drugs for a prolonged period (8 days), and monitor the secretion of newly synthesised proteoglycans into the medium. Five frequently prescribed NSAIDs, indomethacin, diclofenac, ketoprofen, piroxicam and tiaprofenic acid, were tested in these systems over a range of concentrations, spanning three orders of magnitude, which encompassed the physiological range found in the serum and synovial fluid of patients on NSAID therapy [13]. We are unaware of any other report which specifically compares the proteoglycan biosynthetic responses of cartilage cell and explant cultures to NSAIDs under the same experimental conditions.

MATERIALS AND METHODS

(i) *Preparation of primary chondrocyte cultures.* Articular chondrocytes were isolated from the knee joints of 6–8-week-old New Zealand white rabbits, as described [12]. The primary chondrocytes were inoculated into 75 cm^2 tissue culture flasks (Miles Laboratories Inc., Naperville, IL, U.S.A.)

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(2×10^6 cells in 12 mL medium) and grown to confluence (6–8 days). Ham's F12 medium (Flow Labs, McLean, VA, U.S.A.) was used, supplemented with 10% foetal calf serum (FCS) (Commonwealth Serum Laboratories, Parkville, Victoria, Australia), 80 units/mL gentamicin (David Bull Laboratories Pty. Ltd, Rydalmere, NSW, Australia), and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Sigma Chemical Co. St Louis, MO) and is referred to below as F12FCS. The confluent cells were subcultured into 24-well tissue culture plates (Flow Labs.) at high density (10^5 cell/well in 0.5 mL medium) and incubated for 1–3 days to become fully confluent.

(ii) *Preparation of articular cartilage explant cultures.* Full-thickness slices of articular cartilage were cut from the femoral condyles, the tibial plateaux and the patellae of the rabbit knee joints using a scalpel (#11 blade). The rabbits were 6–8 weeks old, as above. The resulting explants had an average wet weight of 3.2 mg and coefficient of variation of 27% and standard deviation of 1.17 mg (N = 120). The explants were cultured individually in the wells of 24-well plates, as used for the cell cultures (see above). The wet weights were determined at the end of the experiment when each explant was lightly blotted and weighed on an Mettler AE240 analytical balance (Mettler Instruments AG, Greifensee, Switzerland).

(iii) *Test cultures.* After preliminary cincubation of the cell and explant cultures the medium was changed and to each well was added 450 μL of F12FCS containing approximately 2.5 μCi $^{35}SO_4^{2-}$ (New England Nuclear Corp., Bedford, MA). Sufficient F12FCS containing the $^{35}SO_4^{2-}$ label was made up in a single batch to last for the entire experiment, in order to minimise variation in the concentration of radionuclide to which the cultures were exposed at each medium change. The test drugs were dissolved and appropriately diluted in phosphate-buffered saline and were dispensed to the wells in 50 μL volumes to give the desired final concentrations. The NSAIDs included in the study were the following: (1) diclofenac sodium (1 μg/mL = 3.18 μM) (Voltaren®, Ciba-Geigy Australia Ltd., Pendle Hill, NSW, Australia); (2) indomethacin (1 μg/mL = 3.58 μM) (Indocid®, Merck Sharp and Dohme Research Labs, Rahway, NJ); (3) ketoprofen (1 μg/mL = 2.54 μM) (Orudis®, May and Baker Australia Pty. Ltd, West Footscray, Victoria, Australia); (4) piroxicam (1 μg/mL = 3.3 μM) (Feldene®, Pfizer Pty. Ltd, West Ryde, NSW, Australia) and (5) tiaprofenic acid (1 μg/mL = 2.60 μM) (Surgam®, Roussel Pharmaceuticals Pty. Ltd, Castle Hill, NSW, Australia). Each drug was tested at concentrations of 0, 0.1, 1, 10, 50 and 100 μg/mL. There were four replicate wells of both cell and explant cultures for each concentration of each drug. Medium was changed and drugs were added after 2, 4 and 6 days of culture. On day 8, medium without drugs or radionuclide was added to all culture wells. On day 10, 450 μL of the medium containing $^{35}SO_4^{2-}$ plus 50 μL of "cold" medium was added to each well. The exhausted medium from each well was collected on days 2, 4, 6, 8 and 12 and stored for proteoglycan assay.

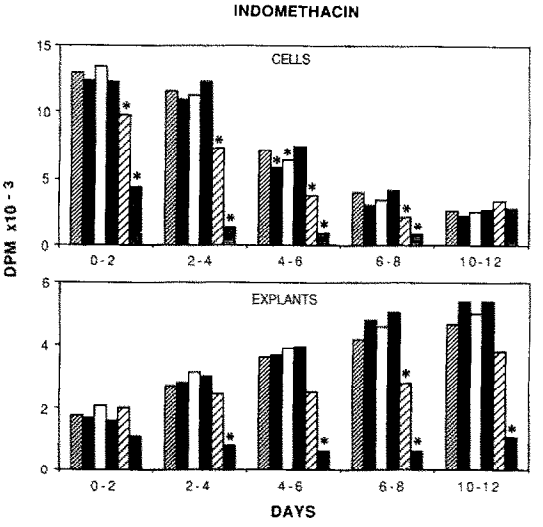

Fig. 1. Effect of indomethacin on the secretion of $^{35}SO_4$-labelled proteoglycans by chondrocyte monolayer cultures (top panel), and cartilage explant cultures (bottom panel) during the indicated 2-day culture intervals. Drug concentrations were: (▨) 0, (■) 0.1, (□) 1, (□) 10, (▨) 50, (▤) 100 μg/mL. Drugs were withdrawn from all cultures on day 8, so the cultures were drug-free during days 8–10 (not assayed) and days 10–12. Asterisks indicate significant differences from the control ($P < 0.05$).

(iv) [$^{35}SO_4$]*Proteoglycan assay.* The [$^{35}SO_4$]-proteoglycans in the medium were separated from the free $^{35}SO_4^{2-}$ using the barium sulphate precipitation method developed by us, which has been described in detail elsewhere [12]. To assay the matrix-bound proteoglycans in cell and explant cultures, after removal of the medium, the cultures were rinsed with 0.5 mL PBS, then digested at 60° with 0.5 mL papain solution [12], and assayed as for the culture supernatant. After separation [$^{35}SO_4$]proteoglycans were quantified by routine β-scintillation counting. Since only relative proteoglycan synthesis by different culture treatments was of interest, results are presented as disintegrations per min (dpm) of [$^{35}SO_4$]proteoglycans. In the case of the cartilage explant cultures, the proteoglycan secretion was expressed as dpm per mg. wet weight.

(v) *Statistical analysis.* For each drug at each timepoint, the [$^{35}SO_4$]proteoglycans synthesised by the control cultures, and the cultures exposed to the five different drug concentrations, were compared by 1-factor analysis of variance. If significant, the differences between the individual means were further analysed by the Student–Newman–Keuls test (5% level) for Experiment 1, and by Dunnett's test (2-tailed, 5% level) in subsequent experiments.

RESULTS

Experiment 1

(i) *Proteoglycan secretion of chondrocyte monolayer cultures. Day* 0–2. A dose-dependent inhibition of proteoglycan secretion was evident for all drugs at high concentrations during the initial 48 hr culture interval (day 0–2) in the presence of drugs (Figs 1–5). Drug concentrations of 50 and 100 μg were

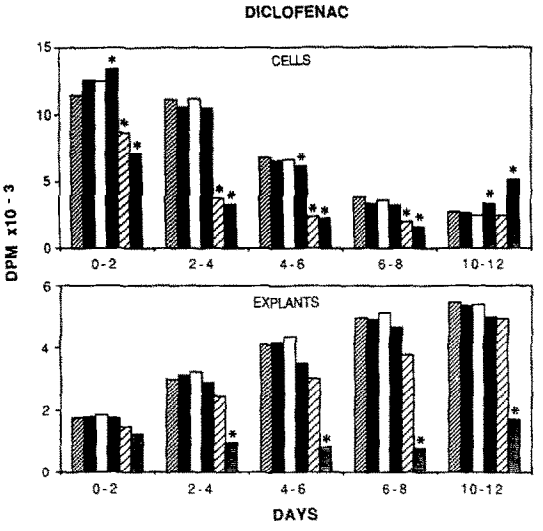

Fig. 2. Effect of diclofenac on the secretion of $^{35}SO_4$-labelled proteoglycans by chondrocyte monolayer cultures (top panel), and cartilage explant cultures (bottom panel) during the indicated 2-day culture intervals. See legend to Fig. 1.

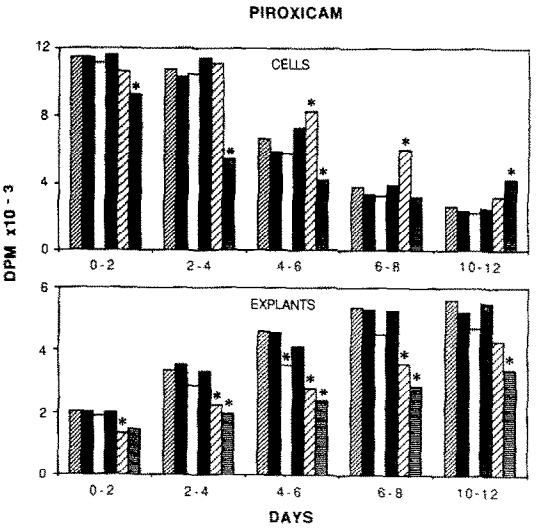

Fig. 4. Effect of piroxicam on the secretion of $^{35}SO_4$-labelled proteoglycans by chondrocyte monolayer cultures (top panel), and cartilage explant cultures (bottom panel) during the indicated 2-day culture intervals. See legend to Fig. 1.

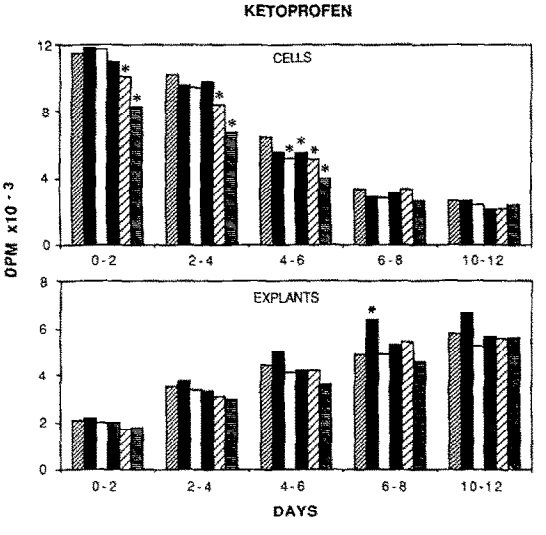

Fig. 3. Effect of ketoprofen on the secretion of $^{35}SO_4$-labelled proteoglycans by chondrocyte monolayer cultures (top panel), and cartilage explant cultures (bottom panel) during the indicated 2-day culture intervals. See legend to Fig. 1.

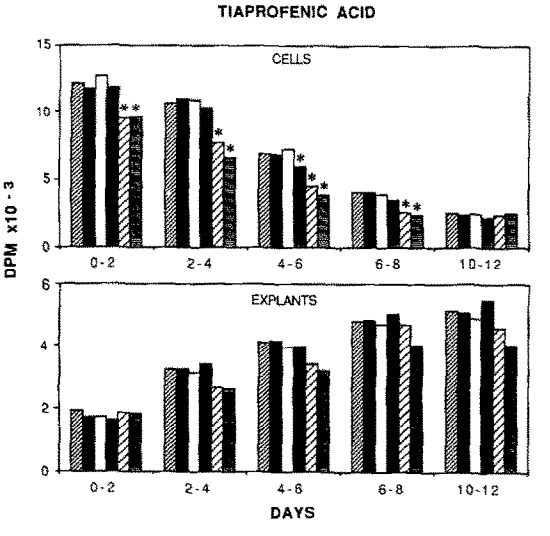

Fig. 5. Effect of tiaprofenic acid on the secretion of $^{35}SO_4$-labelled proteoglycans by chondrocyte monolayer cultures (top panel), and cartilage explant cultures (bottom panel) during the indicated 2-day culture intervals. See legend to Fig. 1.

inhibitory for all drugs except piroxicam which was significantly inhibitory only at 100 μg/mL. No drug was inhibitory at less than 50 μg/mL, and the only significant stimulation of proteoglycan synthesis above the control level was by diclofenac, at 10 μg/mL.

Day 2–4. The pattern of proteoglycan secretion at different drug concentrations established during the initial culture interval were essentially preserved during the 2–4 day period, but the high-dose inhibition was proportionally greater, except perhaps in the case of ketoprofen (Fig. 3). The overall levels of proteoglycan secretion by the control cultures had dropped by an average of approximately 10%. The elevation in proteoglycan synthesis by 10 μg/mL of diclofenac seen at day 0–2 was no longer evident. Diclofenac and indomethacin were strongly inhibitory at 50 and 100 μg/mL, and ketoprofen and

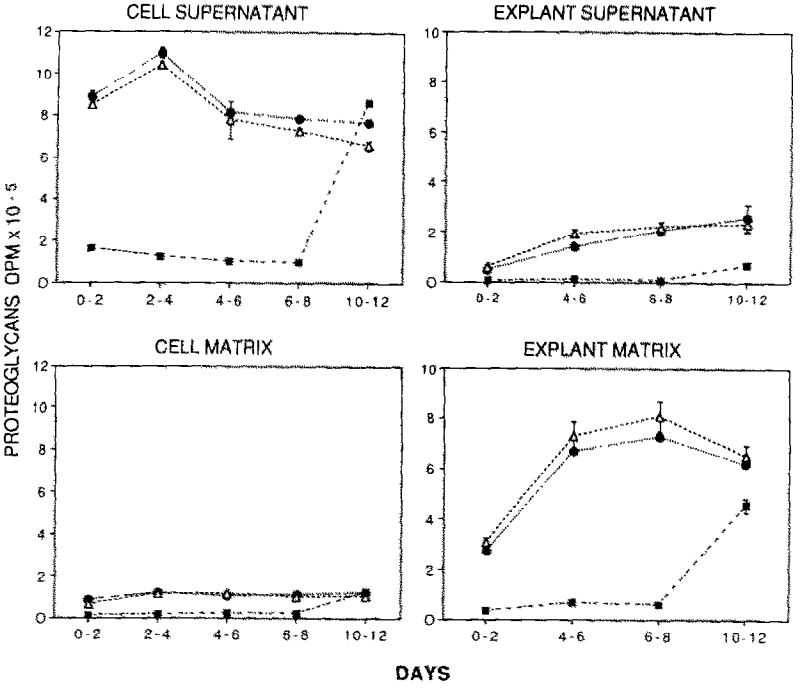

Fig. 6. Effect of diclofenac on the accumulation of $^{35}SO_4$-labelled proteoglycans in the culture supernatants (top panels) and the extra cellular matrix (bottom panels) of chondrocyte monolayer (left panels) and articular cartilage explant (right panels) cultures during successive 2-day culture intervals (x-axis). The drug was removed from the cultures on day 8. Concentrations were: (●) control; (△) 10 μg/mL; (■) 100 μg/mL. Plots are means (N = 4) and standard error.

tiaprofenic acid also were significantly inhibitory at these two concentrations. Proteoglycan secretion was inhibited by piroxicam only at 100 μg/mL, but at that concentration the inhibition was substantial, at approximately 50% of the control value.

Day 4–6. The overall levels of [$^{35}SO_4$]proteoglycans released into the medium during the 4–6 day culture interval had decreased substantially from the levels of the previous culture interval. In addition to the high-dose inhibition seen previously, significant inhibition of proteoglycan secretion was registered for some lower drug concentrations: indomethacin, 0.1 and 1 μg/mL; diclofenac, 10 μg/mL; ketoprofen, 1 and 10 μg/mL; tiaprofenic acid, 10 μg/mL. Interestingly, piroxicam was inhibitory only at 100 μg/mL, as in the two previous culture intervals, but now 50 μg/mL of piroxicam significantly stimulated proteoglycan production, compared with the control cultures, by approximately 125%.

Day 6–8. The dose–response curves had largely levelled out by day 8 as the falling levels of proteoglycan synthesis of the ageing confluent chondrocyte cultures decreased to approach those low levels of cultures which had been strongly inhibited by high drug concentrations since early in the experiment (Figs 1–5). Fifty and 100 μg/mL of diclofenac still resulted in significantly less [$^{35}SO_4$]proteoglycan secretion than the control, as did 50 and 100 μg/mL of indomethacin, but the percentage inhibition was now slight. Ketoprofen had a flat dose–response curve by day 6–8; the analysis of variance comparing the six concentrations of the drug was not significant. Tiaprofenic acid was slightly inhibitory at 50 and 100 μg/mL but there was no difference in the degree of inhibition caused by the latter two concentrations, much like diclofenac at this timepoint. Chondrocyte cultures exposed to piroxicam at 0.1, 1, 10 and 100 μg/mL secreted the same amounts of [$^{35}SO_4$]proteoglycans as the control cultures, but as seen during the 4–6 day culture interval, 50 μg/mL of piroxicam strongly stimulated proteoglycan secretion.

Day 10–12. The accumulation of [$^{35}SO_4$]-proteoglycans in the medium of the chondrocyte cultures during the culture interval day 10–12, i.e. on the 3rd and 4th day after the drugs had been withdrawn from all cultures, was only a little less than that of day 6–8, suggesting that proteoglycan production had stabilised at this low level. There were several notable results in the data shown in Figs 1–5: (i) none of the cultures produced less proteoglycan than their respective controls during this period of culture when the drugs were no longer present; (ii) some cultures produced significantly more [$^{35}SO_4$]proteoglycans than their control, namely, diclofenac at 10 μg/mL and 100 μg/mL and piroxicam at 100 μg/mL; and (iii) some cultures produced more proteoglycans in this drug-free culture interval than they did in the previous 6–8 day culture period in the presence of drugs—diclofenac at 50 and 100 μg/mL, indomethacin at 50 and 100 μg/mL and piroxicam at 100 μg/mL; the increase was most striking for the diclofenac 100 μg/mL culture, the output of which increased more than three-fold.

Table 1.

	Diclofenac (μg/ mL)	Days in culture 0–2	2–4	4–6	6–8	10–12
Chondrocyte monolayers	0	8.9	9.7	8.7	12.5	13.4
Per cent matrix-bound proteoglycans	10	7.3*	10.1	10.1	12.3	13.9
	100	7.9*	12.3*	15.2*	20.9*	12.9
Cartilage explants	0	84.3	—	82.7	78.1	70.3
Per cent matrix-bound proteoglycans	10	84.0	—	78.9	78.6	73.5
	100	80.5*	—	86.3	87.9*	87.1*

Effect of diclofenac on the percentage of [$^{35}SO_4$]proteoglycans, synthesised by chondrocyte cell and cartilage explant cultures, that are incorporated into the extracellular matrix. Drug was present until day 8 then was removed. Asterisks indicate significant ($P < 0.05$) difference from control (0 μg/mL diclofenac).

(ii) *Proteoglycan secretion of cartilage explant cultures*. In contrast to the chondrocyte cultures, the control explant cultures secreted levels of proteoglycan which increased steadily for the duration of the experiment (Figs 1–5, bottom panels). With all drugs, the dose–response patterns of proteoglycan secretion by the explants, once established, were essentially fixed for the rest of the experiment, including the final culture interval (day 10–12) when the drugs were no longer present.

Because of the much greater variability of the explant data compared with the cell data, relatively greater differences between values were required to reach statistical significance. However, as the dose–response curves were reproduced at several timepoints, some drug effects were consistently evident, although they did not reach the 5% significance level at any given timepoint.

Both indomethacin and diclofenac profoundly inhibited proteoglycan secretion of articular cartilage explants at 100 μg/mL, and indomethacin also strongly inhibited at 50 μg/mL. These inhibitory effects persisted even after removal of the drugs. The less pronounced depression of proteoglycan synthesis by diclofenac at 50 μg/mL was largely reversed after removal of the drug (Fig. 2). At 10 μg/mL, diclofenac, but not indomethacin, appeared to slightly suppress proteoglycan secretion. At 1.0 μg/mL piroxicam significantly inhibited proteoglycan secretion ($P < 0.05$) during days 4–6. At the higher concentrations of 50 μg and 100 μg/mL piroxicam significantly inhibited proteoglycan secretion to about 60% of the control values, but in contrast to diclofenac and indomethacin, there was little difference between the levels of inhibition effected by these two highest concentrations of piroxicam. The explant cultures exhibited the same pattern of proteoglycan secretion even after the piroxicam had been removed for 48 hr (Fig. 4). Ketoprofen and tiaprofenic acid did not statistically significantly inhibit proteoglycan secretion of cartilage explants at any dose or timepoint examined in this experiment. Nevertheless, slight inhibition of proteoglycan secretion by tiaprofenic acid at the high concentration of 100 μg/mL was evident from day 2–4 onwards. Of all the drugs, ketoprofen showed the least tendency to produce concentration-dependent inhibition of proteoglycan secretion by cartilage explants. The slight inhibition by 100 μg/mL ketoprofen, most apparent during day 4–6 and day 6–8, disappeared once the drug was removed from the cultures. The most noticeable effect of ketoprofen was a consistent stimulation of synthesis at 0.1 μg/mL of the drug, statistically significant on day 6–8, an effect which persisted after removal of the drug from the cultures.

Experiment 2

In order to take into account the proteoglycans which were retained in the cell layer and cartilage explant matrix, the basic design of the previous experiments was repeated for one drug, diclofenac, at three concentrations only, since the assay was destructive and individual sets of cultures were required for each timepoint.

Cell and explant cultures were incubated with 0, 10 and 100 μg/mL diclofenac, and the $^{35}SO_4$-labelled proteoglycans present in both matrix and supernatant fractions were assayed after 2, 4, 6 and 8 days of culture in the presence of drugs, and for the 10–12 day culture interval, in the absence of the drug (day 8 onwards). Proteoglycan production was highest for cells for the 2–4 day interval, and for explants, days 6–8. Overall, approximately 10% of the proteoglycans produced by cells were retained in the matrix, whereas approximately 80% were retained in the matrix of cartilage explants.

Proteoglycan production was profoundly inhibited for both compartments of both types of cultures by 100 μg/mL of diclofenac (10–20% of control values). However, removal of the drug from the media fully reversed this inhibition in cell cultures and partially reversed it in explant cultures (Fig. 6). The proportions of proteoglycans in the extracellular matrix component increased over time for cell cultures and decreased over time for explant cultures (Table 1). The high dose (100 μg/mL) of diclofenac caused an increase in the proportion of proteoglycans in the matrix compartments of both culture systems which was more evident as time progressed. The biggest shift was noted for day 6–8 for cell cultures and day 10–12 for explant cultures, but the effect

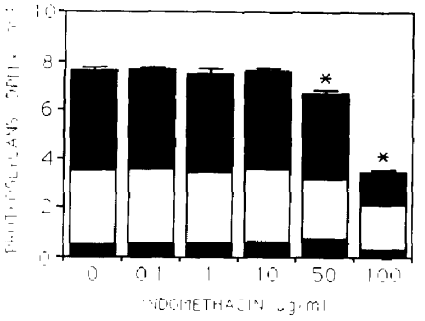

Fig. 7. Effect of indomethacin on proteoglycan production by chondrocyte monolayer cultures. The stacked columns indicate total proteoglycan biosynthesis for the interval days 0–4 (means, N = 4, plus standard errors) comprising the cell layer fraction days 0–4, (■); culture medium fraction day 0–2 (□); and culture medium fraction days 2–4, (▨). Asterisks indicate significant difference from control ($P < 0.05$) by analysis of variance and Dunnett's test. Analyses on the proportion of proteoglycans in the matrix are shown in Table 2.

Table 2. Per cent of [$^{35}SO_4$]proteoglycans in the extracellular matrix

	Drug concentrations µg/mL					
	0	0.1	1	10	50	100
Ketoprofen	7.4	7.6	7.9*	7.5	7.5	8.4*
Tiaprofenic acid	7.2	7.6	8.2	7.3	7.7	11.0*
Piroxicam	7.2	7.5	7.7	7.6	8.5*	9.3*
Indomethacin	7.3	7.9	7.7	8.5*	12.1*	9.0*

Effect of NSAIDs on the percentage of [$^{35}SO_4$]proteoglycans, synthesised by chondrocyte monolayers during the period days 0–4 of culture, that are incorporated into the extracellular matrix. Asterisks indicate significant difference from control (0 µg/mL), $P < 0.05$.

was reversed upon removal of the drug for cell, but not explant cultures.

Experiment 3

In this experiment the four drugs, ketoprofen, tiaprofenic acid, piroxicam and indomethacin, were examined for their effects on the proportion of proteoglycans made by cell cultures which remain in the pericellular/intracellular environment. As proteoglycan biosynthesis by chondrocyte cultures was maximal in the early culture intervals, the period of 0–4 days was selected in this single timepoint experiment. Supernatant proteoglycans were measured for days 0–2 and days 2–4, then the cultures were terminated and assayed for matrix proteoglycans which had accumulated over days 0–4.

Total proteoglycan biosynthesis (day 0–4, supernatant plus matrix) was significantly inhibited by all four drugs at 100 µg/mL. Strongest inhibition was by 100 µg/mL indomethacin which reduced proteoglycans to 50% of the control (Fig. 7). Overall, the proportion of matrix-bound proteoglycans in this experiment was about 7%. This proportion was significantly increased by the high concentrations of all drugs (Table 2). The most notable increases were found with indomethacin at 50 µg/mL, and tiaprofenic acid at 100 µg/mL.

Experiment 4

Experiment 3 was repeated for cartilage explant cultures and all five drugs studied. The single timepoint selected was the interval days 4–8, as proteoglycan production by explant cultures was maximal at this time. The supernatants from the culture intervals day 4–6 and day 6–8 were assayed and the cartilage matrix proteoglycans which had accumulated during days 4–8 also were assayed. Results were expressed as labelled proteoglycans per mg wet weight of cartilage, but variances were high due probably to explant heterogeneity.

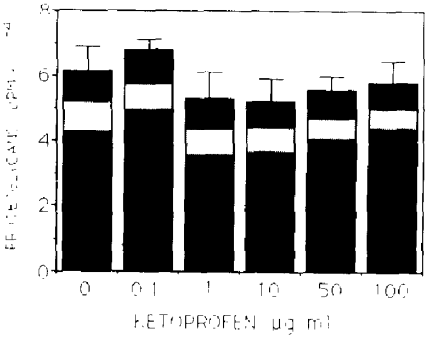

Fig. 8. Effect of ketoprofen on the proteoglycan production by articular cartilage explant cultures. The stacked columns indicate total proteoglycan biosynthesis for the interval days 4–8 (means, N = 4, plus standard errors) comprising: the explant matrix fraction (■); the culture medium fraction for days 4–6 (□); the culture medium fraction for days 6–8 (□). The analysis of variance on total proteoglycans and on the proportion of proteoglycans in the matrix (Table 3) showed no significant differences between ketoprofen concentrations.

The only observed effects on total proteoglycan biosynthesis to reach statistical significance were: inhibition by indomethacin and diclofenac at 100 µg/mL (33 and 10% of their controls, respectively) and stimulation by 1 µg/mL of diclofenac. The proportions of proteoglycans in the matrix and supernatant fractions are shown graphically for one drug, ketoprofen, in Fig. 8. The percentages of matrix-bound proteoglycans are shown for all drugs in Table 3. There was a tendency for per cent matrix proteoglycans to increase at the highest concentrations, as seen with cell cultures, most significantly for indomethacin and diclofenac. There were also significant reductions in the percent matrix proteoglycans by two drugs, indomethacin and piroxicam at 0.1 µg/mL.

Experiment 5

In the above experiments with cell cultures, all wells were initially plated with the same number of chondrocytes but the numbers were not determined

Table 3. Per cent of [$^{35}SO_4$]proteoglycons in the extracellular matrix

	Drug concentration μg/mL					
	0	0.1	1	10	50	100
Indomethacin	70	60*	73	68	76	80*
Diclofenac	69	69	74	67	80*	78*
Piroxicam	80	72*	74	77	77	80
Ketoprofen	68	73	66	69	72	75
Tiaprofenic acid	75	83	76	78	81	84

Effect of NSAIDs on the percentage of [$^{35}SO_4$]proteoglycans, synthesised by cartilage explants during the period days 4–8 of culture, that are incorporated into the extracellular matrix. Asterisks indicate significant difference from control (0 μg/mL), $P < 0.05$.

at the termination of the cultures. Therefore, in Experiment 5, the numbers of cells in the triplicate wells were counted at the end of the experiment (day 11). Care was taken to ensure that all of the wells were highly confluent before the experiment was commenced. As in previous experiments, drugs (10 and 100 μg/mL) were present until day 8 and were then absent from culture media. Proteoglycan secretion into the medium was assayed on days 7–8, and days 10–11. The results are shown in Fig. 9 Cell number was reduced by diclofenac, piroxicam and indomethacin at 100 μg/mL to 62, 81 and 89% respectively of the controls with the latter depression not reaching statistical significance. Presumably the cells detached from the substratum during the experiment and were removed at the time of medium changes. Proteoglycan secretion on day 7–8 was reduced also for these groups, and by more than could be explained by the loss of cells in the case of indomethacin and diclofenac. Ketoprofen and tiaprofenic acid at 100 μg/mL caused no cell loss, but significantly inhibited proteoglycan secretion. Most interestingly, after the drugs had been removed from the cultures, there was a rebound effect, whereby some of the cultures, particularly those where proteoglycan secretion had been inhibited by drugs, responded to the removal of those drugs by the production of proteoglycans well in excess of control levels. Further, the cells exposed to indomethacin and diclofenac at 100 μg/mL, whose numbers had been reduced, showed the strongest rebounds, producing proteoglycans at 222 and 155% control levels on day 10–11. Smaller rebound effects were evident for several other groups (Fig. 9).

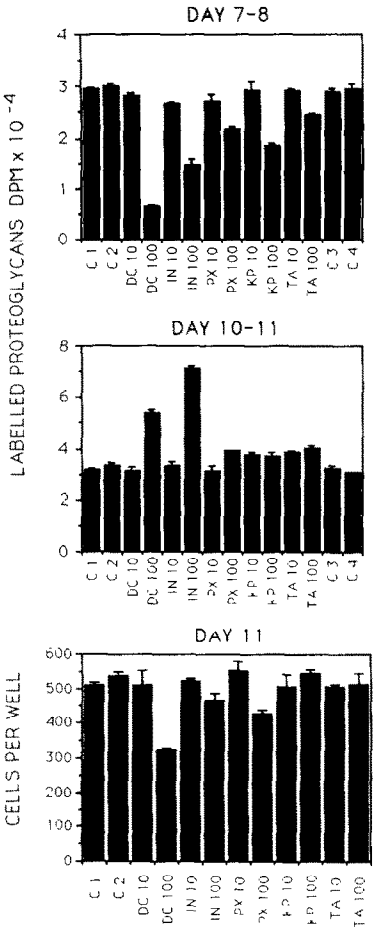

Fig. 9. Effect of long-term culture in the presence of NSAIDs on the final cell number of confluent chondrocyte monolayer cultures. Cultures were grown in the presence of diclofenac (DC), indomethacin (IN), ketoprofen (KP), piroxicam (PX) and tiaprofenic acid (TA) at 10 and 100 μg/mL. Controls (C1, C2, C3, C4) received no drug. Drugs were withdrawn from all cultures on day 8 and cultures were terminated on day 11 when the cells in triplicate wells from each treatment were counted (bottom panel). Proteoglycan secretion into the medium was assayed for the intervals day 7–8 (top panel) and day 10–11 (middle panel). Asterisks indicate significant differences from controls by analysis of variance and Dunnett's test ($P < 0.05$). Shown are means ($N = 3$) and standard errors.

Experiment 6

As NSAIDs are known to be bound by serum proteins, particularly albumin [14, 15], the effects on proteoglycan biosynthesis of 10% foetal calf serum and bovine serum albumin in the medium were examined for diclofenac. Cell cultures were incubated with diclofenac at concentrations of 0, 0.1, 1, 10, 50 and 100 μg/mL. At each drug concentration there were four control wells containing 10% FCS and four wells with 10% FCS plus added bovine serum albumin (BSA, 1 mg/mL). Proteoglycan secretion into the supernatant was measured for the intervals days 0–2 and 2–4. The very similar results were pooled and analyses were done on total (days 0–4) proteoglycan secretion. In the absence of drug, and at 0.1 μg/mL diclofenac, less proteoglycans was released into the medium of cultures containing BSA (89 and 90% of controls, respectively, $P = 0.004$). The BSA-treated groups were not significantly different from the control groups at 1 ($P = 0.08$) and 10 μg/mL ($P = 0.1$) of diclofenac. However the high-dose inhibition of synthesis exhibited by diclofenac was reduced by the presence of 1 mg/mL BSA from 47–63% of the control value with 50 μg/mL diclofenac ($P = 0.0001$), and from 15–24% of the control value with 100 μg/mL diclofenac ($P = 0.0001$).

DISCUSSION

In this study it was our intention to investigate the effects of some of the commonly used NSAIDs on the biosynthesis of proteoglycans by chondrocytes while exposed to the drugs for an extended period *in vitro*. A broad range of drug concentrations was selected and their effects on both chondrocyte monolayer cell cultures, and cartilage explant cultures were measured.

Secretion of newly synthesised proteoglycans into the culture medium could be assayed without destroying the cells and explants, and so was monitored over 12 days in both culture models, for the five NSAIDs, each at six different concentrations (Experiment 1). The most consistent result obtained was the inhibition of proteoglycan secretion by both the cell and explant cultures with high concentrations (50 and 100 μg/mL) of diclofenac and indomethacin at all timepoints studied. Similar findings were obtained with chondrocyte monolayer cultures in a previous study [12], and with indomethacin using human cartilage explant cultures [3]. At lower concentrations of the NSAIDs, which were more comparable with the levels achieved by these drugs in synovial fluid during anti-inflammatory therapy in man, less consistent responses were observed. The results obtained varied over time and between the two models. The chondrocyte monolayer cultures showed significant inhibition of proteoglycan synthesis by indomethacin, at 0.1 μg/mL during the third 48 hr culture interval, and at 1 μg/mL during the third and fourth culture intervals. Ketoprofen at 1 μg/mL significantly reduced proteoglycan secretion of cells during the third culture interval (day 4–6). It may be significant that indomethacin, a drug which is reported to have adverse effects *in vivo* [2] registered the only inhibitory effect on proteoglycan secretion at the lowest concentration tested (0.1 μg/mL). This *in vitro* concentration was well within the range reported in synovial fluid of patients receiving this drug [13].

In the case of cartilage explants, ketoprofen at 0.1 μg/mL had a moderate stimulatory effect on proteoglycan secretion, and piroxicam at 1 μg/mL had a slight inhibitory effect. Both of these results were noteworthy in that they were not part of any apparent "dose–response" trend, however, the results were consistent over the time-course of the experiment. Further experiments are necessary with a range of drug concentrations centred closely around these results of interest. In other studies, piroxicam at concentrations of up to 20 and 25 μg/mL were found not to inhibit proteoglycan biosynthesis of rabbit chondrocytes [9] and porcine cartilage explants [6].

An important difference between the results obtained with the cell and explant cultures was that, after the drugs were removed from the cell cultures, their inhibitory effects on the proteoglycan secretion of chondrocyte monolayers disappeared, whereas with the cartilage explant cultures, the inhibition largely persisted. The stimulatory effect on explants of 0.1 μg/mL ketoprofen also persisted after removal of the drug. These effects were maintained despite a 48 hr "washout" period (day 8–10) preceding the 48 hr drug-free assay period (day 10–12). The most obvious explanation for the difference between the models was that the NSAIDs remained bound to proteins within the cartilage matrix of the explants and continued to influence proteoglycan secretion. It is known that NSAIDs bind strongly to certain proteins [14], and in Experiment 6 of this study, binding of diclofenac by BSA was demonstrated indirectly. If the explant cultures were extended for longer periods after withdrawal of the drugs, a gradual reversal of the drug effects would be expected, and there was indeed evidence that this was underway during the 10–12 day culture interval in Experiments 1 and 2.

Experiment 2 compared the amount of secreted proteoglycan with the amount retained in the cell layer, and explant matrix. Whereas, the majority of proteoglycans produced by explants remain in the matrix, most of the proteoglycans produced by the cell cultures were secreted into the medium. Nevertheless, the patterns observed over time and dose–responses from the matrix and supernatant compartments were surprisingly similar. Proteoglycan levels secreted by explants, although in the minority of total production, were a reasonable reflection of total production in these experiments (2 and 4). Piroxicam and indomethacin at 0.1 μg/mL reduced the proportion of matrix proteoglycans in explants significantly at the single timepoint studied (Table 3). There was no evidence of a reciprocal relationship between matrix and secreted proteoglycans, e.g. an increase in medium proteoglycans and corresponding decrease in matrix proteoglycans, suggesting the release of degraded matrix proteoglycans into the supernatant. Shifts in the proportions of proteoglycans in the two compartments, where they did occur, were modest in magnitude. There were some significant early and transient reductions in the proportions of proteoglycan deposited in the matrix for both cells and explants in the presence of diclofenac (Table 1). The most consistent and greatest effect was the per cent increase in matrix proteoglycans in both cell and explant cultures when incubated with 100 μg/mL of diclofenac. Again, upon removal of the drug, this effect was reversed for cell, but not explant, cultures. This increase in the per cent matrix proteoglycans was also seen at the highest concentrations of the other drugs in cell cultures (Table 2). A similar pattern was evident with all drugs for the explant cultures (Table 3) but due to high variability, not all were statistically significant. Overall, none of the shifts in per cent matrix proteoglycans in the cultures were very large, mostly involving redistributions of less than 10% of the total labelled proteoglycans synthesised.

It was not unexpectedly that the highest concentration of some of the drugs caused detachment and loss of the cells from culture wells, as the levels used could be considered to be approaching toxic concentrations. The greatest reduction in cell number, as well as in proteoglycan secretion (Experiment 5) was observed with diclofenac at 100 μg/mL. What was surprising was that two of the cultures whose cell numbers had been depleted in the presence of high concentrations

of NSAIDs responded to removal of the drugs with secretion of proteoglycans well in excess of control levels (Fig. 9). A smaller "rebound" effect was seen upon removal of several drugs at 10–100 μg/mL, but in contrast to diclofenac, not all of which had previously inhibited proteoglycan secretion. The rebound phenomenon appears not to be a release from the cell layer of stored proteoglycans. There is evidence (Table 1) that diclofenac [and the other drugs (Table 2)] at 100 μg/mL increased the proportion of matrix-bound proteoglycans, however, the total proteoglycan production (matrix plus supernatant) was so low with this drug at this concentration, that the release of stored proteoglycans, upon drug removal, would represent only a fraction of the amounts observed in Figs 6 and 9.

The binding of NSAIDs to serum proteins has been investigated previously in some detail [13, 16] and although 10% FCS was used as a standard supplement to all cultures, experiments were undertaken to evaluate the effects of added albumin on proteoglycan biosynthesis in the presence of various concentrations of diclofenac. These experiments indicated that increasing the medium concentration of albumin by 0.1% could reduce the effective concentration of diclofenac when used at high concentration. This effect was demonstrated by the partial reversal of the dose-dependent inhibition of proteoglycan secretion in cell cultures. However, over the range of concentrations which could be reasonably achieved in synovial fluid (1–10 μg/mL) [13] the addition of BSA to the cultures had little effect on proteoglycan synthesis. As the stoichiometric binding constants of diclofenac to serum albumin are higher than those of indomethacin [16] and NSAIDs of the proprionic acid family [14, 16], it is likely that the results obtained with diclofenac in the present experiments could apply to the other NSAIDs investigated. However, additional studies would be required to confirm this suggestion.

In these studies the most provocative effects of the NSAIDs on the cultures were at concentrations well in excess of the levels generally achieved in synovial fluid. Ten μg/mL was probably the highest drug concentration used *in vitro* with any physiological relevance to the clinical application of these NSAIDs, particularly with regard to the synovial fluid concentration which may reach a steady state of between 2–5 μg/mL [13]. The most interesting result obtained, therefore, was the apparent stimulation of proteoglycan secretion by cartilage explants exposed to 0.1 μg/mL ketoprofen (Fig. 1) which was supported by the experiment shown in Fig. 8. The latter, however, was not significant using analysis of variance. An *in vitro* study on the effects of ketoprofen on the synthesis and catabolism of proteoglycan in normal and osteoarthritic human cartilage [17] was consistent with our observations using this drug. However, although proteoglycan synthesis was reported to be stimulated in young normal tissues, aged and osteoarthritic cartilage were not affected by the drug. Tiaprofenic acid, which is structurally related to ketoprofen (a thiophene ring in place of a benzene ring), also appeared relatively innocuous to cartilage explants, even at superphysiological concentrations (Fig. 5). Similar findings with tiaprofenic acid have been reported by others [5, 7].

Generally we have found confluent chondrocyte monolayer cultures suitable for study over a period of 12 days, at least with NSAID concentrations of ≤10 μg/mL. Chondrocytes are considered to be phenotypically stable for this long *in vitro* [18] and proteoglycan biosynthesis remains fairly stable. While the cell cultures in Experiment 1 showed reduced proteoglycan biosynthesis over the 12 day culture period, fairly steady biosynthetic rates were observed in Fig. 6, and in our previous study [12]. Notwithstanding this limitation, the cell cultures have the advantage that very small variances were obtained. Proteoglycan biosynthesis in explant cultures can be variable (cf. Figs 7 and 8) because of differences in size of specimens, origins of the tissues [19] (e.g. weight-bearing versus non-weight bearing areas, etc.), as well as different proportions of deep and superficial zone cells, the proteoglycan production of which is known to vary [20]. Nevertheless, we regard explant cultures as the better model for studies of this type. Explants are less artificial and they are stable for long periods *in vitro* [21, 22] and the variability can be reduced by increasing the number of replicates per group. Most importantly, however, whereas the dose–response curves of cell cultures changed markedly over time in some cases, the responses of explants were more consistent in long-term culture.

We see a role for studies of this type in screening new and currently used antiarthritic drugs for their effects on proteoglycan metabolism *in vitro*. However, in order to draw valid conclusions from such studies the determined parameters should be consistently and repeatedly demonstrable within the physiological range achieved by the drugs in synovial fluid. Furthermore, the present study shows the importance of monitoring drug effects over several days. This allows for the demonstration of consistent drug effects and is more relevant to the clinical use of NSAIDs which is long-term by nature of the disorders they are used to treat.

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