Inhibition of Tendon Cell Proliferation and Matrix Glycosaminoglycan Synthesis by Non-Steroidal Anti-Inflammatory Drugs in vitro

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INHIBITION OF TENDON CELL PROLIFERATION AND MATRIX GLYCOSAMINOGLYCAN SYNTHESIS BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN VITRO

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The purpose of this study was to investigate the effects of some commonly used non-steroidal anti-inflammatory drugs (NSAIDs) on human tendon. Explants of human digital flexor and patella tendons were cultured in medium containing pharmacological concentrations of NSAIDs. Cell proliferation was measured by incorporation of ^{3}H-thymidine and glycosaminoglycan synthesis was measured by incorporation of ^{35}S-Sulphate. Diclofenac and aceclofenac had no significant effect either on tendon cell proliferation or glycosaminoglycan synthesis. Indomethacin and naproxen inhibited cell proliferation in patella tendons and inhibited glycosaminoglycan synthesis in both digital flexor and patella tendons. If applicable to the in vivo situation, these NSAIDs should be used with caution in the treatment of pain after tendon injury and surgery.

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are often used to manage pain after tendon and ligament injury or surgery. These drugs act by inhibiting cyclooxygenase (prostaglandin synthase) to block the formation of prostaglandins, which are important mediators of the inflammatory process (Vane, 1971). However, there are limited data to support their use for tendon and ligament injuries (Almekinders, 1990; Almekinders and Temple, 1998), and evidence to suggest that some NSAIDs may be harmful to tendon repair (Kulick et al., 1986). These studies were conducted on animals, and their relevance to humans is questionable.

The objectives of this study were to develop an in vitro model of human tendon metabolism, using explant culture techniques similar to those used for the study of NSAIDs and human cartilage matrix metabolism (Dingle, 1991; Dingle, 1996). This model was then used to investigate the effects of four commonly used NSAIDs on tendon cell proliferation and matrix glycosaminoglycan (GAG) synthesis, and to determine whether pharmacological doses of these drugs have deleterious effects on tendon matrix metabolism and the repair process.

MATERIALS AND METHODS

Tendon specimens

Tendon specimens, all waste material, were obtained with consent (and ethical committee approval) from patients during orthopaedic, trauma and plastic surgery procedures. Fourteen specimens of normal digital flexor tendons were obtained from ten patients (age, 24–77 years) during routine hand surgery for trauma or to correct flexion deformities. Fourteen patella tendons were collected from patients (age, 56–77 years) undergoing knee joint replacement for osteoarthritis (OA). Specimens were transported to the laboratory in sterile Hanks buffered salt solution, dissected free of surrounding fat and loose connective tissue, and sub-divided into multiple 2 mm square fragments.

Glycosaminoglycan synthesis

Individual fragments (4–6 replicates) were cultured in 200 µl of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM glutamine, 5% foetal calf serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin containing 10 µCi/ml ^{35}S-SO4 and maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Explants were labelled for up to 20 hours, then rinsed in unlabelled cold media containing 1 mg/ml non-radioactive sulphate, followed by overnight washes in 5% trichloroacetic acid to remove unincorporated radio-label. The explants were lyophilised, weighed, and solubilised at 60°C for 18 hours in a solution of papain (1/200 dilution of a 2x crystallised papain suspension) in 0.1 M phosphate buffered saline pH 7.0 containing 2 mM cysteine and 2 mM EDTA. Triplicate aliquots (20 µl each) of each digest were spotted onto filters, allowed to air-dry, then washed in 10% cetylpyridinium chloride (CPC) for 30 minutes, rinsed in two changes of 1% CPC for 5 minutes and then allowed to air-dry. Filters were saturated in scintillant for scintillation counting (LKB Wallac 1410).

DNA synthesis

Individual explants were cultured in 200 µl medium (see above) containing 10 µCi/ml ^{3}H-thymidine for 20 hours, followed by several washes in fresh medium containing 1 mg/ml non-radiolabelled thymidine, followed by extensive washes in 5% trichloroacetic acid to remove any unincorporated radio-label. Dried explants were solubilised with papain (see above) and duplicate aliquots were counted in a liquid scintillation counter.

Tendon culture and addition of NSAIDs

Four to six replicate pieces of each tendon were placed together in a single well of a 24 well culture plate containing 2.0 ml DMEM supplemented with 1 mM glutamine, 5% FCS, 50 IU/ml penicillin and 50 μg/ml streptomycin. NSAIDs were added to the culture media to give final concentrations in the range of the peak plasma and synovial fluid concentrations (Day et al., 1995; Dingle, 1996). Stock solutions of naproxen, diclofenac and aceclofenac were prepared in DMEM and further diluted in DMEM 5% FCS (plus glutamine and antibiotics). Indomethacin was dissolved in methanol then diluted in DMEM 5% FCS to the working concentration. The final concentrations used were: naproxen, 100 μg/ml; diclofenac, 2 μg/ml; indomethacin, 20 μg/ml; aceclofenac, 10 μg/ml. Explants were cultured for up to 7 days in media containing NSAIDs, alongside control cultures containing media alone, with a complete change of media at day 3 or day 4. At the end of the culture period, the explants were metabolically labelled over a 20 hour period in fresh media (including drugs as appropriate) containing 10 μCi/ml ^{3}H-thymidine and 10 μCi/ml ^{35}S-SO4 and further processed as described above. In some experiments, culture media was retained and frozen for subsequent analysis of prostaglandins (PGE_2) by radioimmunoassay (RIA), or assayed for cytotoxicity using a commercially available kit for detection of the release of cytoplasmic lactate dehydrogenase (Cytotox96, Promega, UK).

Characterisation of the tendon explant culture system

In experiments to characterise the tendon explant culture system, we found no significant loss of collagen (as measured by hydroxyproline release) into the culture medium over 7 days. The explants did not adhere to the culture plastic and there was no significant change in the macroscopic and histological appearance of explants and no cell outgrowth (data not shown). Serum was required for the maintenance of glycosaminoglycan (GAG) synthetic activity beyond 48 hours, with maximal activity obtained using 10% FCS. A concentration of 5% FCS was used in subsequent experiments since this stimulated GAG synthesis to approximately half-maximal levels (data not shown), enabling the detection of any stimulatory effects of NSAIDs as seen in cultures of human articular cartilage (Dingle, 1996). In an experiment to investigate the distribution of the sulphate radiolabel in different compartments, 53% was incorporated into the tendon explant at day 3, with the remaining radiolabelled GAG released into the culture media. After 7 days in culture, approximately twice as much radiolabelled GAG was incorporated into tendon explants compared to day 3, although this represented a smaller proportion (32%) of the total. All subsequent experiments with NSAIDs were conducted using a 7-day culture and 20-hour labelling period so as to maximise the incorporation of radiolabelled glycosaminoglycan into the tendon explant.

Prostaglandin analysis

Concentrations of prostaglandin (PGE_2) in the culture media were determined by radio-immunoassay (RIA), utilising dextran-coated charcoal to separate bound from free ^{3}H-PGE_2, essentially as described elsewhere (Levine et al., 1971). The commercial antiserum used was cross-reactive with PGE_1. Data were calculated as picograms PGE_2/mg tendon dry weight (SD).

Materials

Dulbecco's Modified Eagle's Medium (DMEM), glutamine, antibiotics and FCS were obtained from Life Technologies, Paisley, UK. ^{3}H-thymidine, ^{35}S-SO4 and ^{3}H-prostaglandin E_2 were obtained from Amersham Pharmacia Biotech, Little Chalfont, UK. Naproxen, diclofenac, indomethacin, 2x crystallised papain, ethylenediaminetetraacetic acid (EDTA), L-cysteine HCl, cetylpyridinium chloride (CPC), trichloroacetic acid (TCA), PGE_2 and rabbit polyclonal anti-sera to PGE_2 were obtained from Sigma, Dorset, UK. Aceclofenac was a gift from Prodespharma SA, Spain.

Statistical methods

The incorporation of radioactive thymidine or sulphate was expressed as disintegrations per minute (DPM) per mg of tendon dry weight. Differences between control and drug-treated samples were tested for statistical significance by the Wilcoxon matched pairs signed rank test. A value of $P<0.05$ was taken to indicate statistical significance. Ninety-five percent confidence intervals (CI) are quoted where applicable.

RESULTS

Rates of cell proliferation and GAG synthesis were highly variable within each sample, and the majority of tendons showed a substantial increase in both parameters after 7 days culture (Tables 1 and 2). Four patella specimens were not viable (showed no activity after culture) and were excluded from the analysis. There was no apparent effect of age on the cellular activity of digital flexor tendons, with similar activity in young adults and elderly tendons, both at day 0 and after 7 days culture (Table 1). Although there were significant differences in activity between digital flexor tendons and patella tendons, these could be attributed to a number of variables such as differences in pathology, age or site-related variations and are not shown.

In digital flexor tendons, cell proliferation was inhibited by naproxen to 73% (95% CI, 48–98) of control values. Indomethacin, diclofenac and aceclo

Table 1—Cell proliferation and glycosaminoglycan synthetic activity in normal digital flexor tendons

Patient No	*Age/Sex*	*Cell proliferation DPM ^{3}H-thymidine/mg × 10^{-3}*		*Glycosaminoglycan synthesis DPM ^{35}S-SO_4/mg × 10^{-3}*	
		day 0	*day 7*	*day 0*	*day 7*
1	a) 77 m	0.22	5.95	0.06	0.74
	b) 77 m	0.84	19.47	0.17	0.85
	c) 77 m	0.97	16.36	0.31	1.69
2	a) 31 m	0.38	2.52	0.8	2.33
	b) 31 m	0.72	30.73	0.17	1.38
3	25 m	0.65	2.57	0.08	0.44
4	24 m	1.96	25.58	0.88	5.42
5	26 m	0.07	1.89	0.52	0.96
6	35 m	3.54	8.53	0.47	0.72
7	40 m	0.79	2.41	0.35	0.79
8	65 m	1.00	6.01	0.20	1.15
9	a) 75 m	0.96	2.85	0.045	0.37
	b) 75 m	0.82	1.12	0.07	0.94
10	75 m	0.59	1.10	0.06	0.23
median		0.80	4.4	0.17	0.89

Table 2—Cell proliferation and glycosaminoglycan synthetic activity in patella tendons

Patient No	*Age/Sex*	*Drug treatment at time of surgery*	*Cell proliferation DPM ^{3}H-thymidine/mg × 10^{-3}*		*Glycosaminoglycan synthesis DPM ^{35}S-SO_4/mg × 10^{-3}*	
			day 0	*day 7*	*day 0*	*day 7*
11	59 m	azathioprine	0.69	8.26	0.09	0.48
12	60 f	none reported	0.51	10.55	0.03	0.66
13	69 f	nil	0.26	7.13	0.04	0.18
14	63 f	diclofenac	0.30	15.54	0.08	0.81
15	67 m	indomethacin	0.19	13.28	0.05	2.50
16	77 f	none reported	0.44	7.08	0.07	0.31
17	69 m	nabumetone	0.53	11.31	0.27	1.64
18	56 m	none reported	0.23	28.58	0.07	1.32
19	60 f	none reported	0.17	6.62	0.01	0.35
20	68 m	tenoxicam	0.19	13.45	0.006	0.36
median			0.28	10.93	0.05	0.57

fenac had no significant effect. In patella tendons, cell proliferation was inhibited to 51% (95% CI, 38–64) by naproxen and to 58% (95% CI, 29–87) by indomethacin (Fig 1). GAG synthesis in normal digital flexor tendons was inhibited to 81% (95% CI, 68–94) by naproxen and to 71% (95% CI, 55–87) by indomethacin. In patella tendons, GAG synthesis was inhibited to 66% (95% CI, 46–86) by naproxen and to 78% (95% CI, 58–98) by indomethacin. Diclofenac and aceclofenac had no significant effect (Fig 2).

There was no evidence of cytotoxicity with any of the NSAIDs as measured by lactate dehydrogenase (LDH) release into the culture media. To investigate whether the effects of some NSAIDs were associated with differential activity against cyclooxygenase (COX1 and COX2), levels of PGE_2 were analysed in the culture media by radioimmunoassay. Prostaglandin could be detected in some (but not all) of the control cultures at day 3 and at day 7, but none could be detected in any of the NSAID treated cultures.

DISCUSSION

Our data shows that some NSAIDs have potentially deleterious (inhibitory) effects on human tendon cell proliferation and matrix glycosaminoglycan synthesis in vitro. Our results are consistent with in vivo studies which have shown that ibuprofen and naproxen significantly reduce the repair strength of monkey flexor tendons (Kulick et al., 1986). However, other studies have shown either no effect or even an increase in tendon repair strength after treatment with indomethacin (Carlstedt, 1986; Thomas et al., 1991). Carlstedt showed that after 16 weeks there was a significant increase in tensile strength in repaired rabbit tendons treated with indomethacin. The associated decrease in soluble collagen was ascribed to a higher degree of collagen cross-linking. NSAIDs (diclofenac and flurbiprofen) have also been shown to improve corneal wound strength (McCarey et al., 1995) and indomethacin treatment increases the breaking strength of normal

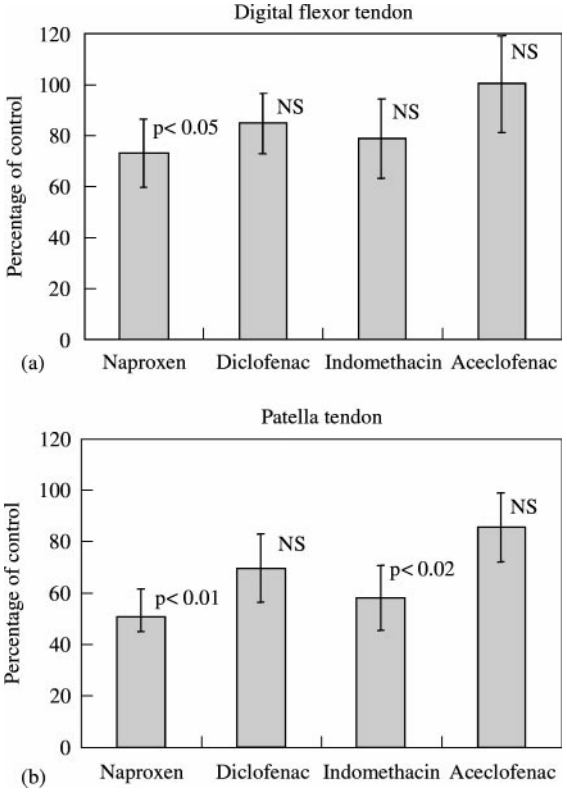

Fig 1 Effect of NSAIDs on tendon cell proliferation. Explants were cultured for 7 days in growth promoting medium with and without pharmacological concentrations of NSAIDs. At the end of the culture period, explants were labelled for 20 hours with 10 μCi/ml ^{3}H thymidine. Incorporated ^{3}H thymidine was calculated as DPM/mg tendon and the effect of NSAIDs was expressed as a percentage of the control. Plotted values represent the mean percentage change (SEM) in 14 normal digital flexor tendons (a) and ten patella tendons from OA patients (b). Statistical significance relative to control values was calculated using the Wilcoxon matched pairs signed rank test.

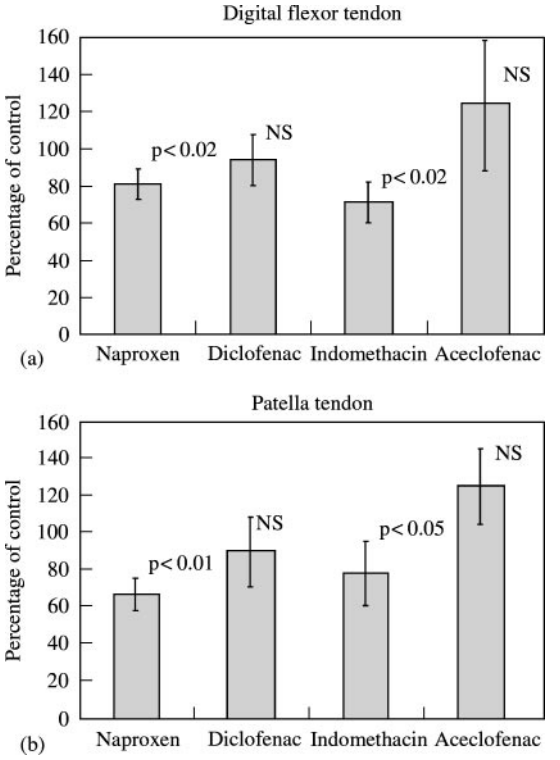

Fig 2 Effect of NSAIDs on tendon glycosaminoglycan synthesis. Explants were cultured for 7 days in growth promoting medium with and without pharmacological concentrations of NSAIDs. At the end of the culture period, explants were labelled for 20 hours with 10 μCi/ml ^{35}S-SO_4. Incorporated ^{35}S-SO_4 was calculated as DPM/mg tendon and the effect of NSAIDs was expressed as a percentage of the control. Plotted values represent the mean percentage change (SEM) in 14 normal digital flexor tendons (a) and ten patella tendons from OA patients (b). Statistical significance relative to control values was calculated using the Wilcoxon matched pairs signed rank test.

rat tendon (Vogel, 1977), although this has no effect on normal rabbit tendons (Carlstedt, 1986). The increased insoluble collagen in treated rat tendons was attributed to a reduction in collagen breakdown (Vogel, 1977), consistent with other studies which have reported an anti-catabolic effect of various NSAIDs (Karpinnen et al., 1995; Ratcliffe et al., 1993). Thus it appears that some NSAIDs can affect both the synthesis and degradation of matrix, and that differences between these studies and our own data can be attributed to a number of factors such as the drug dose, species differences and the use of different NSAIDs.

In agreement with our findings, Almekinders et al. (1995) have shown that indomethacin inhibits human tendon cell proliferation in vitro. This effect was partly offset by mechanical strain, demonstrating that complex cell and matrix interactions may influence the tendon response to NSAIDs in vivo. These authors also reported that indomethacin had a stimulatory effect on tendon protein synthesis (and, therefore, may be beneficial during the remodelling phase of tendon repair), but they did not study its effects on specific proteoglycans or collagens that are important components of the tendon extracellular matrix (Almekinders et al., 1995).

Our studies were conducted with single doses of NSAIDs at approximate peak plasma concentrations, as determined from previous studies and published pharmacological data (Day et al., 1995; Dingle, 1996). Although these plasma concentrations may be obtained for only a few hours in vivo, it has been shown that some NSAIDs can accumulate at a higher concentration in the paratenon and tissues surrounding tendon, at least when applied topically to the skin in the affected area (Rolf et al., 1997).

Unfortunately we were unable to conduct dose-response studies with the limited quantities of tendon available. Although it would have been possible to conduct more wide-ranging experiments on isolated human tenocytes similar to those used by Almekinders et al. (1995), our

explant culture system retains the mixed tendon cell population within the normal matrix and is arguably a better model of the in vivo situation than monolayer cultures on plastic surfaces. Similarly, although more detailed studies could have been conducted on animal tendons, species-related differences in the responses to NSAIDs make any correlation with human tendon questionable (Brandt and Slowman-Kovacs, 1986).

The differential effects of NSAIDs observed in our study were apparently unrelated to inhibition of cyclooxygenase and PG synthesis, and are likely to be a secondary effect of particular NSAIDs. Naproxen and indomethacin for example, but not tiaprofenic acid, have been shown to reduce glucocorticoid receptor expression by synovial cells (Pelletier et al., 1994). Tenidap, but not diclofenac, induces a reduction in IL1 receptor expression (Fernandez et al., 1995; Martel-Pelletier et al., 1996). The inhibitory effect of NSAIDs such as indomethacin and naproxen has been attributed to toxic effects (Dingle, 1996), although we could find no evidence of cytotoxicity in our study. Alternatively it has been suggested that some NSAIDs may possess inhibitory activity against glycosyl transferases, enzymes that are involved in the synthesis of glycosaminoglycans (Hugenberg et al., 1993).

We chose to focus on the synthesis of sulphated glycosaminoglycans, sugars that are major constituents of proteoglycans. Although proteoglycans are quantitatively minor constituents of the tendon matrix, they increase in quantity after tendon injury, modulate the formation of collagen fibres and have a major role in the repair process (Flint, 1972; Gelberman, 1988; Scott, 1988). Our study has shown that NSAIDs such as naproxen and indomethacin significantly inhibit glycosaminoglycan synthesis and (in patella tendons) cell proliferation by tendon explants maintained in vitro. Although caution must be exercised in extrapolating this data to the in vivo situation, the administration of some NSAIDs may be harmful to tendon repair after injury and surgery.

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