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EFFECTS OF INDOMETHACIN ON GLYCOSAMINOGLYCAN METABOLISM IN THE DEVELOPMENT OF EXPERIMENTAL OSTEOARTHRITIS IN RABBITS

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Abstract—The effects of indomethacin on glycosaminoglycan (GAG) metabolism during the development of experimental osteoarthritis produced by the immobilization of the knee in extension were studied in adult rabbits. The rabbits received intraperitoneal injections of indomethacin (10 mg/kg) daily for 17 days. The GAG content of the samples taken from the articular and periarticular connective tissues of the knee was assayed from determinations of hexosamine and uronic acid concentrations following papain proteolysis and subsequent purification. The uptake of ^{35}S-sulphate (calculated as DPM/μg galactosamine) was used as an indicator of the synthesis rate of sulphated GAGs. The controls comprised normal rabbits, rabbits treated with indomethacin, rabbits immobilized for 17 days, and the non-immobilized contralateral leg of the immobilized rabbits receiving indomethacin.

Indomethacin treatment failed to inhibit the uptake of ^{35}S-sulphate in rabbits. The concentration of chondroitin sulphates was normal or elevated in animals receiving indomethacin. The drug did not prevent the loss of GAGs from the weight-bearing cartilage of the immobilized knees, but in tibial marginal tissues it prevented the accumulation of GAGs to some extent.

INTRODUCTION

Immobilization of rabbit hind knee in extension [1] produces a progressive and non-traumatic model of osteoarthritis (OA) with typical macroscopic, radiographic, histological and biochemical changes [1–4]. This experimental disease is "chronic" and leads to joint deformation without the involvement of a primary inflammatory process.

During the development of OA in the rabbit the chondrocyte response to proteoglycan depletion results in an increase in glycosaminoglycan (GAG) synthesis [3, 5], although the magnitude of this response in advanced human disease is debatable [6, 7]. The anti-inflammatory effect of indomethacin is attributed to its inhibition of the biosynthesis of prostaglandins [8], which stimulate the synthesis of both GAG and protein [9, 10]. In animal tissues, including cartilage, anti-inflammatory drugs have been reported to inhibit GAG synthesis, both *in vivo* and *in vitro* [11,12]. Thus it would be interesting to know whether indomethacin treatment is capable of modifying the development of immobilization OA.

The aim of the present study was to investigate the *in vivo* effects of indomethacin on GAG metabolism in connective tissues both in normal rabbits and in animals in the metabolically active stage of developing experimental disease.

MATERIAL AND METHODS

Pilot study. The knee of the right hind leg of 21 adult rabbits was immobilized for up to 7 weeks according to the procedure described by Langenskiöld *et al.* [1]. The rabbits were given 10 mg/(kg.day) of indomethacin (Dumex, Copenhagen, Denmark) intraperitoneally, intramuscularly or rectally from the beginning of immobilization up to the time the rabbits were killed. The control animals not receiving indomethacin consisted of 7 rabbits immobilized for 5 weeks with a subsequent mobilization period of 7 weeks and of 6 rabbits immobilized for 7 weeks without a follow-up period.

The mobility of the knees was estimated weekly using a goniometer. X-ray pictures were taken from the immobilized and contralateral hind limbs at the end of the immobilization and remobilization periods. Degenerative changes were assessed according to established criteria [2]. The knee specimens were also studied with routine histological techniques [1].

Biochemical study. Twenty-six rabbits older than 7 months were used as follows: Group 0 consisted of 9 rabbits living normally in their hutches; group IM of 4 rabbits receiving indomethacin daily for 17 days; group IZ of 8 rabbits with knee immobilization for 17 days; and group IM–IZ of 5 rabbits with knee immobilization and indomethacin administration for 17 days. The drug was dissolved in saline prior to use and given as daily intraperitoneal injections of 10 mg/kg. Twenty-four hours before the animals were killed, they were given 0.3 mCi/kg ^{35}S-sulphate (carrier free, Radiochemical Centre, Amersham, U.K.) intramuscularly. Samples were taken from both the immobilized and the contralateral knee; they consisted of cartilage from the tibial weight-bearing region and the tibial margin, the medial

meniscus and the medial collateral ligament. Samples were deep-frozen if not immediately processed.

ASSAY METHODS

The analyses of tissue samples were carried out as described previously [3]. Briefly, samples were defatted, dried, and digested with papain [13], whereafter proteins were removed by trichloroacetic acid precipitation and centrifugation. After dialysis and lyophilization the GAG-containing retentate was analysed for its uronic acid (UA) content [14]. The acid hydrolysates of the retentate were analysed for their galactosamine (GalN) and total hexosamine contents [15], and the glucosamine (GlcN) content was calculated as the difference between the two. Results were calculated as micrograms per milligram of dry, defatted tissue.

The total radioactivity in separated and purified GAGs was determined with scintillation counting corrected for the decay of the isotope (GAG–DPM/mg dry defatted tissue) and, in the further analyses, was expressed as specific radioactivity in chondroitin sulphates (DPM/μg GalN).

Statistical significances were evaluated by Student's *t*-test. Differences were considered significant when the P-value was <0.05. (The plasma concentrations of indomethacin were assayed at the Dumex Laboratory in Copenhagen by gas chromatography of the samples 2 and 8 hours after the last dosage of indomethacin).

RESULTS

Pilot study

The pilot study was complicated by infections that resulted in 11 of the 15 rabbits receiving indomethacin intramuscularly dying before the planned end of the experiment. The rectal and intraperitoneal administration of the drug was better tolerated, and only 1 rabbit died before the end of the 7-week immobilization period. The plasma concentrations of indomethacin 8 hr after the last intraperitoneal injection ranged from 1.1 to 1.9 μg/ml, and from 0.15 to 0.20 μg/ml after the rectal administration.

No differences were observed between the immobilized indomethacin-treated and immobilized control animals in the X-ray assessment, the degenerative changes [2] being slight or moderate in both groups. The recovery of mobility of the knees after the 5-week immobilization was slightly inhibited in the indomethacin-treated animals in comparison with that of the controls. The histological sections suggested that PAS-positive substances were more abundant in the indomethacin-treated animals than in the controls.

Biochemical study

The main results are presented in Tables 1–4.

Group IM. The daily administration of indomethacin failed to alter the GAG metabolism of cartilage in rabbits living normally as compared with the results of untreated control animals. In the medial meniscus the uptake of ^{35}S-sulphate was enhanced, and in collateral ligaments the concentrations of UA and GalN were elevated and the ratio GlcN/GalN decreased after indomethacin (Tables 3 and 4).

Group IZ. The immobilized knee of the rabbits developed previously described alterations [3, 4] i.e. depletion of GAG in the weight-bearing cartilage of the tibia, increased levels of GAG in other connective tissues of the knee, and an increased uptake of ^{35}S-sulphate in all tissues (Tables 1–4). The only significant changes found in the non-immobilized knee of the contralateral legs, as compared with the legs of group 0, were an increased concentration of GalN in the medial meniscus (Table 3) and a decrease of UA in the collateral ligaments (Table 4).

Group IM–IZ. For the tibial weight-bearing cartilage of the immobilized knees, the results (Table 1) were similar to those obtained in group IZ, except that the GlcN concentration had decreased further and the GalN/UA ratio was higher. In the non-immobilized knees the level of GalN and the ratio GalN/UA rose when compared with that of the controls or with the results from non-immobilized knees in group IZ.

In the tibial marginal tissues of the immobilized legs, the concentrations of UA and GlcN were lower than in group IZ (Table 2). In the non-immobilized legs no significant changes were seen when compared with those of the knees of groups 0 and IM or with the non-immobilized knees of group IZ.

In the medial meniscus of the immobilized knees the ^{35}S-sulphate uptake and GAG concentrations resembled those found in group IZ; only the GlcN concentration was elevated. No significant differences were seen between the immobilized and contralateral legs in group IM–IZ. In the non-immobilized legs the UA, GalN and GlcN concentrations were higher than those in group 0, group IM and in the non-immobilized legs of group IZ. ^{35}S-sulphate uptake was higher than in group 0 or in the non-immobilized legs of group IZ.

In the medial collateral ligaments of the immobilized knees the ^{35}S-sulphate uptake and GAG concentrations were higher than in group 0. These values did not differ significantly from those of group IZ, with the exception of the GlcN concentration, which rose less than usual after immobilization (Table 4). On the non-immobilized side, the UA and GalN concentrations were higher than in group 0 and higher than the values for the non-immobilized knees in group IZ.

The results can be summarized as follows: indomethacin did not inhibit ^{35}S-sulphate uptake in any tissue studied either in normal or in immobilized rabbits. Indomethacin increased or had no effect on GalN concentration, and it inhibited the rise in GlcN concentration (except in the meniscus). Both phenomena were reflected in the lowered GlcN/GalN ratios of the separated GAGs.

DISCUSSION

Indomethacin has been shown to inhibit ^{35}S-sulphate incorporation *in vivo* in inflamed joint tissues in rat adjuvant arthritis [16]. The same inhibition was demonstrated by autoradiography [17] in the chondrocytes of the femoral condyles of healthy rabbits. Anti-inflammatory drugs have also been

Table 1. The radioactivity and content (means and S.D.) of GAG components in tibial articular cartilage (weight-bearing region). For groups IZ and IM–IZ the values for the contralateral (CL) side are listed under those of the immobilized side

	Groups, number (n) and statistical significances (P)											
	0* (n = 9)			IM† (n = 4)			IZ‡ (n = 8)		P	IM–IZ§ (n = 5)		P
DPM/μg GalN	119	±	38	105	±	36	165	± 32	b/CL, 0	188	± 74	a/0
							116	± 42		126	± 39	
GAG-DPM/mg (dry-wt)	3075	±	528	3210	±	1123	4026	± 955	a/0	4428	± 1776	
							3427	± 1114		4133	± 1210	a/0
μg GalN/mg	27.7	±	5.2	30.5	±	4.6	24.4	± 3.4	b/CL	23.6	± 3.7	c/CL
							29.9	± 2.8		33.0	± 1.6	a/0, IZ
μ GlcN/mg	19.7	±	3.2	19.0	±	2.1	17.4	± 2.4		15.2	± 1.6	a/CL, IZ b/0
							20.0	± 4.1		18.7	± 2.8	
GlcN/GalN (molar ratios)	0.75	±	0.25	0.63	±	0.06	0.72	± 0.13		0.65	± 0.11	
							0.68	± 0.16		0.57	± 0.10	c/0
μg UA/mg	33.4	±	4.4	31.8	±	5.2	26.6	± 3.3	b/CL, 0	23.4	± 4.2	b/CL, 0
							32.2	± 3.8		33.0	± 3.1	
GalN/UA (molar ratios)	0.91	±	0.17	1.05	±	0.03	1.00	± 0.10		1.11	± 0.10	a/0, IZ
							1.01	± 0.05		1.10	± 0.10	a/0, IZ

(a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$.
* Group 0: rabbits living normally in their hutches.
† Group IM: normal rabbits getting indomethacin daily for 17 days.
‡ Group IZ: rabbits immobilized for 17 days.
§ Group IM–IZ: rabbits immobilized for 17 days and getting indomethacin daily.

Table 2. The radioactivity and content (means and S.D.) of GAG components in tibial marginal tissue (abbreviations as specified in Table 1)

	Groups, number (n) and statistical significances (P)											
	0 (n = 9)			IM (n = 4)			IZ (n = 8)		P	IM–IZ (n = 5)		P
DPM/μg GalN	338	±	197	314	±	75	788	± 225	c/CL, 0	863	± 203	b/CL, 0
							360	± 203		458	± 221	
GAG-DPM/mg (dry-wt)	1410	±	576	1410	±	945	7401	± 2936	b/IM, c/0	5965	± 2418	b/0, IM
							2070	± 1527		2793	± 2406	
μg GalN/mg	4.90	±	1.50	4.35	±	2.19	9.86	± 4.32	b/CL, 0	6.81	± 1.58	a/0
							5.41	± 1.71		5.59	± 1.85	
μg GlcN/mg	3.84	±	0.78	3.35	±	0.47	5.42	± 0.89	c/0	2.65	± 0.92	a/CL, 0 c/IZ
							4.78	± 1.68		4.02	± 1.35	
GlcN/GalN (molar ratios)	0.93	±	0.42	0.94	±	0.42	0.63	± 0.25	a/CL	0.41	± 0.18	a/CL c/0
							0.93	± 0.38		0.77	± 0.29	
μg UA/mg	6.17	±	1.81	6.44	±	1.45	13.48	± 4.22	b/CL, 0	8.06	± 0.33	a/0 b/IZ
							7.98	± 2.42		7.44	± 1.10	

(a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$.

Table 3. The radioactivity and content (means and S.D.) of GAG components in medial meniscus (abbreviations as specified in Table 1)

	Groups, number (n) and statistical significances (P)					
	0 (n = 9)	IM (n = 4)	P	IZ (n = 8)	P	IM–IZ (n = 5)	P
DPM/μg GalN	220 ± 80	354 ± 155	a/0	496 ± 147	b/CL c/0	564 ± 218	c/0
				264 ± 164		493 ± 138	a/IZ c/0
GAG-DPM/mg (dry-wt)	1006 ± 475	1935 ± 1022		3710 ± 1327	a/IM, c/0	3794 ± 2379	b/0
				1621 ± 1045		3686 ± 1393	a/IZ c/0
μg GalN/mg	4.62 ± 0.79	5.36 ± 1.91		7.59 ± 1.97	a/CL c/0	6.46 ± 1.68	b/0
				6.06 ± 1.48	a/0	7.41 ± 1.22	a/IM c/0
μg GlcN/mg	3.67 ± 0.96	4.05 ± 0.55		3.04 ± 1.05		4.16 ± 0.50	a/IZ
				2.87 ± 0.97		4.83 ± 0.74	a/0 b/IZ
GlcN/GalN (molar ratios)	0.80 ± 0.19	0.80 ± 0.18		0.44 ± 0.26	b/0	0.67 ± 0.15	
				0.50 ± 0.22	b/0	0.66 ± 0.11	
μg UA/mg	5.89 ± 0.66	6.39 ± 1.95		8.06 ± 1.09	b/CL c/0	8.34 ± 1.35	c/0
				6.08 ± 1.62		8.99 ± 1.41	a/IM b/IZ c/0

(a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$.

Table 4. The radioactivity and content (means and S.D.) of GAG components in medial collateral ligament (abbreviations as specified in Table 1)

	Groups, number (n) and statistical significances (P)					
	0 (n = 9)	IM (n = 4)	P	IZ (n = 8)	P	IM–IZ (n = 5)	
DPM/μg GalN	472 ± 192	490 ± 195		847 ± 186	c/CL, 0	980 ± 284	a/CL, 0
				437 ± 242		616 ± 197	
GAG-DPM/mg (dry-wt)	295 ± 65	585 ± 375	a/0	2301 ± 906	b/IM c/0	3216 ± 1016	b/IM c/0
				360 ± 237		975 ± 707	a/0, IZ
μg GalN/mg	0.71 ± 0.22	1.14 ± 0.36	a/0	2.69 ± 0.73	c/CL, 0	3.32 ± 0.66	c/CL, 0
				0.96 ± 0.50		1.47 ± 0.54	b/0
μg GlcN/mg	1.35 ± 0.29	1.36 ± 0.27		2.74 ± 0.80	c/CL, 0	1.63 ± 0.15	a/CL b/IZ
				1.29 ± 0.23		1.39 ± 0.19	
GlcN/GalN (molar ratios)	2.17 ± 0.61	1.25 ± 0.27	b/0	1.04 ± 0.21	a/CL	0.50 ± 0.10	b/CL c/0, IZ
				1.70 ± 0.77		1.01 ± 0.26	a/IZ b/0
μg UA/mg	1.81 ± 0.31	2.29 ± 0.44	a/0	4.44 ± 1.37	c/CL, 0	4.20 ± 0.61	c/CL, 0
				1.45 ± 0.26	a/0	2.67 ± 0.46	c/0, IZ

(a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$.

reported to inhibit GAG synthesis in tissues other than cartilage, both *in vivo* and *in vitro* [11, 12]. In this study we found no suppression of ^{35}S-sulphate uptake in articular connective tissues after the administration of indomethacin. On the contrary, indomethacin seemed to increase ^{35}S-sulphate uptake in the menisci of healthy rabbits and in the menisci and collateral ligaments of the non-immobilized contralateral legs of immobilized rabbits.

In articular cartilage the synthesis rate of keratan sulphate is low [18, 19], and therefore the radioactivities were calculated as DPM/μg galactosamine, corresponding mainly to the radioactivity in chondroitin sulphates, as in the periarticular connective tissues, where the main sulphated GAGs are chondroitin sulphates (CS) [20]. The extent to which our results reflect the real turn-over rate of CS in tissues is not clear, because the elimination rates of inorganic sulphate in animals, as well as its concentration in tissues, was not analysed. A part of the detected increase in the radiosulphate uptake in articular cartilage may also be caused by lowered CS concentration because the newly synthetized material was diluted to a lesser extent than in the controls.

In rats, indomethacin has been found to inhibit fracture healing by delaying new bone formation [21]. In another study [22] retarded bone repair in rats was reported also after the administration of indomethacin. The accumulation of sulphated GAGs precedes the calcification of tissue [23, 24], and osteophyte formation in our animal model is normally seen after only 2 weeks of immobilization [1]. Thus a decreased accumulation of GAGs in the tibial margin of the immobilized knees of rabbits receiving indomethacin may be of importance in the further development of the disease. A comparison of the results from cartilage obtained after immobilization alone with those obtained after immobilization and indomethacin treatment shows that indomethacin failed to prevent the depletion of GAG in weight-bearing cartilage.

Our dosage of indomethacin was high, but the plasma concentrations were comparable to the levels used in earlier studies. The rabbits were killed at a time when the synthesis of GAG in articular tissues was the most enhanced after immobilization [3, 4]. In previous studies histological specimens showed no inflammatory cell infiltration in the knees after immobilization [1], and the system responsible for the activation of GAG synthesis is apparently not caused by prostaglandins, as in immunological arthritis. It remains to be established whether the lack of inhibition of CS synthesis in our experiment with indomethacin is a general phenomenon of osteoarthritis or if it occurs only in immobilization osteoarthritis of rabbits. In view of the widespread use of indomethacin and similar drugs in the treatment of osteoarthritis it is important to elucidate further the effects of these drugs on connective tissue metabolism.

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