[³H]Proline incorporation and hydroxyproline concentration in articular cartilage during the development of osteoarthritis caused by immobilization

A study *in vivo* with rabbits

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(Received 7 August 1981/Accepted 12 August 1981)

Proline metabolism *in vivo* was studied during the development of immobilization osteoarthritis in rabbits. Collagen content was measured as the hydroxyproline concentration of the tissue in question. The incorporation of [³H]proline was used as the indicator for total protein synthesis; collagen synthesis rate was estimated from measurements of the specific radioactivity of hydroxyproline. Cartilage samples from knee and hip joints were analysed after 3, 7, 11, 18, 35 and 56 days of immobilization. The total protein and collagen synthesis rates of the immobilized legs increased and reached a maximum after 11–35 days. Although they decreased thereafter, these rates remained elevated to the end of the experiment. A slight increase in the synthetic activity of the non-immobilized contralateral legs was also detected after 7–18 days of immobilization. The isotope incorporation was markedly higher in tibial marginal tissue than in weight-bearing cartilage. In spite of the increased synthesis, no clear changes were found in the collagen content of the tissues studied during the experiment.

The collagen of articular cartilage is considered to be stable, and, until quite recently, there was no evidence of its being metabolically active. Its turnover is very slow, much lower than that of proteoglycans, the other main macromolecular component of articular cartilage (Maroudas *et al.*, 1976). Earlier reports (Boolet *et al.*, 1963; Mankin & Lippiello, 1970, 1971) have indicated that the total collagen content of the cartilage does not decrease in osteoarthritis as the proteoglycan content does. However, there is now evidence that collagen synthesis is increased in both human (Lippiello *et al.*, 1977) and experimental-animal (Eyre *et al.*, 1975, 1980; Floman *et al.*, 1980) disease, and the synthesis rate is directly related to the severity of the disease process (Lippiello *et al.*, 1977).

Several experimental models have been used for studying degenerative joint diseases (Gardner, 1960; Bentley, 1974). Immobilization of rabbit knee in extension produces progressive osteoarthritic changes with typical roentgenological, histological and macroscopic findings (Langenskiöld *et al.*, 1979). The experimental disease corresponds biochemically to human osteoarthritis, i.e. increased synthesis of sulphated glycosaminoglycans in articular

connective tissues and glycosaminoglycan depletion in weight-bearing cartilage are observed (Eronen *et al.*, 1978; Videman *et al.*, 1979).

The purpose of the present study was to determine the rates of collagen and protein synthesis *in vivo*, as well as the total collagen content of cartilage samples from different sampling sites, during the development of experimental osteoarthritis.

Experimental

Material

The right hind leg of 18 rabbits older than 9 months was immobilized in extension (Langenskiöld *et al.*, 1979) for 3, 7, 11, 18, 35 and 56 days (three animals for each period). At 24 h before the rabbits were killed, they were injected intramuscularly with 0.13 mCi of L-[G-³H]proline/kg. (Intramuscular injection was used instead of intra-articular injection to avoid a possible disturbance in joint metabolism.) The tissue samples consisted of articular cartilage from the following sites of both hind legs: the tibial weight-bearing region, tibial margin (not pure cartilage), femoral condyle and femoral head. The samples were deep-frozen if not immediately processed.

Biochemical methods

The samples were dried and defatted in several changes of acetone and acetone/diethyl ether (1:1, v/v). Constant-weight samples were hydrolysed with 6M-HCl for 16h at 107°C (Stegeman & Stalder, 1967). After evaporation of the acid under vacuum, the samples were analysed for (1) collagen content, by measurement of total hydroxyproline (Stegeman & Stalder, 1967); (2) total radioactivity, by liquid-scintillation counting; and (3) specific radioactivity of hydroxyproline, by measurement with a combined assay using the procedures of Juva & Prockop (1966) and Kivirikko *et al.* (1967).

The assay of specific radioactivity in hydroxyproline consisted of the oxidation of hydroxyproline and primary separation of the oxidation products, mainly by the method of Kivirikko *et al.* (1967). With the exception of the toluene extracts, which were 4 and 3 ml, the volumes were decreased by a factor of 2.5. Chloramine-T solution (0.2M) was prepared in distilled water just before use, and 0.5 mg of proline was added to the samples (up to 0.5 mg of hydroxyproline) for isotope dilution before oxidation. Further purification of extracted hydroxyproline oxidation products was achieved by using silicic acid columns (Bio-Sil A, 200–325 mesh; Bio-Rad Laboratories) (Juva & Prockop, 1966). The radioactivity of the eluate (10 ml) was measured by liquid-scintillation counting and quantified (a diluted sample) by Ehrlich's reagent (Kivirikko *et al.*, 1967).

The total radioactivity (proline incorporated as ^3H d.p.m./mg of dry defatted tissue) was used as the measure of the protein-synthesis rate, and the specific radioactivity of hydroxyproline (as [^3H]-hydroxyproline d.p.m./ μg of hydroxyproline) as the indicator of the collagen-synthesis rate. (All analyses were done in duplicate.)

Statistical methods

Owing to the small number of subjects on each day and the obviously skewed distributions of some of the variables, non-parametric methods were applied.

The values of the measurements at each time point were estimated from medians. The statistical significance of the differences between the treated and untreated legs was evaluated with Wilcoxon's matched-pairs signed-ranks test (Table 1 and Figs. 1–3). In the comparisons between days the Mann-Whitney U-test was used (Table 1).

Results

The results from the immobilized and non-immobilized legs and from different sampling sites are presented in Figs. 1–3, drawn with the medians of the results after each immobilization period. Significances are presented in Table 1 and the Figures.

Table 1. Medians of the differences between the immobilized and non-immobilized legs

	Time (days)	...	3–7		11–18		35–56
	n	...	6		6		6
[Hydroxyproline] ($\mu\text{g}/\text{mg}$)							
Tibial							
Marginal tissue			–3.78	← a →	–13.34*	← b →	0.83
Weight-bearing cartilage			1.14		1.16		–3.70
Femoral							
Head cartilage			1.64		–3.78		–2.95
Condylar cartilage			1.28		–0.44		–6.82
[^3H]Proline incorporation (d.p.m./mg)							
Tibial							
Marginal tissue			561*		1295*		802*
Weight-bearing cartilage			34		40*		104*
Femoral							
Head cartilage			80		32		24
Condylar cartilage			100		158*		188*
Specific radioactivity of hydroxyproline (d.p.m./ μg)							
Tibial							
Marginal tissue			0.884	← b →	2.292*		1.312*
Weight-bearing cartilage			–0.016		0.040	← a →	0.205*
Femoral							
Head cartilage			0.034		0.066		0.125*
Condylar cartilage			0	← b →	0.225*		0.370

* $P < 0.05$ (Wilcoxon's matched-pairs signed-ranks test). $P < 0.05 = b$ and $P < 0.01 = a$ (Mann-Whitney U-test; differences between days).

Fig. 1. *Hydroxyproline concentration in immobilized and non-immobilized legs*

Shown are individual values and the median curves of hydroxyproline concentrations (as µg/mg of dry defatted tissue) in tibial weight-bearing cartilage of immobilized (●) and non-immobilized (○) legs and in tibial marginal tissue of immobilized (■) and non-immobilized (□) legs (a); in femoral head cartilage of immobilized (●) and non-immobilized (○) legs and in femoral condylar cartilage of immobilized (■) and non-immobilized (□) legs (b). The hydroxyproline concentrations were determined by the method of Stegeman & Stalder (1967).

Hydroxyproline concentration

The hydroxyproline content of the tibial weight-bearing cartilage remained constant after a slight transient increase. It varied between 55 and 65 µg/mg during the experiment, and the corresponding values of the immobilized and non-immobilized knees did not differ (Fig. 1a).

In the tibial marginal tissue of the immobilized legs the hydroxyproline concentration decreased gradually, reaching a steady state in 18 days. The content in the corresponding tissue of the non-immobilized legs remained unchanged for up to 18 days and thereafter decreased to the value shown by the immobilized legs (Fig. 1a).

In femoral head and condylar cartilage the hydroxyproline concentration and its slight changes were similar in the immobilized and non-immo-

bilized legs. The values varied from 50 to 75 µg/mg (Fig. 1b).

[³H]Proline incorporation

The incorporation of [³H]proline in the tibial weight-bearing cartilage of the immobilized knees was constant after an initial increase around day 11. From day 11 on, the amount of [³H]proline incorporation in the tibial weight-bearing cartilage of the non-immobilized knees remained below that of the immobilized knees (Fig. 2a).

In the tibial marginal tissue of the immobilized knees the [³H]proline incorporation increased rapidly, reaching a maximum around day 11, whereafter it slightly decreased. It was clearly higher than that of the corresponding tissue of the non-immobilized knees, which also showed an activation

Fig. 2. $[^3\text{H}]$ Proline incorporation in immobilized and non-immobilized legs

Shown are individual values and the median curves of $[^3\text{H}]$ proline incorporation (as ^3H d.p.m./mg of dry defatted tissue) in tibial weight-bearing cartilage of immobilized (●) and non-immobilized (○) legs and in tibial marginal tissue of immobilized (■) and non-immobilized (□) legs ($P < 0.001$) (a); in femoral head cartilage of immobilized (●) and non-immobilized (○) legs and in femoral condylar cartilage of immobilized (■) and non-immobilized (□) legs ($P < 0.001$) (b). The total $[^3\text{H}]$ proline incorporation was measured by liquid-scintillation counting after acid hydrolysis of the tissue.

of protein synthesis after shorter immobilization periods (Fig. 2a).

The articular cartilage of the examined femoral heads showed a $[^3\text{H}]$ proline incorporation that was parallel in the immobilized and non-immobilized legs. In femoral condylar cartilage the $[^3\text{H}]$ proline incorporation was clearly higher on the immobilized than on the contralateral side (Fig. 2b). The maximum $[^3\text{H}]$ proline incorporation of the immobilized femoral cartilage occurred at day 18, whereas that of the tibial cartilage had appeared already at day 11.

Specific radioactivity of hydroxyproline

In the tibial weight-bearing cartilage the specific radioactivity of hydroxyproline had increased 4-fold by day 35 but thereafter decreased. At days 35 and 56 the specific radioactivity in corresponding tissue of the non-immobilized legs was clearly lower than that of the immobilized legs (Fig. 3a).

The specific radioactivity of hydroxyproline in the tibial marginal tissue was more than ten times higher than in the weight-bearing cartilage. The radioactivity had increased to its maximum in the

Fig. 3. *Hydroxyproline specific radioactivity in immobilized and non-immobilized legs*

Shown are individual values and the median curves by hydroxyproline specific radioactivity (as ^3H hydroxyproline d.p.m./μg of hydroxyproline) in tibial weight-bearing cartilage of immobilized (●) and non-immobilized (○) legs and in tibial marginal tissue of immobilized (■) and non-immobilized (□) legs ($P < 0.001$) (a); in femoral head cartilage of immobilized (●) and non-immobilized (○) legs ($P < 0.01$) and in femoral condylar cartilage of immobilized (■) and non-immobilized (□) legs ($P < 0.01$) (b). For full experimental details of the assay of hydroxyproline specific radioactivity, see under 'Biochemical methods' in the text.

immobilized knees by day 11 and in the non-immobilized knees by day 7. The difference between the immobilized and non-immobilized legs was clear (Fig. 3a).

For femoral condylar and femoral head cartilage the difference between the immobilized and non-immobilized knees was clear after 18 and 35 days of immobilization respectively. The increase in specific radioactivity in the legs was parallel.

The results can be summarized as follows. Immobilization caused an increase in both the total protein- and collagen-synthesis rate of cartilaginous tissues. Increased synthetic activity reached a maximum generally after 7–18 days, and the effect was seen also in the tissues of the non-immobilized knees. For the most part, however, the collagen content of the tissues remained unaffected during the experiment.

Discussion

Our present study shows clearly that, in the model of osteoarthritis produced by immobilization, the protein- and, especially, collagen-synthesis rates increase in articular cartilage, at least during the development of the disease. We found previously, in studies based on radi sulphate incorporation in separated glycosaminoglycans (Eronen *et al.*, 1978; Videman *et al.*, 1981), that in the model used, glycosaminoglycan synthesis is enhanced in the beginning of the disease. When considered together, our former and present findings indicate that immobilization is not followed by alterations in glycosaminoglycan or collagen synthesis alone, but, instead, result in an overall increase in the protein synthesis as a chondrocyte response to the harmful effects of immobilization. The same kind of results have been obtained with a model in which osteoarthritis is provoked in dogs by a severing of the anterior cruciate ligament (Eyre *et al.*, 1975, 1980) or in which osteoarthritis follows partial or complete meniscectomy in rabbits (Floman *et al.*, 1980); such results have also been obtained in human osteoarthritis (Lippiello *et al.*, 1977).

The collagen content of the articular cartilage remained rather unchanged during our experiment; this result could be expected on the basis of former studies of osteoarthritis (Mankin & Lippiello, 1970; Lippiello *et al.*, 1977). The lack of material loss, as occurs for glycosaminoglycan in weight-bearing cartilage (Eronen *et al.*, 1978), shows that reparative processes in cartilage can maintain the normal collagen content of the tissue. In tibial marginal tissue the collagen content decreased, but the decrement could have partly been caused by an increased content of other cartilage substances, e.g. glycosaminoglycan and, especially, chondroitin sulphates (Videman *et al.*, 1981).

As in previous studies we found that after the immobilization of one knee the synthetic activity increased in the contralateral non-immobilized knee. Such bilateral changes have been found in studies concerning glycosaminoglycan metabolism (Videman *et al.*, 1981), scanning electron microscopy (Candolin & Videman, 1980) and radiography (Videman, 1980). These bilateral effects could simply be the result of increased mechanical stress in the non-immobilized leg after the immobilization of the other leg. Another possible explanation could be the release of a factor activating connective tissue

from the immobilized joint. The same kind of bilateral effect has also been seen in collagen synthesis in the meniscectomy model of osteoarthritis in rabbits (Floman *et al.*, 1980). The nature of this possible factor and its role in the pathogenesis of osteoarthritis are entirely unknown, however.

This study was supported by grants from the Sigrid Jusélius Foundation and the Finnish Ministry of Education. We wish to express our gratitude to Mrs. Sisko Asp, M.Soc.Sci., for her help in statistical analyses of the results.

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