Proteoglycans of the Knee-Joint Cartilage of Young Normal and Lame Pigs

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Intensive rearing, and restricted activity, induce rapid growth in pigs, but they often become lame. Groups of normal and lame pigs reared intensively were killed when 10 or 25 weeks old. Although there were no differences in the overall composition of the knee-joint cartilage of lame and sound animals, the proteoglycans in the cartilage of the lame pigs were extracted more easily by a standardized sequential procedure and contained a higher proportion of molecules of smaller size as assessed by gel chromatography on 6% agarose and Sepharose 4B. These increased at the expense of both the larger and medium-sized molecules. Differences were most evident at 10 weeks of age, when there was twice as much of the smaller proteoglycans in the cartilage of lame pigs. Despite these size-differences, the compositions of the proteoglycans in corresponding sequential extracts of cartilage of lame and normal groups were the same, as were the changes in chemical composition that accompany development. Proteoglycans from lame animals may have undergone limited proteolysis, thus decreasing their size without changing their composition detectably. As the differences between normal and lame groups were greater at 10 weeks than at 25 weeks of age, the first weeks after birth (when the greatest changes occur in the proteoglycans and in the cartilage) may be a critical period in the maturation of articular cartilage in this species. At this time, rapid gain in weight produced by intensive rearing may be too great for the immature cartilage to bear.

The composition of articular cartilage of the knee joint (stifle joints) of pigs changes both quantitatively and qualitatively from foetal life to old age (Šimůnek & Muir, 1972) and like other kinds of cartilage it contains a varied population of proteoglycans (Brandt & Muir, 1969, 1971*a,b*; Šimůnek & Muir, 1972). Since differences were greatest between foetal cartilage and that of animals only a few weeks old, it was suggested (Šimůnek & Muir, 1972) that in the pig, a species whose young are born fully mobile, the first weeks after birth may be a critical period in the development of articular cartilage. When reared by intensive methods, where weight is gained rapidly and activity is restricted, pigs often become lame, the condition being popularly known as 'leg-weakness'. The present study was undertaken to determine whether there were any differences in the overall composition or in the constituent proteoglycans of the articular cartilage of lame and sound pigs aged 10 weeks and 25 weeks reared under intensive conditions. Some of the results have been published in a preliminary form (Šimůnek & Muir, 1971).

Materials and Methods

Reagents

All reagents were of analytical grade, with the exception of 9-aminoacridine hydrochloride, glucosamine hydrochloride and acetylacetone, which was redistilled (b.p. 133–134°C). For chemical analysis all samples were dried to constant weight at 80°C *in vacuo* over P₂O₅. All the analytical procedures used here were exactly the same as described previously (Šimůnek & Muir, 1972).

Animals

The pigs were the progeny of Large White boars mated with sows produced by crossing Saddleback sows with Landrace boars. A diet containing 16% crude protein was fed *ad libitum*. Four days after birth, the piglets were removed from their mothers and reared in a controlled environment in Biehl units on metal slats until they were 10 weeks old and weighed about 27 kg. Some animals were killed after being classified by a veterinarian as being normal or having the clinical signs of 'leg-weakness', particularly anterior curvature of the tarsus or 'sickle

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hocks' (Vaughan, 1969). The remaining animals were transferred to separate pens (1.83 m × 0.91 m) in which movement was restricted, where they were reared until they were 25 weeks old and weighed about 110 kg. These pigs were also classified in the same way as being lame or sound before they were killed. Four groups of six animals were thus obtained, two groups of sound and two groups of lame pigs, 10 weeks and 25 weeks old.

As soon as the pigs were killed, one knee joint was taken from each animal and frozen in solid CO₂ until required. After thawing, the joint capsule was opened, synovial fluid wiped away and the cartilage shaved from femoral condyles and femoral patellas and finely diced. The cartilage from the six animals of each group was pooled, weighed and a weighed sample removed for the determination of dry weight and for overall analysis as described by Brandt & Muir (1969). The dry tissue was digested with activated papain (EC 3.4.4.10) and portions of the digest were removed for the determination of uronic acid and of hexosamine and hydroxyproline after hydrolysis. Proteoglycans were extracted from the remaining cartilage by a standard sequential procedure, exactly as described previously (Šimůnek & Muir, 1972), in which the cartilage was first extracted with 0.15 M-sodium acetate and then twice with 2 M-CaCl₂ and the uronic acid contents of the extracts were determined. The proteoglycans in each extract were precipitated and purified as described by Tsiganos & Muir (1969a) before analysis and gel chromatography. The residual glycosaminoglycans remaining in the cartilage after the third extraction were obtained after proteolysis (Šimůnek & Muir, 1972). The proteoglycans and glycosaminoglycans thus obtained were examined on gel chromatography exactly as described before (Šimůnek & Muir, 1972).

Results

There were no differences in the overall composition of the cartilages between normal and lame groups at either age (Table 1) and the same proportions of the dry weight were unaccounted for by the analyses.

The physical state of the proteoglycans in the cartilage was assessed by their ease of extraction, by application of the standard procedure used previously (Šimůnek & Muir, 1972). The proteoglycans were more readily extracted from the cartilage of lame animals, particularly of those 10 weeks old, where almost 40% of the total uronic acid was in the first sodium acetate extract, compared with just over a quarter from normal animals (Table 2). The second and third extractions with 2 M-CaCl₂ also removed somewhat more from the cartilage of lame pigs 10 weeks old and hence there was less uronic acid in the cartilage residue compared with normal pigs (Table 2). There were similar though less marked differences in the extractability of proteoglycans from the cartilage of normal and lame pigs at 25 weeks of age and again the most noticeable difference was in the amount in the first extract (Table 2). The hydrodynamic sizes of the proteoglycans in each of the three extracts of the four groups of cartilages were compared by gel chromatography on 6% agarose (Fig. 1) and Sepharose 4B (Fig. 2). The proteoglycans extracted from the cartilage of lame pigs, particularly in the first two extracts, consisted of more smaller molecules. Indeed the total proportion of smallest proteoglycans retarded by 6% agarose in the cartilage of lame pigs was at least twice as high as that of normal animals (Table 3). Moreover, the large proteoglycans excluded from Sepharose 4B were almost lacking in the first two extracts of cartilage from lame animals 10 weeks old (Table 4), and there were also somewhat diminished amounts of medium-sized proteoglycans (Table 3). These differences in molecular size between the normal and lame groups were less marked at 25 weeks of age, but nevertheless persisted throughout (Table 4). The differences in size were not, however, reflected in differences in the chemical composition of the proteoglycans of lame and sound groups. The proteoglycans in sequential extracts changed in composition, but the change was similar in both groups of the same age (Table 5). Moreover, in corresponding extracts of younger and older cartilage, the proteoglycans showed similar differences in composition with age in lame and normal groups.

Table 1. *Composition of knee-joint cartilage of normal and lame pigs 10 weeks and 25 weeks old*

Experimental details are given in the text.

Age of pigs ...	10 weeks		25 weeks	
	Normal	Lame	Normal	Lame
Dry wt. (% of wet wt.)	25.5	26.1	29.9	27.3
Uronic acid (% of dry wt.)	3.05	3.12	3.04	2.96
Collagen (% of dry wt.)	38.2	39.5	49.0	51.8
Glucosamine/galactosamine molar ratio	1:30.2	1:31.1	1:17.0	1:17.7

Table 2. Amount of uronic acid in proteoglycan extracts and residue

The proteoglycans of knee-joint cartilage of normal and lame pigs 10 weeks and 25 weeks old were extracted sequentially with 0.15M-sodium acetate, pH 6.8, and twice with 2M-CaCl₂, pH 6.7. The uronic acid content of each extract was determined, and the uronic acid remaining in the residue was determined after proteolysis. For details see the text.

Age (weeks)	Condition of pigs	% of total uronic acid in cartilage			
		Cartilage extract			Cartilage residue
		0.15M-Sodium acetate	2M-CaCl ₂ (1)	2M-CaCl ₂ (2)	
10	Normal	27.8	23.6	18.2	20.3
10	Lame	39.1	25.0	22.2	12.5
25	Normal	15.8	50.2	6.8	24.6
25	Lame	20.4	45.6	10.1	21.8

Table 3. Proteoglycans in three sequential extracts (see Table 2) retarded on gel chromatography in 6% agarose and Sepharose 4B

The conditions of gel chromatography are given in the legend to Fig. 1. For further details see Šimůnek & Muir (1972). The proportions of gel-chromatographic fractions (measured as uronic acid) are relative to the total uronic acid in the cartilage.

		% of total uronic acid in cartilage					
Age (weeks)	Condition of pigs	Sodium acetate extract	CaCl ₂ extract (1)	CaCl ₂ extract (2)	Total (A)	Total (B)	(B)-(A)
Gel chromatography in 6% agarose							
10	Normal	13.06	7.55	2.18	22.8		
10	Lame	28.15	19.25	7.55	54.95		
25	Normal	4.27	8.03	0.82	13.11		
25	Lame	7.14	17.34	2.22	26.7		
Gel chromatography in Sepharose 4B							
10	Normal	22.5	16.6	9.46	—	48.56	22.76
10	Lame	38.3	23.75	15.31	—	77.36	22.41
25	Normal	11.53	30.6	3.13	—	45.26	32.15
25	Lame	16.32	28.75	5.96	—	51.03	24.33

Table 4. Proteoglycans in three sequential extracts (see Table 2) excluded from Sepharose 4B on gel chromatography

The conditions of gel chromatography are given in the legend to Fig. 1. For further details see Šimůnek & Muir (1972). The proportions of the gel-chromatographic fractions (measured as uronic acid) are relative to the total uronic acid in the cartilage.

Age (weeks)	Condition of pigs	% of total uronic acid in cartilage			
		Sodium acetate extract	CaCl ₂ extract (1)	CaCl ₂ extract (2)	Total
10	Normal	5.28	7.08	8.74	21.1
10	Lame	0.78	1.25	6.88	8.91
25	Normal	4.27	19.9	3.67	27.84
25	Lame	4.08	16.87	4.14	25.09

Fig. 1. Elution of proteoglycans from 6% agarose

A column (2.5 cm $\times$ 100 cm) of 6% agarose was eluted with 0.5 M-sodium acetate, pH 6.5. The uronic acid content of each 6.5 ml fraction was determined. V_0 indicates the void volume of the column. About 15 mg of proteoglycan dissolved in the acetate buffer was applied to the column. Extracts of proteoglycans from cartilage of lame pigs (left-hand curves) or of normal pigs (right-hand curves), (a) 10 weeks old or (b) 25 weeks old, were made sequentially in (1) sodium acetate, and (2) and (3) calcium chloride solutions, as described in the text.

In all four groups, sodium acetate extracted a higher proportion of the smaller molecules (Table 3), which contained more galactosamine and less protein and hexose than proteoglycans extracted afterwards with CaCl_2 (Table 5). In proteoglycans of cartilage,

Fig. 2. Elution of proteoglycans from Sepharose 4B

The conditions were the same as given for Fig. 1 except that Sepharose 4B was used in the column. V_0 indicates the void volume. Left-hand curves, cartilage proteoglycans from lame pigs; right-hand curves, cartilage proteoglycans from normal pigs. See Fig. 1 for other details.

galactosamine is attributable to chondroitin sulphate and glucosamine to keratan sulphate and hence the proteoglycans in corresponding extracts of cartilage from normal and lame animals contained the same proportions of each glycosaminoglycan. The small degree of contamination with collagen, shown by the presence of hydroxyproline, would not account for the differences in protein content (Table 5). As previously noted (Šimůnek & Muir, 1972), the glucosamine/galactosamine molar ratios of the whole tissue (Table 1) and of the extracted proteoglycans (Table 5) in all four groups suggested that there was some material other than proteoglycan in the tissue, which contained predominantly galactosamine.

The lengths of chondroitin sulphate chains of proteoglycans extracted with sodium acetate from cartilage of lame pigs, 10 weeks old, were somewhat shorter than those of normal animals as assessed by gel chromatography on Sephadex G-200, although in

Table 5. Composition of proteoglycans extracted sequentially from pig knee-joint cartilage of normal and lame pigs 10 weeks and 25 weeks old

The extractions were with: (1) 0.15M-sodium acetate, pH 6.8; (2) 2M-CaCl₂, pH 6.7; (3) 2M-CaCl₂, pH 6.7. The proteoglycans in each extract were precipitated and purified (Tsiganos & Muir, 1969a) before analysis.

Age (weeks)	Condition of pigs	Extract no.	Composition (% of dry wt.)					Hydroxyproline	Glucosamine/galactosamine molar ratio
			Uronic acid	Hexosamine	Protein	Hexose	Pentose		
10	Normal	1	28.4	28.3	4.5	5.4	1.3	0	1:29.5
		2	28.1	27.9	7.5	5.5	1.2	0.03	1:12.5
		3	27.0	27.5	8.0	5.9	1.3	0.04	1:12.4
10	Lame	1	28.2	28.0	6.8	5.4	1.2	0.04	1:33
		2	28.3	28.4	7.3	5.2	1.1	0.03	1:14.4
		3	27.4	28.0	7.9	6.1	1.3	0.03	1:11.6
25	Normal	1	27.2	26.6	6.7	4.9	1.2	0.05	1:19.2
		2	27.5	26.2	10.4	5.7	1.1	0.08	1: 8.7
		3	26.9	25.8	10.7	5.4	1.2	0.10	1: 6.5
25	Lame	1	28.0	26.2	6.9	5.2	1.3	0.04	1:18.1
		2	27.3	26.8	7.6	4.8	1.0	0.09	1:10.4
		3	26.8	26.0	8.4	5.4	1.2	0.09	1: 5.6

both groups the elution profiles were symmetrical and similar in shape.

Discussion

Since there were no differences in the overall composition of cartilage from lame and normal groups (Table 1), the material unaccounted for by the analyses, previously noted in human (Muir *et al.*, 1970) and pig (Šimůnek & Muir, 1972) articular cartilage, was similar in amount in both groups of the same age.

There is a varied population of proteoglycans in pig articular cartilage, which changes with age and development (Brandt & Muir, 1969, 1971a,b; Šimůnek & Muir, 1972); those of smaller hydrodynamic size are extracted by neutral iso-osmotic sodium acetate more readily than the larger ones from laryngeal (Tsiganos & Muir, 1969b) or articular cartilage (Brandt & Muir, 1971a; Šimůnek & Muir, 1972). Since there was no difference in the overall composition of the cartilage, including collagen content, the greater ease of extraction of proteoglycans from the cartilage of lame pigs (Table 2) was presumably caused by a higher proportion of smaller proteoglycans in the cartilages of the lame groups, particularly at 10 weeks of age (Figs. 1 and 2; Table 3), where over half were small enough to penetrate 6% agarose and were largely extracted with sodium acetate.

Some of the proteoglycans in cartilage exist as aggregates (Sajdera & Hascall, 1969); these would be

present amongst the proteoglycans prepared by the procedure used here and would be excluded from Sepharose 4B since they are excluded from Sepharose 2B (Tsiganos *et al.*, 1971). The diminished proportions of proteoglycans excluded from Sepharose 4B in the cartilages of the lame groups (Table 4), particularly at 10 weeks of age, suggest that fewer aggregates were present, either because there was less of the aggregating component (Hascall & Sajdera, 1969) or because there was a greater proportion of smaller proteoglycans, which appear to be unable to form aggregates (Hardingham & Muir, 1972).

In the sodium acetate extracts of 10-week-old pigs, the proteoglycans from the lame group had somewhat shorter chondroitin sulphate chains, but this difference was not enough to be detected by a difference in uronic acid content (Table 5) and would probably not wholly account for their smaller molecular size. Xylose is the terminal sugar linking chondroitin sulphate chains to the protein core in proteoglycans (Lindahl & Rodén, 1966) and hence the pentose/uronic acid ratio of proteoglycans is inversely related to the chain length of the chondroitin sulphate. Since the proteoglycans in the various extracts of cartilage from lame and sound groups had the same pentose and uronic acid contents (Table 5), differences in the average length of the chondroitin sulphate chains in the proteoglycans would not seem to be sufficient to account for differences in their molecular size. Pentose was determined by a colorimetric procedure designed to minimize interference from the other sugars that are

present (Tsiganos & Muir, 1966), which gave values for pentose close to theoretical in a proteoglycan from guinea-pig Kurloff cells (Dean & Muir, 1970). Moreover, with a highly purified preparation of chondroitin sulphate good agreement was found between the values of pentose determined by this method and values of xylose determined by gas chromatography (Ewins, 1972).

In normal cartilage at a given age larger proteoglycans contain more glucosamine and protein than smaller ones (Tsiganos & Muir, 1969b; Šimůnek & Muir, 1972). The differences in molecular size found here, however, must have arisen from rather subtle changes in structure because the proteoglycans in corresponding extracts of cartilage of normal and sound animals had the same chemical composition (Table 5). The protein core of the proteoglycans from the lame groups may have undergone limited proteolysis where only a few peptide bonds were cleaved. From the structure of proteoglycans (Mathews & Lozaityte, 1958; Partridge *et al.*, 1961), even very limited proteolysis would produce a large change in hydrodynamic size without detectable changes in chemical composition.

The keratan sulphate and protein contents of proteoglycans from lame and normal groups increased to the same extent between 10 weeks and 25 weeks of age (Table 5), and hence the changes in composition that accompany development (Šimůnek & Muir, 1972) do not appear to have been affected in the lame animals, despite a disturbance in the normal size-range of proteoglycans. Since smaller proteoglycans are more readily extracted by iso-osmotic sodium acetate than are larger ones, the differences between lame and normal groups were most apparent in proteoglycans extracted with sodium acetate. At the various ages examined, the first sodium acetate extract contained a more varied population of proteoglycans than subsequent extracts (Šimůnek & Muir, 1972). Proteoglycans of smallest size may be able to diffuse or leach out of cartilage *in vivo*, which might result in a somewhat faster turnover of proteoglycans in the cartilage of lame animals.

The differences in molecular size of the proteoglycans shown here appeared to be associated with the different clinical state of the lame and sound groups of animals, but it is not known what causes this disease ('leg-weakness'). It is probably attributable to a combination of factors, amongst which rapid growth is a predisposing one subjecting the weight-bearing joints to undue stress (Vaughan, 1971), particularly as bone growth does not keep pace with increasing body weight (Thurnley, 1965). There is no evidence of an infective agent, nutritional deficiency or disturbance in the central nervous system, and affected pigs, whose growth is unimpeded, show no systemic upset or loss of appetite (Vaughan, 1971). The tendency to develop the disease is partly inherited

and occurs in the three breeds, Large White, Saddleback and Landrace, which were crossed to produce the pigs used here (Vaughan, 1971).

Previous work with normal animals showed that most of the quantitative and qualitative changes in pig knee-joint cartilage were mainly finished by 25 weeks of age, the population of proteoglycans being most varied at 10 weeks of age (Šimůnek & Muir, 1972). Since in the lame animals disturbance in the normal range of sizes of proteoglycans was more extensive at 10 weeks than at 25 weeks of age (Tables 3 and 4), it is possible that the first few weeks after birth may be a critical period in the pig, during which articular cartilage is maturing rapidly. At this time intensive rearing may produce a gain in weight that is too rapid for the immature cartilage to bear; the result is perhaps a slow release of lysosomal protease (Dingle, 1961; Fell & Dingle, 1963), particularly cathepsin D, which attacks the proteoglycans of the matrix (Weston *et al.*, 1969; Dingle *et al.*, 1971) giving products of comparatively high molecular weight (Morrison, 1970) in contrast with papain. In other species whose young are not fully mobile at birth articular cartilage may mature more slowly, so that the critical period may be different, as for instance in man where it may be delayed until the child begins to walk. The topographical distribution of collagen, chondroitin sulphate and keratan sulphate in the cartilage of human femoral condyles, which shows a pattern characteristic of the individual irrespective of age or sex (Maroudas *et al.*, 1969; Muir *et al.*, 1970; Kempson *et al.*, 1972), may be determined at some critical period in development. The particular pattern of distribution of these constituents possessed by an individual may be a factor predisposing to degenerative joint disease in later life.

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