The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids

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Lubricin was isolated from bovine ankle, metacarpophalangeal and knee and human knee synovial fluids. The lubricins isolated from the bovine joint fluids had the same amino acid and carbohydrate compositions, but differences were observed in the relative molecular masses. The M_r values of bovine metacarpophalangeal and ankle lubricin determined by light-scattering measurements were about 200 000, whereas values of 132 000 and 143 000 were obtained for the bovine knee lubricin. The human knee lubricin had a similar carbohydrate composition to bovine knee lubricin except for the higher glucosamine content, and the amino acid composition differed slightly. The human sample had a lower glutamic acid content and a leucine/isoleucine ratio of 2:1 compared with 1:1 in the bovine. The M_r value of the human knee lubricin (166 000) was also lower than that of the bovine metacarpophalangeal and ankle samples. The M_r value of the bovine knee lubricin determined by sedimentation-equilibrium measurements was 171 000. The length measurements determined by electron microscopy and also the sedimentation measurements showed considerable polydispersity and indicate that the degree of extension of lubricin molecules can vary. Friction measurements showed that the human knee synovial-fluid lubricin had equivalent lubricating ability in a test system *in vitro* to that observed for lubricin isolated from normal bovine synovial fluids. The lubricating ability of lubricin was concentration-dependent, and each lubricin sample was able to act as a lubricant *in vitro* in an equivalent manner to whole synovial fluid at concentrations that are thought to occur *in vivo*.

In previous studies (Swann *et al.*, 1981a,b) lubricin was isolated from bovine metacarpophalangeal-joint synovial fluid, and at concentrations that are thought to occur *in vivo* this glycoprotein was an extremely efficient lubricant for articular cartilage. Experiments carried out on pooled human synovial fluids obtained from different joints of patients with different joint diseases (Swann *et al.*, 1981c) showed that a similar molecule to bovine metacarpophalangeal lubricin was present, but the chemical composition of these preparations varied. It was not clear from these experiments whether the variations in composition were due to differences in the chemical structure of human lubricin obtained from patients with joint

diseases, to joint-to-joint variations in structure or to the occurrence of other constituents present in human pathological fluids (that were not present in normal bovine fluids) that co-fractionated with the lubricin. Other studies have shown (Swann *et al.*, 1984) that most human synovial fluids from patients with joint diseases were good lubricants, but 8% of the fluids tested had decreased lubricating ability. It therefore became important to determine whether there are structural differences in lubricin isolated from different joints and whether human lubricin has a similar structure and lubricating ability to bovine lubricin.

In order to answer these questions we have isolated lubricin from bovine ankle- and knee-joint

synovial fluids and from the human knee synovial fluids that were good lubricants (Swann *et al.*, 1984), obtained from patients that had degenerative or traumatic joint disease, and determined the structures of these molecules.

Experimental procedures

Synovial fluids

Bovine synovial fluid was aspirated from the ankle and knee joints of adult cattle within 15 min after death. After collection, the fluids were examined visually, and those samples free from blood contamination were frozen and stored at -20°C .

Human synovial fluids were collected and treated as previously described (Swann *et al.*, 1984). Fluids from the knee joints of patients with degenerative or traumatic joint disease that had equivalent lubricating ability to normal metacarpophalangeal synovial fluid were used in this study.

Preparation and purification of lubricin

Two methods were used to isolate lubricin. Method I employed the procedures used in earlier studies (Swann *et al.*, 1977, 1981b) and involved three sequential CsCl-density-gradient procedures followed by two gel-chromatography fractionation steps, with first a Bio-Gel A-5m column and then a Bio-Gel A-15m column. In the present experiments, the chromatographic conditions were modified slightly in that 0.5M-guanidinium chloride/50mM-Tris/HCl buffer, pH 7.2, was used as the eluent for both columns, and before application on to the Bio-Gel A-15m column the lubricin samples were reduced with dithiothreitol (15mg/ml of sample) instead of the 2-mercaptoethanol and iodoacetamide reduction/alkylation procedure used previously.

Method II involved treatment of the synovial-fluid supernatant fraction with *Streptomyces* hyaluronidase (Calbiochem) (100 units of enzyme/l of synovial fluid, pH adjusted to 5.5 with acetic acid) at 37°C for 16h in the presence of proteinase inhibitors (1mM-phenylmethanesulphonyl fluoride, 1mM-p-chloromercuribenzoic acid and 10mM-disodium EDTA). After dilution with distilled water to 3 times the volume, the enzyme digestion mixture was fractionated batch-wise with DEAE-cellulose (Whatman DE-52) (400ml settled volume). The DEAE-cellulose was washed repeatedly with 50mM-sodium acetate buffer, pH 5.8 (total volume 1.2 litres), and then with 1M-NaCl. The 1M-NaCl fraction (1 litre) was concentrated on an Amicon PM-30 membrane and then fractionated by gel-permeation chromatography on a column (4.5 cm $\times$ 100 cm) packed with Bio-Gel

A-5m and eluted with 1M-NaCl. A high- M_r glycoprotein fraction (GP-I, Fig. 1a) was concentrated (Amicon PM-30 membrane filter) and re-fractionated on a Sepharose CL-6B column (2.5 cm $\times$ 100 cm) eluted with 3M-guanidinium chloride/50mM-Tris/HCl buffer, pH 7.2. The GP-II fraction obtained (Fig. 1b) was reduced by the addition of dithiothreitol (15mg/ml of solution) and re-fractionated on a column (2.5 cm $\times$ 200 cm) packed with Bio-Gel A-15m and eluted with 0.5M-guanidinium chloride/50mM-Tris/HCl buffer, pH 7.2. The GP-III fraction (Fig. 1c) was concentrated (Amicon PM-30 membrane filter) and fractionated by CsCl-density-gradient sedimentation [at 125000g (r_{av} , 6.3 cm) for 48 h at 4°C , initial density 1.50 g/ml] (Fig. 1d). Lubricin was isolated from the low-density fractions as indicated in Fig. 1(d).

Analytical procedures and physical methods

The gradient and column fractions were analysed to determine the content of uronic acid (Bitter & Muir, 1962), the absorbance at 280 nm and 230 nm and sialic acid by a manual resorcinol method (Jourdain *et al.*, 1971). Isolated fractions were analysed to determine the amino acid composition by ion-exchange chromatography and the carbohydrate composition by g.l.c. as described previously (Swann *et al.*, 1977, 1981b). Sedimentation-velocity and sedimentation-equilibrium measurements were performed on samples that had been dialysed against 0.5M-guanidinium chloride/50mM-Tris/HCl buffer, pH 7.2. The distribution of solute after equilibrium sedimentation was determined with a microprocessor-controlled automated scanning device (Margosian & Stafford, 1982) and the point-average effective reduced relative molecular masses [$M_r(1 - \bar{v}\rho)\omega^2/RT$] were calculated by using the computer program of Roark & Yphantis (1969). A value of 0.69 was used for the partial specific volume of lubricin. Intrinsic-viscosity measurements were also performed after dialysis of samples against 0.5M-guanidinium chloride/50mM-Tris/HCl buffer, pH 7.2.

Light-scattering measurements

M_r values were determined from the reciprocal of Kc/R_0 extrapolated to zero concentration, measured by using a Chromatix KMX-6 light-scattering device and light at a wavelength of 633 nm, where K , c and R_0 are an optical constant, weight concentration and excess Rayleigh factor respectively. All measurements were made at 20°C at sample concentrations between 0.2 and 1.4 mg/ml in 0.5M-sodium acetate/10mM-Tris/HCl buffer, pH 7.0, that had been filtered through a $0.45\mu\text{m}$ -pore-size Millipore filter. The particle scattering factor was taken as the ratio of the

Fig. 1. Fractionation of bovine ankle synovial fluid by method II (Table I)

(a) Chromatography of the DEAE-cellulose 1M-NaCl fraction on the Bio-Gel A-5m column. The column fractions indicated were pooled to yield the fraction GP-I. (b) Chromatography of the GP-I fraction on the Sepharose CL-6B column after reduction with dithiothreitol. The column fractions indicated were pooled to yield the fraction GP-II. (c) Chromatography of the GP-II fraction on the Bio-Gel A-15m column. The column fractions indicated were pooled to yield fraction GP-III. (d) CsCl-density-gradient sedimentation of fraction GP-III. The gradient fractions indicated were pooled to yield proteoglycan (PG) and lubricin (LUB) fractions. The arrows marked V_0 indicate the column

excess Rayleigh factor obtained at 175.5° divided by that measured at 4° . Particle scattering factors were converted into molecular lengths assuming a rigid-rod model by using previously published Tables (Silver, 1981).

Translational diffusion coefficients were determined from intensity fluctuations of scattered light as a scattering angle of 4.0° as previously described (Silver, 1981; Swann *et al.*, 1981b).

Electron microscopy

Samples of lubricin were prepared for electron microscopy by two procedures (Slayter, 1983). In procedure a, lubricin at 0.05mg/ml in 0.15M-ammonium acetate, pH 7.0, was sprayed as an aerosol at freshly cleaved mica and, after being maintained in a vacuum evaporator overnight at $1.3\mu\text{Pa}$ (10^{-8} Torr), was shadowed with platinum. In procedure b, lubricin was prepared as 0.05mg/ml solution in 50% (v/v) glycerol and the molecules were shadowed with tungsten ($0.1\mu\text{g}/\text{cm}$). After backing with 2.5 nm carbon and coating with Parlodian, the replicas were mounted on standard grids.

Friction measurements

These were performed on samples that had been dialysed against 0.15M-sodium Veronal buffer. A rotating cartilage on glass apparatus was used to measure the relative lubricating ability of fractions and purified lubricin preparations as described previously (Swann *et al.*, 1984). Normal bovine synovial fluid and Veronal buffer were used as a positive and a negative control respectively.

Results and discussion

The yields of lubricin obtained from bovine metacarpophalangeal, ankle and knee synovial fluids by method I in one experiment were 13.1, 12.4 and 14.3mg/l. The yield of lubricin isolated by method II from bovine ankle synovial fluid was 11.5mg/l. The uncorrected yield from the human knee synovial fluid was 29mg/l, which indicates that lubricin probably occurs at higher concentrations in fluids from patients with degenerative or traumatic joint disease. As indicated in Fig. 1(d), fractionation of synovial fluids by method II also led to the isolation of high-density proteoglycans,

void volume and V_i the elution position of bovine serum albumin. The column and gradient fractions were analysed to determine the A_{230} ($\circ$), A_{280} ($\square$) and the sialic acid content ($\triangle$, A_{630}). The density ($\bullet$) of the gradient fractions was also measured. See the text for further experimental details.

the properties of which are outside the scope of the present paper.

The results obtained by analysis of the different lubricin preparations isolated from bovine ankle-joint and bovine and human knee-joint synovial fluids are shown in Table 1. Also included in Table 1 are the chemical compositions and properties of lubricin isolated from bovine metacarpophalangeal synovial fluid. The data for metacarpophalangeal lubricin are the average of previously published values (Swann *et al.*, 1977, 1981*b,c*) and two new preparations. The amino acid compositions are the average of analyses performed on two samples, and the carbohydrate compositions are from analyses performed on single samples. The errors associated with individual amino acid and carbohydrate analysis (not including losses incurred during hydrolysis) are $\pm 3\%$.

Although there are variations in the chemical compositions of the different bovine lubricin samples, they have the same overall chemical composition. The lubricin samples prepared by methods I and II also had essentially the same compositions, and the human knee lubricin had a similar chemical composition to bovine lubricin. The human lubricin, however, differs in that it has

a lower content of glutamic acid and a 2:1 leucine/isoleucine ratio, whereas the bovine samples had a 1:1 leucine/isoleucine ratio. The human lubricin also had a higher glucosamine content.

The physical properties of the bovine knee and ankle and human knee lubricin samples are shown in Table 2 and are compared with previously reported values obtained for bovine metacarpophalangeal lubricin (Swann *et al.*, 1981*b*). The M_r value obtained for the bovine ankle lubricin (method I) sample calculated from laser-light-scattering measurements was 216000 and the length was 197 nm. The M_r values of the bovine knee samples were 132000 and 143000 and the human knee lubricin sample had an M_r of 166000. The calculated length of the bovine and human knee lubricin samples was 173 nm. The M_r (weight-average) of the bovine knee lubricin (method I) sample calculated from sedimentation-equilibrium measurements was 171000. The standard deviation calculated from the average M_w and M_z values was 84000.

A relatively broad range of molecule lengths was obtained from electron-microscope measurements, and the ranges of calculated number-average and weight-average lengths of the lubricin samples

Table 1. Chemical properties of bovine and human lubricin samples isolated from metacarpophalangeal, ankle and knee joints

Joint Method of isolation . . .	Bovine					Human knee I
	Metacarpo- phalangeal I	Ankle		Knee		
		I	I	II	I	
Amino acids (residues/1000 residues)						
Aspartic acid	35	33	29	43	38	38
Threonine	224	229	235	213	219	234
Serine	56	59	62	62	61	60
Glutamic acid	134	140	142	140	133	106
Proline	238	235	237	230	241	244
Glycine	24	26	26	27	26	32
Alanine	90	94	91	93	86	93
Half-cystine	—	—	—	—	—	—
Valine	18	14	12	13	14	15
Methionine	4	3	2	3	4	5
Isoleucine	16	15	14	16	13	11
Leucine	15	15	14	16	14	22
Tyrosine	4	3	3	2	5	3
Phenylalanine	6	4	3	4	7	4
Lysine	119	116	117	124	118	117
Histidine	3	2	2	2	4	7
Arginine	14	12	11	12	17	9
Total amino acids (% w/w)	42.3	44.0	38.6	40.5		40.0
Carbohydrates (% w/w)						
Mannose	0.2	0.2		0.2		0.1
Galactose	14.8	16.7		16.0		15.9
Glucosamine	0.8	0.4		0.5		2.0
Galactosamine	14.1	15.6		13.3		16.9
N-Acetylneuraminic acid	18.6	16.1		17.1		16.5

Table 2. Physical properties of lubricin samples

Joint	Bovine				Human knee I
	Metacarpo- phalangeal I	Ankle I	Knee I II		
Method of isolation . . .					
Intrinsic viscosity* (ml/g)	92			120	
Sedimentation					
$s_{20,w}^0$ * (S)	4.84			5.35	
$M_r \times 10^{-3} \parallel$	200		171 (84)		
Light-scattering					
$M_r \times 10^{-3} \dagger$	206	216	132	143	166
$A_2 \times 10^3 \ddagger$		-3.25			-2.21
$D_{20,w} \S$	1.10	0.97			1.01
$P(175.5^\circ) * \S$		0.55	0.60	0.61	0.61
Length (nm)		197		173	173
Electron microscopy					
Length $\parallel$ (nm)					
(a) $\bar{L}_n$ (i)	195 (53)		164 (61)		172 (43)
$\bar{L}_w$ (ii)	210		186		182
(b) $\bar{L}_n$ (i)		119 (22)	125 (25)		120 (24)
(ii)			135 (59)		155 (49)
$\bar{L}_w$ (i)		123	130		125
(ii)			160		173

* The errors associated with these values are estimated to be $\pm 5\%$.
† The errors associated with these values are estimated to be $\pm 10\%$.
‡ Second virial coefficient.
§ The $D_{20,w}$ and particle scattering factors [$P(175.5^\circ)$] are intensity-average values.
|| The numbers in parentheses are standard deviations.

observed by procedure b were 119–155nm and 123–173nm respectively. The average length values obtained for the bovine and human knee lubricin samples were somewhat larger when samples were processed for electron microscopy by procedure a. The average lengths of the bovine and human knee lubricin samples were slightly smaller than the values obtained previously for bovine metacarpophalangeal lubricin. The intrinsic viscosity of a bovine knee lubricin (method II) sample was 120ml/g and the $s_{20,w}^0$ value of this sample was 5.35S.

When the lubricating abilities of lubricin preparations were determined (Fig. 2), the bovine knee lubricin and a human knee sample at concentrations of 100µg/ml had equivalent lubricating abilities to whole bovine synovial fluid. At lower concentrations there were increases in friction values, particularly at the lower speeds, and the values approached those obtained with Veronal buffer, used as a negative control. Essentially the same results were obtained with bovine knee lubricin isolated by methods I and II and with lubricin isolated from bovine metacarpophalangeal and ankle-joint fluids.

General discussion

The previous studies on the structure and properties of lubricin were performed on samples

isolated from normal adult bovine metacarpophalangeal-joint fluids by method I (Swann *et al.*, 1977, 1981*a,b*). An alternative isolation procedure (II) was also used in the present studies, but this possessed no overall advantage and procedure I is considered to be the best method currently available for the preparation of lubricin.

The chemical analyses of lubricin isolated from bovine metacarpophalangeal, ankle- and knee-joint synovial fluids show that this molecule has a similar structure and occurs in similar concentrations in these joint fluids. Lubricin isolated from the knee synovial fluid of patients with degenerative or traumatic joint disease, however, occurred in higher concentrations, had a slightly different amino acid composition and a consistently higher content of glucosamine. It therefore appears that there are species-related differences in the structure of the peptide and oligosaccharide side chains of lubricin molecules. Variations were also observed in the M_r values of the different lubricin samples and in the lengths of the molecules calculated from electron-microscope measurements.

One of the contributing factors to the apparent differences in the length and M_r values obtained by the different physical methods is thought to be the occurrence of molecules with different sizes, the relative distributions of these molecules in the

Fig. 2. Friction measurements (detector response) at various speeds of rotation with bovine knee (a) and human knee (b) lubricin samples as test lubricants in the cartilage on glass apparatus

The curves obtained with Veronal buffer (◆) and bovine synovial fluid (●) used as control lubricants are shown. The curves obtained with the lubricin samples at 100 µg/ml were the same as those obtained with bovine synovial fluid. The curves obtained with lower concentrations of lubricin at 75, 50, 30, 20 and 10 µg/ml are shown by the curves with △, ■, □, ▲ and ○ symbols respectively.

samples examined and the apparent ability of lubricin to occur in extended and less-extended forms. This view is supported by the observation that lower length values were obtained when samples were prepared for electron microscopy as a solution in 50% glycerol (Table 2). Our interpretation of these data is that lubricin occurs in synovial fluids as a polydisperse population of molecules and that differences can occur in the M_r and conformation of the molecules.

The physiological significance of these differences, however, is difficult to determine. On the basis of the friction measurements (Fig. 2), we could not distinguish between the lubricating abilities of lubricins isolated from the different bovine and human joint fluids. It therefore appears that each of the lubricin samples contained a sufficient number of molecules with the required structures to function efficiently as a boundary lubricant for articular cartilage in the test system *in vitro*.

The present study provides additional data that lubricin is the synovial-fluid constituent responsible for the boundary lubrication of articular cartilage. Lubricin with apparently the same structure has now been isolated from three different bovine joint fluids, and lubricin from human synovial fluids has a similar chemical structure and specific activity as a lubricant *in vitro* to bovine lubricin.

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