Role of hyaluronan chain length in buffering interstitial flow across synovium in rabbits

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1. Synovial fluid drains out of joints through an interstitial pathway. Hyaluronan, the major polysaccharide of synovial fluid, attenuates this fluid drainage; it creates a graded opposition to outflow that increases with pressure (outflow 'buffering'). This has been attributed to size-related molecular reflection at the interstitium–fluid interface. Chain length is reduced in inflammatory arthritis. We therefore investigated the dependence of outflow buffering on hyaluronan chain length.

2. Hyaluronan molecules of mean molecular mass ~2200, 530, 300 and 90 kDa and concentration 3·6 mg ml^{-1} were infused into the knees of anaesthetized rabbits, with Ringer solution as control in the contralateral joint. Trans-synovial drainage rate was recorded at known joint pressures. Pressure was raised in steps every 30–60 min (range 2–24 cmH_2O).

3. With hyaluronan-90 and hyaluronan-300 the fluid drainage rate was reduced relative to Ringer solution ($P < 0{\cdot}001$, ANOVA) but increased steeply with pressure. The opposition to outflow, defined as the pressure required to drive unit outflow, did not increase with pressure, i.e. there was no outflow buffering.

4. With hyaluronan-530 and hyaluronan-2000 the fluid drainage rate became relatively insensitive to pressure, causing a near plateau of flow. Opposition to outflow increased markedly with pressure, by up to 3·3 times over the explored pressures.

5. Hyaluronan concentration in the joint cavity increased over the drainage period, indicating partial reflection of hyaluronan by synovial interstitium. Reflected fractions were 0·12, 0·33, 0·25 and 0·79 for hyaluronan-90, -300, -530 and -2200, respectively.

6. Thus the flow-buffering effect of hyaluronan depended on chain length, and shortening the chains reduced the degree of molecular reflection. The latter should reduce the concentration polarization at the tissue interface, and hence the local osmotic pressure opposing fluid drainage. In rheumatoid arthritis the reduced chain length will facilitate the escape of hyaluronan and fluid.

The volume of synovial fluid in most healthy joints is very small, namely ~50 μl in the rabbit knee and 300 μl in the dog knee. Its turnover time, however, is an order of magnitude faster than that of the general interstitial fluid pool, with a turnover time of ~2 h in rabbit knee. The maintenance of a small volume of synovial fluid in the face of rapid turnover implies a close coupling between drainage rate and input rate, since even a small, sustained imbalance would quickly lead to joint swelling or fluid depletion. Sustained flexion is a particular threat to volume homeostasis because it raises intra-articular pressure, driving fluid out of the joint cavity (Levick *et al.* 1999). Hyaluronan in joint fluid partly counters this threat by 'buffering' the fluid drainage rate. That is to say, hyaluronan generates a dynamic, graded opposition to fluid loss that increases as joint pressure is increased (McDonald & Levick, 1995). As a result the drainage rate attains a plateau, and the plateau flow is very low, namely 3–5 μl min^{-1} in the rabbit knee.

The buffering of outflow by hyaluronan is thought to arise as follows. The flexible hyaluronan chain, stiffened by H-bonds and expanded by internal electrostatic repulsion, encompasses a very large, roughly spherical domain of solvent of radius 100–200 nm (Fujii *et al.* 1996; Gribbon *et al.* 1999). Adjacent molecular domains overlap at $\geqslant$ 1 mg ml^{-1}, which causes chain–chain entanglement and other interactions. These create a quasi-infinite, dynamic

network of loosely linked polymer chains (Cowman *et al.* 1998; Wik & Wik, 1998; Scott & Heatley, 1999). As a result, hyaluronan molecules do not escape through the interstitial drainage pores in the synovial lining (radius 30–90 nm) as easily as water or albumin, and hyaluronan is partially reflected during fluid drainage (Coleman *et al.* 1997; Scott *et al.* 1998). It has been suggested that the reflected hyaluronan forms a concentrated layer at the tissue–fluid interface (a 'concentration polarization' boundary layer), and that this layer exerts sufficient osmotic pressure to oppose fluid escape. Since the boundary layer concentration increases when drainage rate is increased, the osmotic opposition to fluid drainage from the cavity should increase too, in conformity with the experimental observations. Numerical analysis and work *in vitro* have supported this mechanistic hypothesis (Barry *et al.* 1996; Coleman *et al.* 1999).

The buffering action of hyaluronan has been evaluated at concentrations ranging from those in normal joints (up to 4 mg ml^{-1}) to those in severe inflammatory arthropathies (down to 0·2 mg ml^{-1}). Reductions in concentration cause loss of outflow buffering, in conformity with the osmotic boundary layer hypothesis (Scott *et al.* 2000*a*).

Severe arthropathies are associated with a reduction in both concentration and chain length of hyaluronan (Balazs *et al.* 1967; Dahl *et al.* 1985). The reduced chain length is attributed to the synthesis of shorter chains (Castor & Dorstewitz, 1966; Vuorio *et al.* 1982; Dahl & Husby, 1985) and/or cleavage by free oxygen radicals (McCord, 1974; Greenwald & Moak, 1986; Baker *et al.* 1989; Halliwell, 1995; Haubeck *et al.* 1995; Schenck *et al.* 1995). Chain length, as well as concentration, can be expected to determine the drainage-attenuating action of hyaluronan, because shorter chains occupy smaller volume domains and should therefore be less well reflected by the interstitial pores. In support of this, early results from our laboratory using human umbilical hyaluronan of subnormal size, namely of weight-average molecular mass $\sim 0{\cdot}7 \times 10^6$ Da (McDonald & Levick, 1995), indicated weaker buffering than did later results with rooster comb hyaluronan of $\sim 2 \times 10^6$ Da (Coleman *et al.* 1999). The aim of the present investigation was, therefore, to examine directly the role of chain length in the buffering of fluid drainage by hyaluronan, using a set of 'tailor-made' chain lengths constructed from rooster comb hyaluronan. A preliminary report has been published (Scott *et al.* 1999).

METHODS

Materials

Rooster comb hyaluronan (Sigma Chemical Co.) was used throughout at 3·6 g l^{-1}, a concentration characteristic of rabbit and young human knee joints (Balazs, 1982; Price *et al.* 1996). The vehicle was Baxter Ringer solution (147 mM Na^+, 4 mM K^+, 2 mM Ca^{2+}, 156 mM Cl^-, pH 7·2; Baxter Healthcare Ltd, Thetford, Norfolk, UK) adjusted to pH 7·4 with drops of NaOH solution.

Sonication of hyaluronan to specified average chain length

Controlled, progressive cleavage of hyaluronan chains can be induced by ultrasound (Orvisky *et al.* 1993). Preliminary studies defined the relation between sonication time, ultrasound intensity and reduction in chain length. Samples comprising 10 ml of 3·6 mg ml^{-1} rooster comb hyaluronan of weight-average molecular mass $\sim 2{\cdot}2 \times 10^6$ Da were sonicated at amplitudes of 14 or 22 μm for up to 1200 s in a Soniprep (MSE Scientific Instruments, Crawley, UK) and analysed by size-exclusion high performance liquid chromatography (HPLC; see below). The column retention time, R_{min} (in minutes), was found to increase as a negative exponential function of sonication time, S (in seconds), indicating progressive, limited cleavage of the chains. Our results were well described by the expression $R_{min} = k_1 + k_2(1 - e^{-S/z})$, where $k_1 = 7{\cdot}25$ and 7·33, $k_2 = 1{\cdot}55$ and 1·53, and $z = 408$ and 250 for 14 and 22 μm, respectively (correlation $r^2 = 0{\cdot}99$). These expressions were used to predict the times needed to generate hyaluronan molecules of chain lengths from ~100 to 2200 kDa for infusion *in vivo*. Sonicated products were checked by HPLC.

Hyaluronan analysis by HPLC

Mean molecular masses and concentrations were determined by HPLC (Waters Ltd, Watford, UK), using a size-exclusion TosoHaas TSK G6000 PW_{XL} column (Anachem Ltd, Luton, UK) and absorbance at 206 nm (Beaty *et al.* 1985). Hyaluronan standards of average molecular mass 210, 790, 900, 1200, 2000, 3900 and 5500 kDa were generously donated by Dr O. Wik (New Pharmacia, Uppsala, Sweden). Since the standard molecular masses were determined by light scattering, the HPLC-determined values are taken to represent the weight-average molecular mass, M_w. The column retention time was found to be related to M_w by $R_{min} = 16{\cdot}22 - 1{\cdot}416 \log M$ (correlation $r^2 = 0{\cdot}99$).

Viscometric evaluation of molecular domain volume, radius and critical overlap concentration

The relative viscosity η_r of each sonicate was measured in an Ostwald viscometer at 23–26 °C at concentrations (C) of 0·9–3·6 mg ml^{-1}, with Ringer solution as the reference fluid. Intrinsic viscosity $[\eta]$ was determined by linear extrapolation of the plot of reduced viscosity, $(\eta_r - 1)/C$, *versus* C to zero concentration. Intrinsic viscosity represents the space occupied by a gram of solute at infinite dilution, and is a sensitive index of chain length.

The quasi-spherical domain of solvent occupied by a flexible polymer chain is usually characterized by the radius of gyration R_g (root mean square of segment distances from molecular centre of gravity). R_g was determined from the measurements of $[\eta]$ and molecular mass M using the relation $R_g^3 = M[\eta]/8{\cdot}84N_A$, where N_A is Avogadro's number (Flory, 1971).

The 'critical concentration' C^* at which adjacent molecular domains overlap was defined by de Gennes (1979) as the concentration at which the number of chain segments per unit volume of solution is the same as the number of chain segments per unit volume of molecular domain at extreme dilution. This definition, in combination with Flory's model, gives the simple expression $C^* = 2{\cdot}1/[\eta]$. The latter was used to evaluate C^* for each sonicate from its intrinsic viscosity.

Physiological methods *in vivo*

Trans-synovial fluid drainage from the cavity of rabbit knees was measured at controlled, incremental intra-articular pressures in the presence of 3·6 mg ml^{-1} hyaluronan of nominal molecular mass 90 kDa (7 rabbits), 300 kDa (5 rabbits), 530 kDa (6 rabbits) and

2200 kDa (12 rabbits). The last of these were a subset of the results reported by Coleman *et al.* (1999). Contralateral knees were infused with Ringer solution as a control.

Animal preparation and measurement of pressure and volume drainage rate. New Zealand White rabbits weighing 2–3 kg were anaesthetized with 30 mg kg^{-1} sodium pentobarbitone plus 500 mg kg^{-1} urethane I.V. and tracheostomized. Anaesthesia of sufficient depth to abolish the corneal blink reflex was maintained by 15 mg sodium pentobarbitone plus 250 mg urethane I.V. every 30 min. The infusion and recording systems were as described by Coleman *et al.* (1999). Briefly, an intra-articular cannula connected to a water-calibrated differential pressure transducer measured intra-articular fluid pressure P_j. A second intra-articular cannula was connected to an infusion reservoir, the height of which controlled P_j. Flow of solution into the joint cavity from the reservoir, $\dot{Q}_{in}$, was recorded by a photoelectric drop counter. An initial infusion raised P_j from a subatmospheric pressure in extensions to ~2–3 cmH_2O to generate a measurable trans-synovial drainage rate. P_j was increased in steps of ~2–4 cmH_2O by raising the infusion reservoir at 30–60 min intervals, at which times the flows were in a steady state. At the end of each period the net trans-synovial drainage rate, $\dot{Q}_s$, was calculated from $\dot{Q}_{in}$ by subtracting the volumetric creep of the cavity walls as described previously (Coleman *et al.* 1999). Experiments continued to ~24 cmH_2O, which is in the range found in arthritic effusions.

Procedures conformed to UK legislation. Animals were killed by an overdose of I.V. sodium pentobarbitone at the end of the experiment.

Measurement of hyaluronan reflection by the synovial lining. At the end of the experiment samples were aspirated from the infusion line and from the joint cavity after mixing the joint fluid by a series of flexion–extension cycles. Hyaluronan concentrations and molecular masses were analysed by HPLC. The hyaluronan reflected fraction, namely (mass in filtrand − mass in filtrate)/mass in filtrand, was calculated from the increase in the intra-articular concentration and cumulative fluid drainage during the experiment, with corrections for the slight secretion of hyaluronan *de novo* and the small quantity of endogenous hyaluronan, as described by Scott *et al.* (1998).

Statistical methods

Slopes (conductances) were fitted by linear regression analysis. Sets of paired conductances were compared by Student's paired t test. Since P_j varied a little between experiments, flows were interpolated to standard pressures at 2·5 cmH_2O intervals by linear interpolation between the two bounding measurements to enable comparisons at identical pressures. Flows were compared by two-way analysis of variance (ANOVA), with repeated measures and Bonferroni's *post hoc* test as appropriate. $P < 0{\cdot}05$ was accepted as a significant difference. Means are followed by S.E.M. throughout.

RESULTS

Average molecular masses after sonication

Sonication caused a graded reduction in the average chain length (Fig. 1). The spread of the HPLC peak showed little change, indicating that polydispersity was not much affected. The products used for infusion *in vivo* had weight-average molecular masses of 88 ± 6 kDa ($n = 7$), 305 ± 6 kDa ($n = 4$), 527 ± 56 kDa ($n = 4$) and 2230 ± 47 kDa ($n = 16$). Since unit disaccharide mass is 379 Da the average chains comprised ~232, 805, 1390 and 5884 disaccharides, respectively. Chain lengths are given in Table 1. For convenience these preparations are referred to as hyaluronan-90, hyaluronan-300, hyaluronan-530 and hyaluronan-2200, respectively, based on rounded molecular masses. Hyaluronan-660 refers to umbilical hyaluronan of mass ~662 kDa as used by McDonald & Levick (1995).

Viscosity, domain radius and overlap concentration of sonicates

The relative viscosities of 3·6 mg ml^{-1} solutions in the Ostwald viscometer were 3·46 for hyaluronan-90, 5·18 for hyaluronan-300, 9·36 for hyaluronan-530 and 107 for hyaluronan-2230. The solvent volume occupied by a gram of

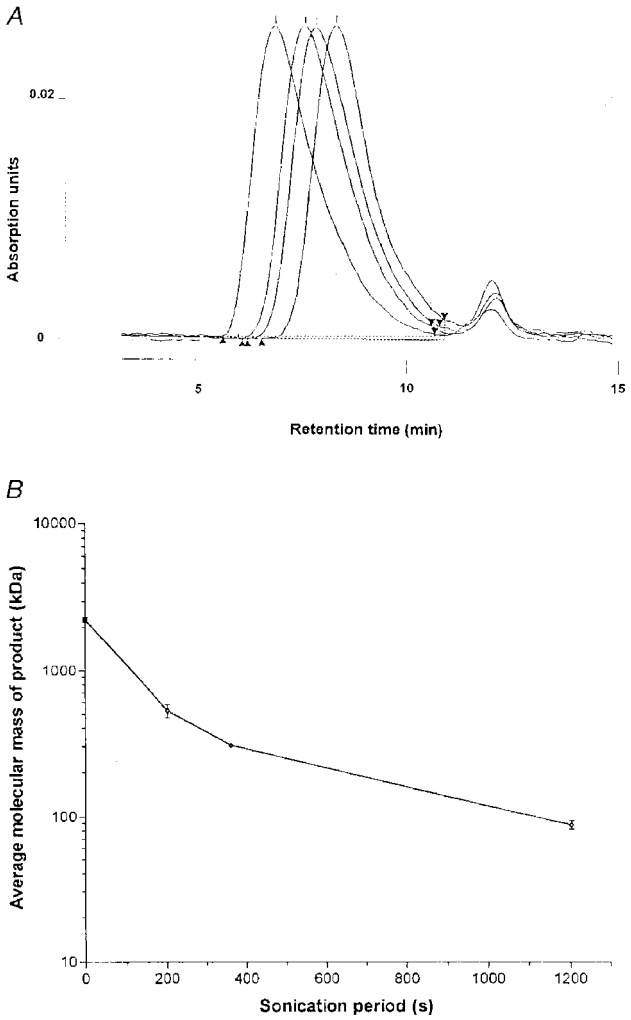

Figure 1. Relation between sonication period and average molecular mass of infused hyaluronan

A, exclusion column elution profiles for starting material (left) and progressively sonicated products (3·6 mg ml^{-1}). *B*, relation between sonication time and hyaluronan size. Samples of weight-average molecular mass (M_w) 2230, 527, 305 and 88 kDa were produced by 0 s sonication, 200 s sonication at 14 μm, 360 s sonication at 14 μm, and 1200 s sonication at 22 μm, respectively.

Table 1. Average molecular parameters of infused hyaluronan molecules

	Hyaluronan-90	Hyaluronan-300	Hyaluronan-530	Hyaluronan-660 [a]	Hyaluronan-2200
M_w (kDa)	88 ± 6	305 ± 6	527 ± 56	662	2230 ± 47
Chain length (nm) [b]	220	765	1320	1659	5590
D_{20} (10^{-7} cm^2 s^{-1}) [c]	1·93	0·82	0·56	0·48	0·21
$[\eta]$ (ml g^{-1})	524	625	873	1456	2953
R_g (nm)	20	33	44	57	107
C^* (mg ml^{-1})	4·0	3·4	2·4	1·4	0·7
Reflected fraction	0·12	0·33	0·25	—	0·79

M_w, weight-average molecular mass; D_{20}, diffusion coefficient; $[\eta]$, intrinsic viscosity; R_g, radius of gyration; C^*, critical overlap concentration. [a] Median of estimates for umbilical hyaluronan from McDonald & Levick (1995). [b] Unit disaccharide mass = 379 Da, length = 0·95 nm. [c] Extrapolated from Laurent *et al.* (1960) using $D_{20} = 41{\cdot}79M^{0{\cdot}6867}$.

hyaluronan, $[\eta]$, increased with chain length from 524 ml g^{-1} for hyaluronan-90 to 2953 ml g^{-1} for hyaluronan-2200 (Table 1). The Flory radius of gyration of the smallest sonicate, 20 nm, was less than a fifth of that of the starting material, 107 nm. The de Gennes overlap concentration fell with increasing molecular size from 4 mg ml^{-1} for hyaluronan-90, through 3·4 mg ml^{-1} for hyaluronan-300 and 2·4 mg ml^{-1} for hyaluronan-530, to 0·7 mg ml^{-1} for hyaluronan-2200. Since the infused concentration was 3·6 mg ml^{-1} throughout, hyaluronan-2200 and hyaluronan-530 were well above de Genne's overlap concentration, hyaluronan-300 was on the borderline and hyaluronan-90 was a little below overlap concentration.

Effect of hyaluronan-90 on fluid drainage

Hyaluronan-90 caused moderate reductions in the rate of fluid escape, namely by up to 40 % compared with the Ringer controls ($P < 0{\cdot}0003$, ANOVA; Fig. 2). Hyaluronan-90 failed to induce the quasi-plateau of flow that is characteristic of physiological chain lengths, and the shape of the pressure–flow relation was similar to that for Ringer solution. The latter steepens with pressure at 7–12 cmH_2O (yield pressure), with little or inconsistent curvature above or below this (Edlund, 1949; Levick, 1984). Four out of seven joints containing hyaluronan-90 showed a steepening with pressure, but the slopes were always depressed relative to the control joints. The average of seven conductances (slopes) with Ringer solution below yield pressure, 1·33 ± 0·36 μl min^{-1} cmH_2O^{-1}, was reduced by hyaluronan-90 to 0·62 ± 0·22 μl min^{-1} cmH_2O^{-1} ($P = 0{\cdot}054$, $n = 7$, paired t test). Similarly, the average of seven conductances with Ringer solution above yield pressure, 2·22 ± 0·50 μl min^{-1} cmH_2O^{-1}, was reduced by hyaluronan-90 to 1·11 ± 0·17 μl min^{-1} cmH_2O^{-1} ($P = 0{\cdot}085$, $n = 7$, paired t test).

In analysing the above effects it is helpful to calculate the 'opposition to outflow' at each point along the curve. Opposition to outflow is defined here as the pressure required to generate unit outflow, i.e. $P_j/\dot{Q}_s$. As noted in the Introduction, long-chain hyaluronan causes the opposition to outflow to increase with pressure. Rearrangement of the equation for flow across a membrane shows that the opposition term $P_j/\dot{Q}_s$ depends on four factors, namely the osmotic pressure across the membrane, the solute reflection coefficient, the membrane resistance and the intramembrane fluid viscosity (Coleman *et al.* 1999).

In the Ringer-infused joints the opposition almost halved as pressure was raised (Fig. 2*B*). This was expected, because synovial membrane resistance is known to fall with pressure, the effective osmotic pressure of Ringer solution across interstitium is zero and its relative viscosity is unity (Edlund, 1949; Levick *et al.* 1999). In the contralateral joints hyaluronan-90 increased the opposition to outflow by 56 % on average compared with Ringer solution ($P < 0{\cdot}0001$, ANOVA). However, the opposition showed a slight downward trend with pressure overall (negative regression slope; $P = 0{\cdot}08$), and was marginally lower at 20 cmH_2O than at 5 cmH_2O, namely 1·04 ± 0·23 and 1·29 ± 0·22 cmH_2O min μl^{-1}, respectively. This contrasted sharply with the effect of chains of ⩾ 530 kDa (see below).

Effect of hyaluronan-300

Hyaluronan-300 reduced the fluid drainage rate to 54–57 % of the Ringer control ($P < 0{\cdot}0001$, ANOVA; Fig. 3). Reductions at a given pressure reached individual significance at > 12·5 cmH_2O ($P < 0{\cdot}01$, Bonferroni test). In no case did hyaluronan-300 induce a flow plateau; a steepening with pressure was seen in 3/5 joints and the relation was virtually linear in the other two joints. The average of the slopes for Ringer solution below yield pressure, 1·02 ± 0·46 μl min^{-1} cmH_2O^{-1}, was almost halved by hyaluronan-300 to 0·58 ± 0·21 μl min^{-1} cmH_2O^{-1} ($P = 0{\cdot}41$, $n = 5$, paired t test). The average slope for Ringer solution above yield pressure, 2·52 ± 0·45 μl min^{-1} cmH_2O^{-1}, was reduced by hyaluronan-300 to 1·73 ± 0·56 μl min^{-1} cmH_2O^{-1} ($P = 0{\cdot}06$, $n = 5$, paired t test). The effects of hyaluronan-300 were thus broadly similar to those of hyaluronan-90.

In the five Ringer-infused joints the opposition to outflow fell by 54% as pressure was raised (Fig. 3*B*). In the contralateral joints hyaluronan-300 raised the opposition to outflow ($P = 0{\cdot}04$, ANOVA) but, as with hyaluronan-90, the opposition showed an overall downward trend with pressure (negative regression slope; $P = 0{\cdot}004$), falling by 56% between 5 and 20 cmH_2O.

Effects of hyaluronan-530 and hyaluronan-660

Hyaluronan-530 reduced the fluid drainage rate markedly ($P < 0{\cdot}0001$, ANOVA). Reductions at a given pressure reached individual significance at $> 12{\cdot}5$ cmH_2O ($P < 0{\cdot}05$, Bonferroni test), and the drainage rate was a third of the Ringer control value at the highest pressure. Unlike the shorter chains, hyaluronan-530 caused a striking change in the shape of the pressure–flow relation. In all five joints the relation developed a virtual plateau at $\geqslant 12{\cdot}5$ cmH_2O, with the flows fixed at ~10 μl min^{-1} (Fig. 4). The average of the slopes of the quasi-plateau, $0{\cdot}05 \pm 0{\cdot}04$ μl min^{-1} cmH_2O^{-1}, was 3% of that for Ringer solution over the same pressure range, namely $1{\cdot}96 \pm 0{\cdot}30$ μl min^{-1} cmH_2O^{-1} ($P < 0{\cdot}01$, paired t test). Over the lower pressure range the reduction in slope was less striking, namely $0{\cdot}34 \pm 0{\cdot}20$ μl min^{-1} cmH_2O^{-1} for hyaluronan-530 compared with $1{\cdot}01 \pm 0{\cdot}29$ μl min^{-1} cmH_2O^{-1} for Ringer solution ($P = 0{\cdot}3$, t test).

As well as increasing the opposition to outflow relative to Ringer solution ($P < 0{\cdot}001$, ANOVA), hyaluronan-530 caused the opposition to increase as a function of pressure at $\geqslant 12{\cdot}5$ cmH_2O (Fig. 3*B*). The opposition had increased by 69% at 22·5 cmH_2O. This contrasted with the decrease in opposition with pressure in the contralateral Ringer-infused joints and in joints infused with hyaluronan-330 or hyaluronan-90 (Figs 2*B* and 3*B*).

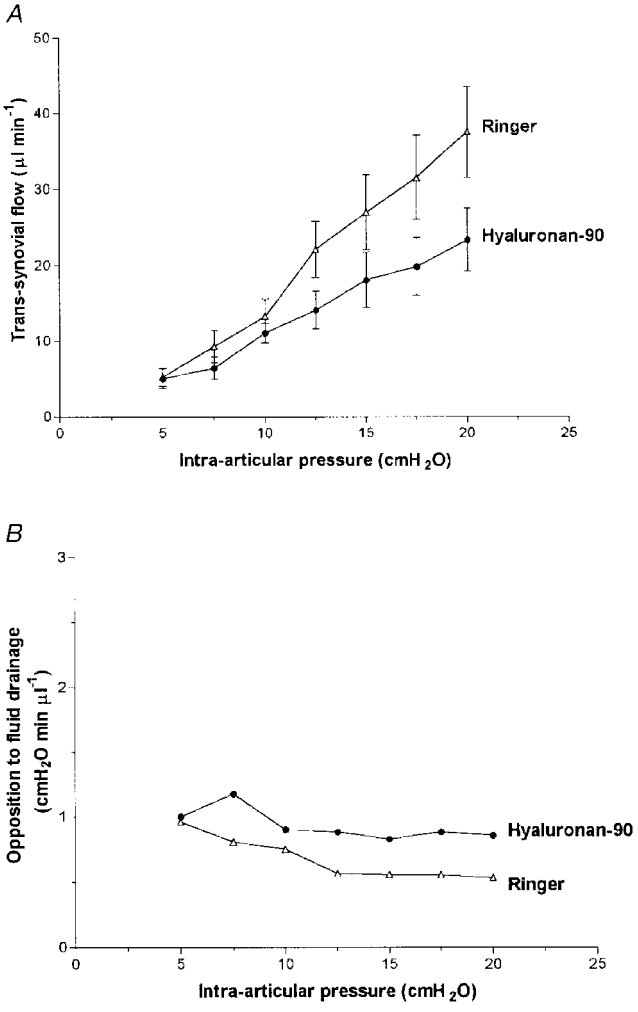

Figure 2. Effect of hyaluronan-90 on fluid drainage from joint cavity

Hyaluronan-90 average mass 88 kDa. *A*, effect on pressure–flow relation (mean flow ± S.E.M., $n = 7$). Contralateral joint received Ringer solution. *B*, effect on average opposition to outflow (pressure needed to drive unit flow across lining).

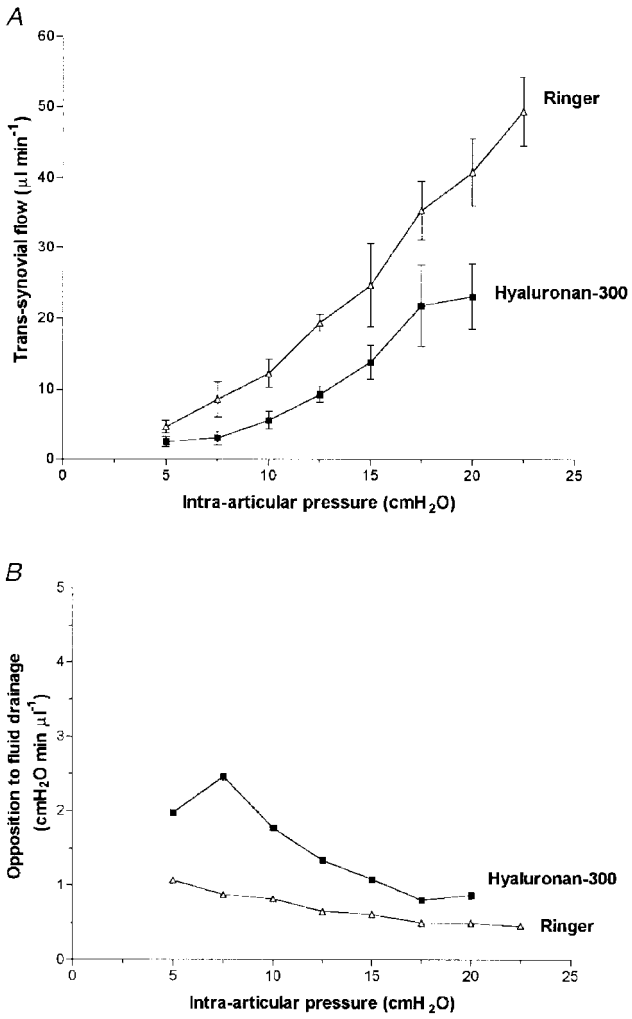

Figure 3. Effect of hyaluronan-300 on fluid drainage from joint cavity

Hyaluronan-300 average mass 305 kDa. *A*, effect on pressure–flow relation (mean flow ± S.E.M., $n = 5$). *B*, effect on opposition to outflow.

Figure 4 also shows for comparison some results from our laboratory using hyaluronan-660 (McDonald & Levick, 1995). The plateauing curves generated by hyaluronan-530 and hyaluronan-660 are of similar form but the flows in the presence of hyaluronan-660 were, overall, lower than with hyaluronan-530, plateauing out at just under 8 μl min^{-1} ($P < 0{\cdot}0001$, ANOVA).

Effect of hyaluronan-2200

Hyaluronan-2200 greatly attenuated the fluid drainage rate when compared with Ringer solution ($P < 0{\cdot}0001$, ANOVA). The flows reached a virtual plateau at ~3·6 μl min^{-1} (Fig. 5). The plateau flow was approximately one-third of that for hyaluronan-530 solution ($P < 0{\cdot}0001$, ANOVA) and was 8% of the Ringer drainage rate at the highest pressure. The average of the slopes of the quasi-plateau, 0·013 ± 0·016 μl min^{-1} cmH_2O^{-1}, was 0·5% of the Ringer slope (2·30 ± 0·05 μl min^{-1} cmH_2O^{-1}; $P < 0{\cdot}01$, t test) and approximately one-quarter of the slope obtained with hyaluronan-530 ($P = 0{\cdot}12$, t test).

The opposition to outflow increased steeply with pressure over the entire pressure range in the presence of hyaluronan-2200 (Fig. 5*B*). The 3·3-fold rise in opposition with pressure contrasted with the smaller, 1·7-fold rise with hyaluronan-530 and the declining opposition in the joints containing hyaluronan-330, hyaluronan-90 or Ringer solution.

Molecular mass and concentration of aspirated hyaluronan solutions

Analysis of the aspirated fluid showed that the average molecular mass of the intra-articular hyaluronan at the end

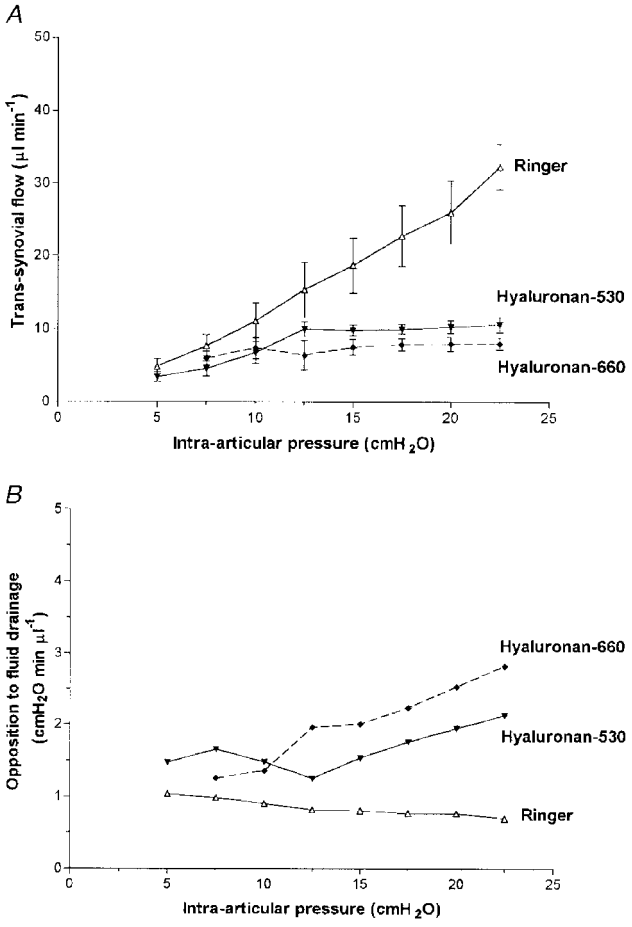

Figure 4. Effect of hyaluronan-530 on fluid drainage from joint cavity

Hyaluronan-530 average mass 527 kDa. *A*, effect on pressure–flow relation, with development of a near plateau (mean flow ± S.E.M., $n = 6$). Dashed line shows data for umbilical hyaluronan of average molecular mass 660 kDa ($n = 5$; McDonald & Levick, 1995). *B*, effect on opposition to outflow; opposition increases with pressure ('buffering' of outflow).

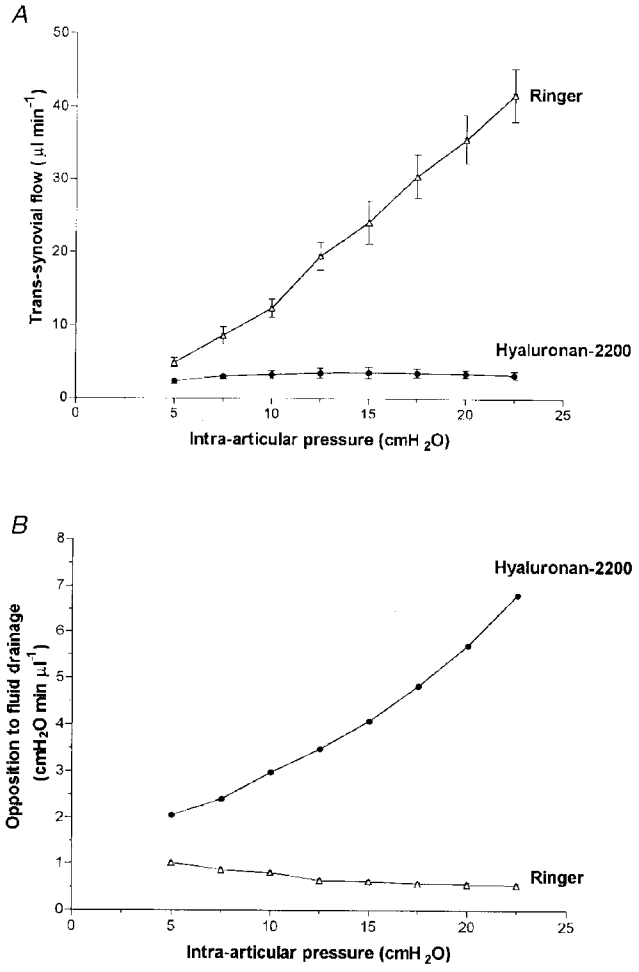

Figure 5. Effect of hyaluronan-2200 on fluid drainage from joint cavity

Hyaluronan-2200 average mass 2230 kDa. *A*, effect on pressure–flow relation (mean flow ± S.E.M., $n = 12$). *B*, effect on opposition to outflow.

of an experiment was not significantly different from that infused ($P = 0{\cdot}5$, $n = 15$, paired t test). The values were as follows: infused hyaluronan $87{\cdot}8 \pm 6{\cdot}4$ kDa, aspirated hyaluronan $77{\cdot}5 \pm 6{\cdot}1$ kDa; infused hyaluronan $304{\cdot}7 \pm 6{\cdot}3$ kDa, aspirated hyaluronan $349{\cdot}2 \pm 7{\cdot}4$ kDa; infused hyaluronan $526{\cdot}9 \pm 55{\cdot}7$ kDa, aspirated hyaluronan $544{\cdot}8 \pm 24{\cdot}6$ kDa. Likewise, an earlier study of hyaluronan-2200 showed that the infused and aspirated molecular masses, namely 2267 ± 84 and 2252 ± 73 kDa, respectively, were not significantly different ($P = 0{\cdot}8$, $n = 16$, paired t test; Scott *et al.* 1998). Thus the average intra-articular chain length was not distorted significantly by factors such as endogenous hyaluronan admixture, intra-articular degradation or hyaluronan secretion *de novo* over 3–5 h (5–6 μg h^{-1}, Coleman *et al.* 1997).

The concentration of hyaluronan in the aspirated fluid was greater than that in the infusate in every case for hyaluronan-90, hyaluronan-300 and hyaluronan-530 ($P < 0{\cdot}01$, $n = 15$, paired t test). Concentrations increased by 22 ± 3, 44 ± 11 and 25 ± 7%, respectively. We have reported previously that hyaluronan-2200 is concentrated by 26 ± 8% ($P = 0{\cdot}02$, $n = 6$, paired t test; Scott *et al.* 1998). The reflected fractions, calculated from the mean concentration increase and filtered volume (see Methods), were 0·12, 0·33, 0·25 and 0·79 for hyaluronan-90, hyaluronan-300, hyaluronan-530 and hyaluronan-2200, respectively. The relation between reflected fraction and radius of the polymer domain is plotted in Fig. 6. The observations reinforce a previous study of the size dependence of hyaluronan permeation by Brown *et al.* (1991). The latter group found that the half-life of labelled hyaluronan in rabbit knees decreased as molecular mass was reduced from 6000 to 90 kDa.

DISCUSSION

Chain length is clearly a major determinant of the effect of synovial fluid hyaluronan on fluid drainage through synovial interstitium. The effect of hyaluronan changed strikingly between 305 and 527 kDa. Hyaluronan of mass $\geqslant 527$ kDa caused outflow buffering, i.e. increasing opposition to drainage with pressure, whereas hyaluronan of mass $\leqslant 305$ kDa reduced drainage without outflow buffering, which profoundly altered the shape of the pressure–flow relation compared to longer chains. Studies of transperitoneal drainage show that hyaluronan of mass 85 or 280 kDa has only a small effect on transperitoneal flow whereas 500 and 4000 kDa hyaluronan markedly attenuates drainage (Wang *et al.* 1999).

Mechanisms underlying the effect of chain length

Reduction of hyaluronan concentration, like reduction of chain length, can abolish outflow buffering (Scott *et al.* 2000*a*). The effect of reduced concentration can be explained largely by a fall in the osmotic pressure of the concentration polarization layer that forms during fluid drainage. There are several reasons for arguing that shortened chains should likewise produce a less concentrated polarization layer, as follows. (i) Short polymer chains are less well reflected by a porous membrane than are long chains, as is evident in Fig. 6 and as shown *in vitro* by Munch *et al.* (1979). This is in keeping with the change in hydrodynamic radius of hyaluronan with chain length; the hydrodynamic radius

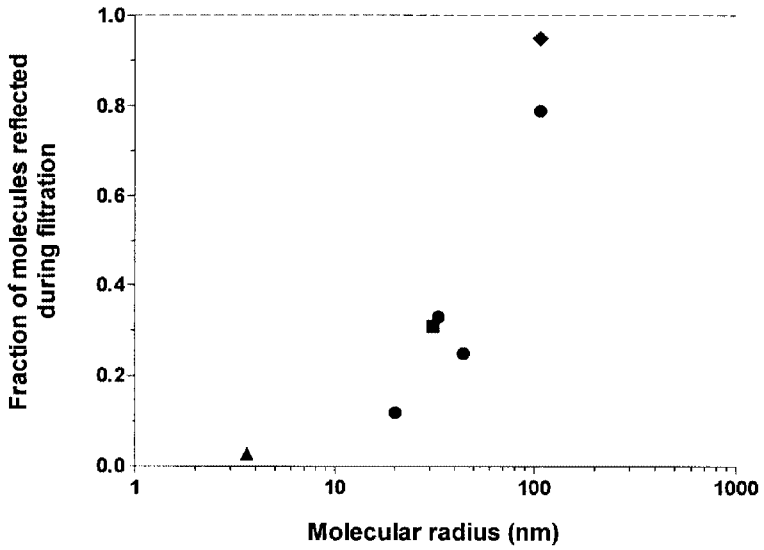

Figure 6. Relation between size of molecular domain and fraction of molecules reflected during drainage through synovial interstitium

The abscissa is the Flory radius of gyration R_g for the polysaccharides. The dashed line of value 1·0 represents the upper limit for the reflected fraction. ●, results for hyaluronan-90 to -2200. Additional data: ◆, hyaluronan-2200 at 2 mg ml^{-1} (Scott *et al.* 1998); ■, dextran of molecular mass 2×10^6 Da (Scott *et al.* 2000*b*); ▲, albumin (J. R. Levick & S. Sabaratnam, unpublished results).

falls from ~103 nm at 2000 kDa to ~14 nm at 90 kDa (Fujii *et al.* 1996). A hydrodynamic radius of 14 nm is smaller than the equivalent pore radius of 30–90 nm for synovial interstitium. Lindholm *et al.* (1996) by contrast observed no difference between the half-lives of radiolabelled 600 and 2500 kDa hyaluronan in equine joints; but the drainage rates and pressures were not recorded, and may have been low. (ii) Experiments with infused dextran of mass 2000 kDa support the argument that molecular domain volume is crucial to outflow buffering. Dextran-2000 fails to buffer the trans-synovial flow despite its large mass (Scott *et al.* 2000*b*). Its reflected fraction of 0·31 and R_g of 31–34 nm are very similar to those of hyaluronan-300 (0·33 and 33 nm, respectively), which likewise fails to buffer outflow. (iii) Chain length affects diffusivity, both directly and through chain–chain interactions. The diffusion coefficient increases ~9-fold as chain length is reduced from 2200 to 88 kDa (Table 1). Increased diffusivity will greatly reduce the degree of concentration polarization, as shown by the theoretical polarization curves in Fig. 9*A* of Coleman *et al.* (1999). The combined effect of increased back-diffusion in the concentration polarization layer and reduced reflection at the interface is to reduce the interfacial hyaluronan concentration to a point where its colloid osmotic pressure is little more than in the bulk phase, namely 1 cmH_2O.

The sensitivity of outflow buffering to chain length may relate partly to the degree of chain–chain interaction. The critical concentration C^* for overlap of adjacent molecular domains depends on the chain length. Consequently, the infused hyaluronan molecules of mass 2200–530 kDa were above C^* (Table 1), whereas those of mass 300–90 kDa were below C^*. Hyaluronan-300 and hyaluronan-90 were not, therefore, in the mutually entangled, quasi-continuous network state that characterized the longer chains. Moreover, measurable chain–chain interactions are often found to begin only at a concentration greater than C^*, referred to here as C^{**}. For rooster comb hyaluronan C^{**} was $1{\cdot}9 \times C^*$ (Scott *et al.* 2000*a*). Hyaluronan-90 and hyaluronan-300 were well below C^{**} while hyaluronan-660 and hyaluronan-2200 were well above it.

Effect of low molecular weight hyaluronan (⩽ 305 kDa)

Although the smaller hyaluronan chains failed to generate outflow buffering, they did reduce outflow substantially, namely by almost half compared with Ringer solution. This could be due to the increased viscosity of the interstitial fluid as non-reflected hyaluronan chains permeate the interstitial void. The bulk phase viscosity of hyaluronan-90 and hyaluronan-300 solutions reduced the macroscopic flow in the viscometer capillary tube by 71% and 81%, respectively, relative to Ringer solution. The viscosity of the intramembrane fluid, however, is probably less than in the bulk phase, due partly to the partial reflection of hyaluronan at the tissue–fluid interface and partly to the phenomenon of anomalous viscous flow in narrow channels (Levick, 1994).

Pathophysiological significance

Inflammatory arthritis is associated with a fall in hyaluronan concentration and chain length, as described in the Introduction. Normal human hyaluronan, of molecular mass 6000–7000 kDa (Dahl *et al.* 1985; Lee & Cowman, 1994), is bigger than rabbit hyaluronan, which has a mass of 2400–2900 kDa. The subnormal average molecular masses in human arthritis span a wide range, namely 1284–4800 kDa (Balazs *et al.* 1967; Kofoed & Barcelo, 1979; Bjelle *et al.* 1982; Dahl *et al.* 1985), and individual values as low as 849 kDa have been reported in acute synovitis (Praest *et al.* 1997). Even so, such chains would still generate outflow buffering according to the results in Fig. 4, unless the reflective properties of the lining are also impaired. The latter has never been studied directly; however, the intra-articular half-life of ^{3}H-labelled hyaluronan in sheep hock joints is approximately halved during acute synovitis (Fraser *et al.* 1993), and one of several possible explanations for this could be increased synovial lining permeability.

Based on the present results and those of Scott *et al.* (2000*a*), it seems likely that the fall in hyaluronan concentration in human arthritis, often to < 1 mg ml^{-1}, will have a greater effect on fluid drainage than does the fall in molecular size. Reduced chain length and reduced reflection may contribute, however, to the rise in plasma hyaluronan concentration observed in rheumatoid arthritis after exercise (Engström-Laurent & Hallgren, 1987). Exercise causes cyclical rises in intra-articular pressure and thus promotes drainage from the joint cavity. Hyaluronan chain length also influences synovial cellular activities and lubrication, as reviewed by Strachan *et al.* (1990).

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