

# Relationship between lymph and tissue hyaluronan in skin and skeletal muscle

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**Armstrong, Shayn E., and Donald R. Bell.** Relationship between lymph and tissue hyaluronan in skin and skeletal muscle. *Am J Physiol Heart Circ Physiol* 283: H2485–H2494, 2002. First published August 1, 2002; 10.1152/ajpheart.00385.2002.—The size of hyaluronan was compared between tissue and lymph using a combination of agarose gel electrophoresis and radiometric assay. Prenodal lymph was collected from heel skin and the gastrocnemius muscle in anesthetized rabbits. The major fraction of hyaluronan in both tissues had a molecular weight  $>4$  million. Lymph contained primarily low-molecular-weight hyaluronan ( $<0.79 \times 10^6$ ), which was absent from tissue. Volume loading produced a preferential increase in the flux of low-molecular-weight hyaluronan, indicating that tissue contains a small quantity of mobile, low-molecular-weight hyaluronan. The maximum daily removal of hyaluronan by lymph was  $<1\%$  of the tissue content. The amount of lysosomal hyaluronidase activity in tissue was more than enough to account for a rapid turnover of hyaluronan. The data support the conclusion that lymph drainage is not significant in the normal catabolism of hyaluronan and may represent a small amount that becomes detached from the pericellular and extracellular matrixes.

extracellular matrix; hyaluronan catabolism; hyaluronidase activity; molecular weight

HYALURONAN IS A MAJOR COMPONENT of pericellular and extracellular matrixes (19, 30). It is a linear,  $\beta$ -1,4-linked polymer of the disaccharide D-glucuronic acid (1- $\beta$ -3)-N-acetyl-D-glucosamine. The molecular weight is considered polydisperse depending on the number of repeating disaccharide units in the chain. It is synthesized at cell membranes as a very large polymer with a molecular weight of  $\sim 6 \times 10^6$  (4, 12). Besides modulating tissue hydration and transvascular fluid balance, it can stabilize the extracellular matrix by binding to specific proteins called hyaladherins. During tissue development or injury, hyaluronan helps regulate cell motility, invasion, and proliferation by binding to cell surface receptors and activating intracellular signaling pathways. Many of these diverse functions depend on the size of the polymer. Low-molecular-weight hyaluronan appears stimulatory for cell proliferation and migration, whereas high-molecular-weight hyaluronan is inhibitory (19). Although high-molecular-weight hyaluronan inhibits angiogenesis, low-mo-

lecular-weight fragments induce tube formation by cultured endothelial cells through binding to the cell surface receptor CD44 (21, 28). These observations suggest that both the size and amount are important determinants of hyaluronan function within tissues even though the size has not been well characterized.

Skin contains approximately one-half of the hyaluronan in the body, and the turnover is rapid with a half-life of  $\sim 2$  days (23, 29). Hyaluronan is specifically involved with the migration of basal keratinocytes toward the outer cornified layer during wound healing (20). Although the catabolism is not well understood, the presence of hyaluronan in lymph leads to the proposal that lymphatic drainage was an important catabolic pathway (15, 30). On the basis of measurements of the hyaluronan content in skin lymph and tissue and the rapid removal of subcutaneously injected low-molecular-weight hyaluronan, Reed and colleagues (8, 24) proposed that the extravascular space contains two distinct pools of hyaluronan. One pool represented recently synthesized hyaluronan attached to matrixes, whereas the other pool represented hyaluronan released from the matrixes and drained by the lymphatics. Increasing lymph hyaluronan flux would increase the turnover of the free pool without influencing the bound pool. In a more recent study (22), they questioned the significance of this proposal because the removal rate of labeled high-molecular-weight hyaluronan after a subcutaneous injection did not increase during a condition known to increase lymph flux.

Our hypothesis was that the size distribution of hyaluronan in skin would reflect the two pools of hyaluronan. The major fraction of hyaluronan, corresponding to the matrix pool, would have a high molecular weight. A smaller fraction in the tissue would have a lower molecular weight and a size distribution similar to that found in lymph. We further proposed that increasing lymph hyaluronan flux with volume expansion would decrease the amount of low-molecular-weight hyaluronan in the tissue without altering the amount of high-molecular-weight hyaluronan. With the use of a combination of gel electrophoresis and a radiometric assay, we measured the size of hyaluronan in lymph and tissue to test this hypothesis. Volume

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expansion was used to increase lymph hyaluronan flux. Besides skin, we studied skeletal muscle because the much lower hyaluronan content and the absence of keratinocytes may influence the relative size of the two pools.

We used anesthetized rabbits because lymph can be collected separately from either skin or skeletal muscle of the hind leg. A difficulty in comparing lymph hyaluronan flux with tissue hyaluronan content is estimating the weight of tissue drained by the cannulated lymphatic. In a previous study using rabbits (33), the weight of heel skin drained by the cannulated lymphatic was estimated by comparing the transvascular clearance of labeled albumin with the lymphatic clearance of endogenous albumin. In the present study, we estimated the weight of skeletal muscle drained by the cannulated lymphatic using the same procedure. The values for the weight of skin or skeletal muscle drained by the lymphatic was used to compare the lymph flux of hyaluronan with the amount in the tissue.

## METHODS

**Animal preparation.** A detailed account of the surgical procedures has been previously reported (18, 33). Protocols for animal use conformed with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee at Albany Medical College. In brief, New Zealand White rabbits of either sex, weighing from 2.0 to 3.0 kg, were anesthetized with pentobarbital sodium (25–35 mg/kg) and were given supplemental doses as required. Body temperature was maintained at 39°C with a heating blanket and a Yellow Springs Instruments rectal probe thermometer. After tracheotomy, a heparinized cannula was placed into the right carotid artery for blood collections and arterial blood pressure recordings using a Statham pressure transducer connected to a Grass polygraph. Cannulas were placed in the right external jugular vein for intravenous infusions.

For collection of skin lymph, the lymphatics located near the calcaneal tributary vein of each leg were tied off, and a single, prenodal, popliteal lymphatic was cannulated using polyethylene tubing heated and pulled to the desired size. The collected lymph was from an area of heel skin weighing 1.6 g dry wt (6 g wet wt) with negligible contamination from other tissues (33). For collection of skeletal muscle lymph, T1824-bound albumin was injected into the heel skin of each leg to identify contamination from skin lymph. In each leg, the lymphatics within the femoral sheath were ligated, and a single lymphatic was cannulated distal to the inguinal node with Silastic tubing (0.3 mm inner diameter and 0.64 mm outer diameter). The initial lymph collected was blue from the T1824 injection. The popliteal node was exposed, and the efferent lymph duct was ligated. If lymph flow remained elevated or if the collected lymph did not become clear after 1–2 h, data collection from that leg was terminated. The procedure collects lymph predominately from the gastrocnemius and soleus muscles (18), although the weight of tissue has not been determined.

Lymph flow (in  $\text{ml}\cdot\text{h}^{-1}\cdot\text{g dry wt}^{-1}$ ) was calculated by dividing the sample volume by the collection time and the weight of tissue drained by the cannulated lymphatic. Lymph was collected for 30-min intervals in weighed, heparinized vials. Sample volume was determined using weight and assuming that the density of lymph was 1. For both prepara-

tions, lymph collection was done with the rabbit lying prone, and the legs were moved passively at 60 times/min to promote lymph flow. A heparinized sample of blood (0.8 ml) was collected at the midpoint of each lymph collection.

The concentration of hyaluronan in lymph was measured using a radiometric assay. The concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$  or  $4 \times 10^6$  was measured using agarose gel electrophoresis. The concentration of low-molecular-weight hyaluronan ( $<0.79 \times 10^6$ ) was calculated as the concentration of hyaluronan, measured using the radiometric assay, minus the concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$ . The concentration of intermediately sized hyaluronan was calculated as the concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$  minus the concentration of hyaluronan with a molecular weight  $>4 \times 10^6$ . The lymphatic flux for each species was calculated by multiplying the lymph flow by the respective lymph concentration.

Lymphatic flux of hyaluronan was increased by volume expanding the animals with lactated Ringer solution (Baxter). An intravenous infusion of 150 ml/kg body wt was given at a rate of  $1.3 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg body wt}^{-1}$ . The duration of the infusion was 2 h. After the infusion was ended, lymph and plasma samples were taken for an additional 2 h. The animal was killed by an overdose of pentobarbital sodium, and either the heel skin or gastrocnemius muscle was removed. Separate animals that did not receive an infusion were controls. After the tissue was removed, a section was weighed and dried to a stable weight at 60°C to calculate the wet weight-to-dry weight ratio. The rest of the tissue was frozen at  $-80^\circ\text{C}$  for later analysis. Although the heel skin was shaved with electric clippers at the beginning of the experiment, additional hair was removed from the dried skin sample with a razor, and the weight was corrected for the residual hair.

**Tissue digestion and radiometric assay.** Frozen sections of tissue (300–350 mg) were weighed and placed in 2 ml of 0.15 M Tris, 0.15 M NaCl, 0.01 M  $\text{CaCl}_2$ , and 5 mM deferoxamine mesylate, pH 8.3, containing 40 units of protease (Sigma) for 8 h at 55°C. Deferoxamine was included to inhibit any hyaluronan depolymerization by iron released during the digestion procedure (1). After incubation, the samples were centrifuged at 53,000 g for 15 min at 4°C, and the supernate was collected for analysis. For the radiometric assay, an aliquot of the tissue digest was placed in a boiling water bath for 20 min to inactivate the protease. Digestion buffer containing protease and no hyaluronan was used as a blank. The influence of the protease on the binding of labeled hyaluronan binding proteins (HABP) to the hyaluronan-Sepharose was always  $<5\%$  after boiling. For both assays, a 3-fold dilution for skeletal muscle and a 12-fold dilution for skin was made using 0.15 M Tris, 0.15 M NaCl, 0.01 M  $\text{CaCl}_2$ , and 5.0 mM deferoxamine mesylate; pH 8.3.

The tissue content of hyaluronan irrespective of size, hyaluronan with a molecular weight  $>0.79 \times 10^6$ , and hyaluronan with a molecular weight  $>4 \times 10^6$  was calculated. The tissue content ( $\mu\text{g/g wet tissue wt}$ ) was calculated by multiplying the digest concentration by the volume of fluid present during digestion and dividing by the weight of the tissue sample. These values were converted to units of micrograms per gram dry weight by multiplying by the wet weight-to-dry weight ratio.

The concentration of hyaluronan irrespective of size was measured using a radiometric assay as described previously (1, 9). Hyaluronan from *Streptococcus* (Calbiochem) was used as a standard. Procedures for the isolation of HABP from cartilage and the preparation of hyaluronan-conjugated Sepharose were described previously (1, 32). HABP were

labeled with  $^{125}\text{I}$  ( $^{125}\text{I}$ -HABP) using the lactoperoxidase method (14). To protect the binding site, 300  $\mu\text{g}$  HABP was incubated with 225  $\mu\text{g}$  rooster comb hyaluronan in 0.05 M phosphate buffer, pH 7.5, before labeling with 2 mCi (74 MBq)  $^{125}\text{I}$ . The labeled HABP were dialyzed (25,000 mol wt cutoff) against 4 M guanidine HCl and applied to a Sephacryl S-400 gel filtration column equilibrated in 4 M guanidine HCl to separate labeled HABP from hyaluronan. Fractions containing the peak activity were mixed with 10 mg BSA, concentrated using an Amicon Ultrafiltration unit with a YM10 membrane, and stored at 4°C. The specific activity was between 25 and 45 kBq/ $\mu\text{g}$ .

**Agarose gel electrophoresis.** The concentration of hyaluronan with determined molecular weight characteristics was measured in samples of lymph and tissue digest using gel electrophoresis as previously described (1, 10). A 0.5% agarose (GIBCO-BRL) gel was made in Tris-sodium acetate-sodium EDTA (TAE) buffer (40 mM Tris, 5 mM sodium acetate, and 0.8 mM sodium EDTA; pH 7.9). To each lane, 10  $\mu\text{l}$  lymph, diluted tissue digest, or a standard was applied. The samples were electrophoresed at 50 V for 3 h in TAE buffer using a DNA Plus Horizontal Gel system from USA Scientific. Hyaluronan was transferred from the gel to Hybond H<sup>+</sup> (Amersham Pharmacia) membranes using upward capillary blotting. Optimum transfer required the overnight passage of 550 ml TAE buffer into a stack of blotting pads that was 6 cm thick. After transfer, membranes were blocked by incubation in 0.1 M sodium acetate-1 M NaCl containing 0.05% Tween 20, 2% nonfat dry milk, and 0.1% sodium azide, pH 6.0, for 6 h at 37°C. Membranes were subsequently transferred to 20 ml fresh solution containing 400 kBq  $^{125}\text{I}$ -HABP and incubated overnight at 4°C. After being washed, X-ray film was exposed to the membrane for 4–6 h at –80°C and developed. A Bio-Rad GS-700 Imaging Densitometer and Molecular Analyst software were used for quantitative analysis of the autoradiograph.

Both molecular weight and concentration standards were applied to each gel. A series of hyaluronan standards with defined molecular weights was generously donated by Dr. O. Wik (Pharmacia; Uppsala, Sweden). The weight-averaged molecular weights of these standards were 0.20, 0.48, 0.79, 1.2, 2.0, 3.9, and  $5.0 \times 10^6$ . Each standard was applied to different lanes. A calibration curve for molecular weight was constructed from the autoradiograph. For each lane containing a standard, the mobility of the peak (maximum lane intensity) was plotted against the logarithm of the peak molecular weight and a straight line fit using least squares. To verify the molecular weight corresponding to the peak, the standards were chromatographed on a Sephacryl S-1000 column (1.6  $\times$  97 cm). The column was calibrated using the weight-averaged molecular weight as described by Laurent and Granath (7). For each standard, the molecular weight corresponding to the peak in the chromatogram was estimated from the standard curve and used for the molecular weight of the peak in the electrophoretic profiles on the autoradiograph.

We calculated the concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$  or  $4 \times 10^6$  in lymph and tissue digests. Concentration measurements were made by making serial dilutions of the  $3.9 \times 10^6$  molecular weight standard and determining the concentration using the radiometric assay. Each dilution was applied to separate lanes of the gel. A standard curve for concentration was constructed from the autoradiograph. For each standard, the total lane intensity was plotted against its concentration, and a straight line was fit using least squares. The relationship was linear between 1 and 10  $\mu\text{g}/\text{ml}$  (10–100 ng applied amount). For lymph and

tissue samples, the lane intensity on the autoradiograph was measured after setting the length of the lane to correspond to a molecular weight of  $0.79 \times 10^6$  or  $4 \times 10^6$ , determined from the molecular weight calibration. For each sample, the lane intensity was converted to hyaluronan concentration using the standard curve. Because the concentration standards used in gel electrophoresis were calibrated with the radiometric assay, the measurements from the two methods were directly comparable.

**Tissue hyaluronidase activity.** Hyaluronidase activity was measured in the liver and skin by determining the initial velocity of end-product production as described previously (2, 3). Tissue (0.5 g) was homogenized in 1.5 ml of 0.01 M sodium acetate and 0.15 M NaCl, pH 5.0, at 4°C using a Brinkman Polytron homogenizer. Hyaluronidase activity was measured in the supernate after centrifugation at 53,000  $g$  for 15 min. Aliquots of tissue supernate (0.2 ml) were incubated with 0.4 ml of 0.15 M sodium acetate, 0.15 M NaCl, and 0.75 mg/ml rooster comb hyaluronan, pH 4.0, for varying lengths of time. Isotonic saline instead of tissue supernate was used as a blank. The reaction was neutralized by the addition of 15  $\mu\text{l}$  of 5 M NaOH. After centrifugation, the concentration of *N*-acetyl-glucosamine at the reducing end of hyaluronan was measured using the method of Reissig et al. (25). The increase in the concentration was linear with time up to 8 h, and the initial velocity was measured as the slope during the first 6 h. Hyaluronidase activity was calculated as the initial velocity multiplied by the dilution factor and volume of tissue supernate and divided by tissue weight. Tissue weight was converted to dry weight using the wet weight-to-dry weight ratio.

We used the diffusion plate assay described by Richman and Baer (26) to measure the hyaluronidase activity in skeletal muscle, plasma, and lymph due to the low activity present in these samples. We assumed that the diffusion characteristics for the enzymes in the sample were the same as those for the enzymes in skin and used serial dilutions of skin supernate as a standard. The procedure for homogenization of skeletal muscle was the same as skin. A 1% agarose gel containing 0.5 mg/ml rooster comb hyaluronan was made in 0.1 M sodium acetate and 0.15 M NaCl (pH 4.0) buffer. Samples of 10  $\mu\text{l}$  were applied to wells in the gel and incubated for 18 h at 37°C. The undigested hyaluronan was precipitated by placing the gel into 0.28 M cetylpyridinium chloride overnight. The diameter of clear circles, representing digested hyaluronan, was related to the logarithm of the concentration of enzyme in the applied sample. Sample concentrations were converted to activities by multiplying the concentration by the activity measured in the skin supernate that was used as a standard.

**Weight of skeletal muscle drained by lymph.** In a separate experiment, the weight of skeletal muscle drained by the cannulated lymphatic was estimated by comparing the transvascular clearance of labeled albumin with the lymphatic clearance of endogenous albumin as we described previously for skin (33). A 1-h infusion of labeled albumin was started after obtaining a stable lymph flow. A bolus of 135  $\mu\text{Ci}$  (5 MBq) of  $^{125}\text{I}$ -labeled albumin was injected intravenously followed by an infusion to maintain the plasma concentration constant with time. Plasma samples were taken at 15 and 45 min after the beginning of the tracer infusion to be certain that the plasma tracer activity was not changing with time. Three minutes before the experiment was ended, 200  $\mu\text{Ci}$  (7 MBq) of  $^{131}\text{I}$ -labeled albumin were injected intravenously to measure the plasma volume in tissue. After the 3-min mixing time, a final plasma sample was taken, the animal was killed by an overdose of pentobarbital sodium, and the gastrocn-



Table 1. Estimation of the weight of skeletal muscle drained by the cannulated lymphatic

Lymph flow per leg, $\mu\text{l/h}$	$50 \pm 4$
Lymph clearance of endogenous albumin, $\mu\text{l/h}$	$32 \pm 3$
Lymph clearance of labeled albumin, $\mu\text{l/h}$	$1.8 \pm 0.3$
Transvascular clearance of labeled albumin, $\mu\text{l}\cdot\text{h}^{-1}\cdot\text{g dry wt}^{-1}$	$6.0 \pm 0.3$
Dry weight drained by lymphatic, g	$5.2 \pm 0.7$

Values are means  $\pm$  SE,  $n = 7$  legs.

mus and soleus muscles were removed. Samples of the muscles were weighed, counted for radioactivity with plasma and lymph samples, and dried to a stable weight at  $60^\circ\text{C}$ . The wet weight-to-dry weight ratio was calculated as the fresh tissue weight divided by the dry weight. The concentration of non-protein-bound activity was determined in plasma using ultrafiltration with an Amicon Centricon-30.

The concentration of endogenous albumin in lymph and plasma samples was measured using rocket electroimmunoassay as described previously (33). Antibody to rabbit albumin was purchased from Cappel Laboratories, and purified rabbit albumin was used as a standard. As described previously (18, 33), rabbit albumin was purified using affinity and gel filtration chromatography. It was labeled with  $^{125}\text{I}$  or  $^{131}\text{I}$  using a modified chloramine-T procedure. The labeled protein was placed in dialysis (Spectropore 6; 25,000 mol wt cutoff) for at least 5 days to reduce the fraction of non-protein-bound activity below 0.3%.

For each tissue sample, the volume of plasma ( $V_P$ ) was calculated as the amount of  $^{131}\text{I}$  in the tissue sample (in disintegrations  $\cdot\text{min}^{-1}\cdot\text{g wet wt}^{-1}$ ) divided by the plasma concentration (in disintegrations  $\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$ ). The transvascular clearance of labeled albumin ( $C_T$ ) was calculated using Eq. 1, where  $R_{WD}$  is the wet weight-to-dry weight ratio and  $T$  is the number of hours the labeled protein was in the circulation

$$C_T = [(\text{tissue } ^{125}\text{I in disintegrations} \cdot \text{min}^{-1} \times \text{g wet wt}^{-1}) / (\text{plasma } ^{125}\text{I in disintegrations} \times \text{min}^{-1} \cdot \text{ml}^{-1}) - V_P] (R_{WD}) / T \quad (1)$$

The weight drained by the cannulated lymphatic ( $W$ ) was calculated using Eq. 2. This equation provides a minimum value for the weight because it assumes that transvascular transport for albumin is by convection only (33). Lymph clearance of endogenous albumin ( $C_{LEA}$ ) was calculated as lymph flow times the lymph-to-plasma concentration ratio for endogenous albumin. Lymph clearance of labeled albumin ( $C_{LLA}$ ) was calculated as the total radioactivity in lymph divided by the plasma concentration

$$W = (C_{LEA} - C_{LLA}) / C_T \quad (2)$$

**Statistics.** Data are presented as means  $\pm$  SE. A one- or two-way ANOVA with repeated measures and Scheffé's test were used to test for significance between groups. Significance was set at  $P < 0.05$ .

## RESULTS

**Weight of skeletal muscle drained by lymph.** Lymph was collected from seven legs in six rabbits to estimate the weight of skeletal muscle drained by the cannulated lymphatic. After ligation of the popliteal node, lymph was collected for 4 h before the infusion of labeled albumin was started. Lymph flow and the

lymph clearance of endogenous albumin remained constant with time during the last 2 h of the experiment. Values measured during the 1-h infusion of labeled albumin are presented in Table 1. Plasma albumin was  $32 \pm 2$  mg/ml. The plasma concentration for the labeled albumin did not change with time. The difference between the initial and final concentrations was  $5 \pm 4\%$ . The amount of non-protein-bound activity in the final plasma sample was  $0.30 \pm 0.08\%$ . The specific activity in the final lymph sample relative to plasma was  $6 \pm 1\%$ . Values for the transvascular clearance of labeled albumin were not different between the gastrocnemius and soleus muscles and were averaged. The wet weight-to-dry weight ratio was  $4.3 \pm 0.1$ , and the vascular volume in the tissue samples was  $31 \pm 2$   $\mu\text{l/g dry wt}$ . The tissue weight drained by the cannulated lymphatic was equivalent to 17 g wet wt.

**Skin hyaluronan.** Electrophoresis of digested skin showed a single major peak that had a molecular weight greater than our highest standard. Figure 1 shows the electrophoretic pattern for a sample of digested skin and skin lymph. For the tissue digest, the concentration of hyaluronan irrespective of size was 100  $\mu\text{g/ml}$ . The concentration of hyaluronan with a molecular weight  $>4 \times 10^6$  was 64  $\mu\text{g/ml}$ . The concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$  was 92  $\mu\text{g/ml}$ . The pattern for lymph showed a shift toward lower-molecular-weight hyaluronan and greater polydispersity. For the lymph sample, the concentration of hyaluronan irrespective of size was 7.7  $\mu\text{g/ml}$ . The concentration of hyaluronan with a molecular weight  $>4 \times 10^6$  was 1.7  $\mu\text{g/ml}$ , which corresponds to only 22% of the hyaluronan in lymph. The concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$  was 5.0  $\mu\text{g/ml}$ , so that 35% of the hyaluronan in the lymph sample had a molecular weight  $<0.79 \times 10^6$ . Control measurements were obtained from eight legs in four rabbits that were not volume

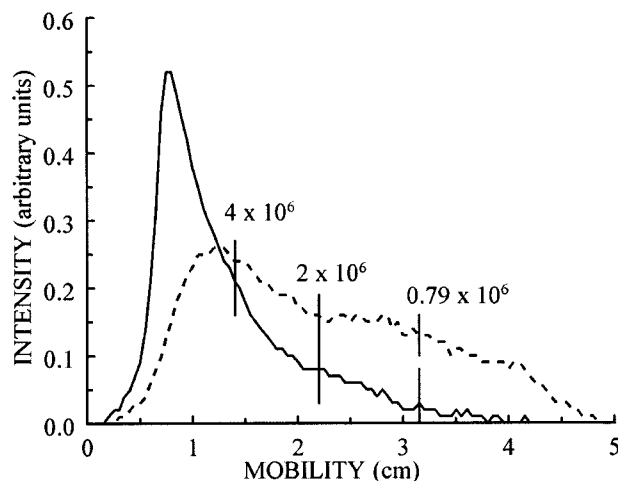


Fig. 1. Densitometric scan of an autoradiograph of skin tissue digest (solid line) and skin lymph (dashed line) after electrophoresis on 0.5% agarose gels. Radiolabeled hyaluronan binding proteins were used to detect hyaluronan specifically after transfer to a nylon membrane. Vertical lines show the mobility for the indicated molecular weight, obtained from the calibration curve.

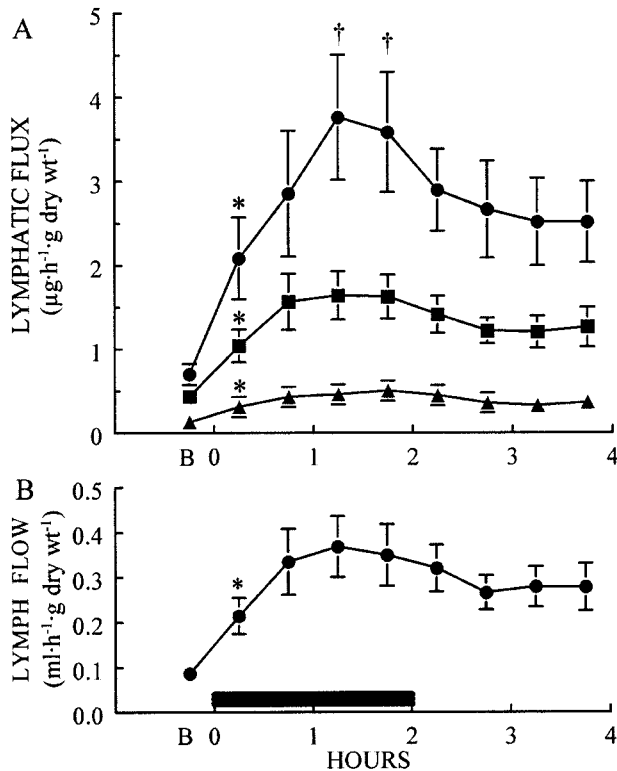


Fig. 2. Skin lymphatic flux of hyaluronan (A) and lymph flow (B) with volume expansion. Values are plotted against time after the start of the intravenous infusion of lactated Ringer solution. Time represents the midpoint of a 0.5-h collection. B, baseline; solid bar, duration of the infusion. In A, closed circles represent hyaluronan measured with the radiometric assay, closed squares represent hyaluronan with a molecular weight  $>0.79 \times 10^6$ , and closed triangles represent hyaluronan with a molecular weight  $>4 \times 10^6$ . Values are means  $\pm$  SE;  $n = 7$ . \* $P < 0.01$  compared with baseline from 0.25 to 3.75 h; † $P < 0.05$  compared with the final (3.75 h) value.

expanded. Lymph was collected for 4 h, during which lymph flow did not significantly change with time. Mean arterial blood pressure was  $79 \pm 8$  mmHg. Lymph flow was  $0.08 \pm 0.01$  ml·h<sup>-1</sup>·g dry wt<sup>-1</sup> using a value of 1.6 g dry wt for the weight of tissue drained by the cannulated lymphatic (33). The lymph concentration of hyaluronan irrespective of size was  $8.1 \pm 0.5$  µg/ml. Lymphatic flux of hyaluronan was  $0.6 \pm 0.1$  µg·h<sup>-1</sup>·g dry wt<sup>-1</sup>. The fraction of hyaluronan with a molecular weight  $>4 \times 10^6$  was only  $21 \pm 2\%$ . The fraction of low-molecular-weight hyaluronan ( $<0.79 \times 10^6$ ) was  $31 \pm 2\%$ .

As shown in Fig. 2, volume expansion produced a sustained increase in skin lymph flow and hyaluronan flux. Measurements were obtained from seven legs in four volume-expanded rabbits. Mean arterial blood pressure did not change during the experiment and was not different from the control group. The baseline values for lymph flow and lymphatic flux of hyaluronan were not significantly different from the values in the control group. Lymph flow increased threefold from baseline during the volume expansion and remained elevated for the 2 h after the infusion was ended. Lymphatic flux of hyaluronan increased to a peak

value of 5.4 times the baseline value during the infusion. It decreased from the peak to a value 3.6 times the baseline value by the end of the experiment when tissue samples were taken. The lymphatic flux of hyaluronan with a molecular weight  $>0.79 \times 10^6$  was three times the baseline value both during and after the infusion. A smaller increase was observed for hyaluronan with a molecular weight  $>4 \times 10^6$ .

The increase for low-molecular-weight hyaluronan was significantly greater than that for high-molecular-weight hyaluronan. Figure 3 shows lymphatic fluxes of differently sized hyaluronan from skin. Baseline values were not different from the control values. The lymphatic flux of low molecular weight hyaluronan increased fivefold. The lymphatic flux of high-molecular-weight hyaluronan was only 2.7 times baseline.

In contrast to lymph, skin did not contain a significant quantity of low-molecular-weight hyaluronan. Figure 4 shows a summary of the content of hyaluronan in heel skin. The values from the expanded animals were not different from the control values. Measurements of hyaluronan with a molecular weight  $>0.79 \times 10^6$ , using gel electrophoresis, were not different from those using the radiometric assay. This observation suggests the absence of a significant quantity of low-molecular-weight hyaluronan in the tissue. The fraction of hyaluronan with a molecular weight  $>4 \times 10^6$  was  $57 \pm 4\%$  and  $57 \pm 8\%$  for control and volume-expanded animals, respectively. These fractions were significantly greater than those found in lymph. The wet weight-to-dry weight ratio was  $3.78 \pm 0.05$  for the control group. The value for the volume-expanded group was  $4.26 \pm 0.07$  and significantly larger than control.

*Skeletal muscle hyaluronan.* Control measurements for skeletal muscle were obtained from seven legs in seven rabbits that were not volume expanded. Lymph

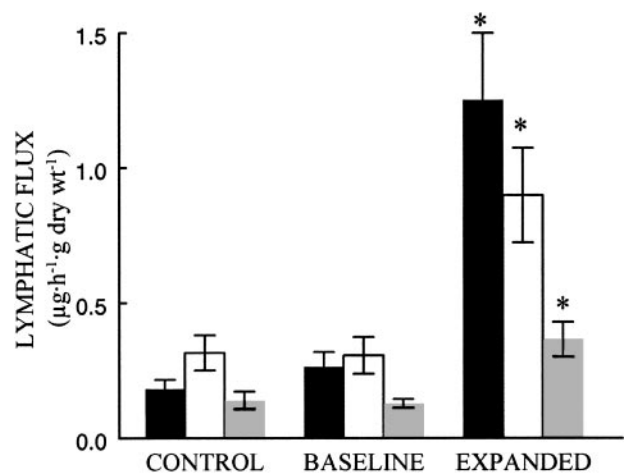


Fig. 3. Skin lymphatic flux of differently sized hyaluronan. Solid bars, hyaluronan with a molecular weight  $<0.79 \times 10^6$ ; open bars, hyaluronan with molecular weights between  $0.79 \times 10^6$  and  $4 \times 10^6$ ; shaded bars, hyaluronan with a molecular weight  $>4 \times 10^6$ . Values are means  $\pm$  SE;  $n = 8$  separate control animals and 7 animals for the baseline and final (3.75 h) values from the experimental group. \* $P < 0.05$  compared with baseline.

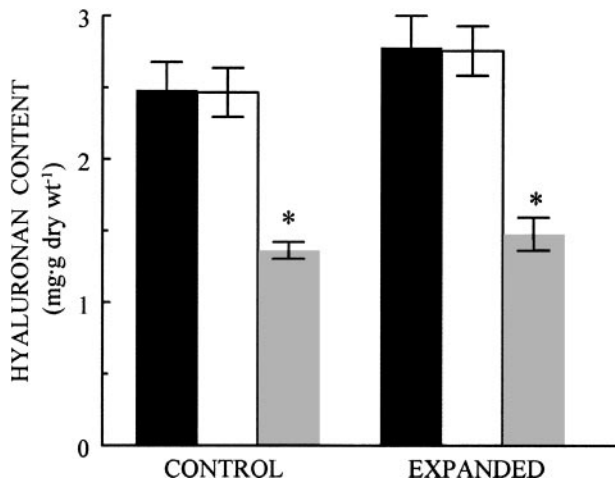


Fig. 4. Summary of hyaluronan content in heel skin. Solid bars, hyaluronan content measured using the radiometric assay; open bars, hyaluronan with a molecular weight  $>0.79 \times 10^6$ ; shaded bars, hyaluronan with a molecular weight  $>4 \times 10^6$ . Values are means  $\pm$  SE;  $n = 8$  control and 7 volume-expanded animals. \* $P < 0.01$  compared with content measured with the radiometric assay.

was collected for 4 h after ligation of the popliteal node. Measurements of hyaluronan in lymph were made on the last 30-min collection just before the tissue samples were taken. Mean arterial blood pressure was  $90 \pm 9$  mmHg. Lymph flow was  $0.012 \pm 0.002$  ml·h<sup>-1</sup>·g dry wt<sup>-1</sup> using a value of 5.2 g dry wt for the weight of tissue drained by the cannulated lymphatic (Table 1). The lymph concentration of hyaluronan irrespective of size was  $4.1 \pm 0.6$   $\mu$ g/ml and was significantly less than that in skin lymph. Lymphatic flux of hyaluronan was  $0.050 \pm 0.008$   $\mu$ g·h<sup>-1</sup>·g dry wt<sup>-1</sup>. The lymph from skeletal muscle contained a higher fraction of low-molecular-weight hyaluronan than lymph from skin. The fraction of low- and high-molecular-weight hyaluronan was  $54 \pm 6\%$  and  $7 \pm 2\%$ , respectively.

Measurements after volume expansion were obtained from five legs in five separate rabbits. Mean arterial blood pressure did not change during the experiment and was not different from the control group. We did not obtain reliable baseline measurements due to the time required to remove the residual skin lymph from the lymphatics after ligation of the popliteal node. The measurements after volume expansion were compared with the separate control group. Figure 5 presents a summary of the lymphatic flux of hyaluronan from skeletal muscle. Lymph flow was  $0.047 \pm 0.006$  ml·h<sup>-1</sup>·g dry wt<sup>-1</sup>, which was significantly greater than the control value. Hyaluronan flux after expansion was five times the control value. Similar to skin, volume expansion produced a preferential increase in the lymphatic flux of low-molecular-weight hyaluronan. The flux of low-molecular-weight hyaluronan was  $0.22 \pm 0.03$   $\mu$ g·h<sup>-1</sup>·g dry wt<sup>-1</sup> and eight times the control value. The flux of high-molecular-weight hyaluronan was not significantly different from the control.

Although skeletal muscle contained much less hyaluronan than skin, the electrophoretic pattern was very similar to that shown for skin tissue in Fig. 1.

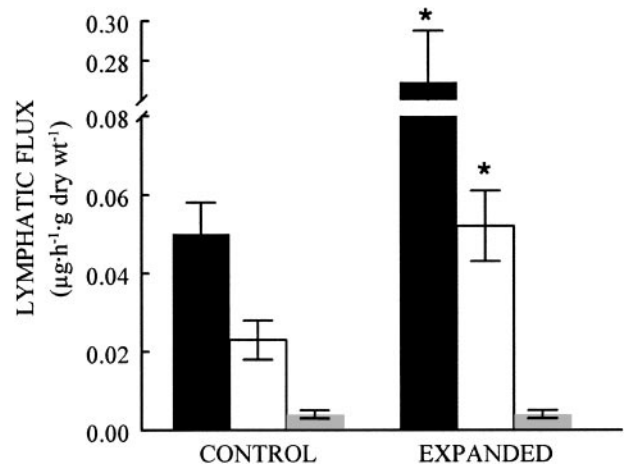


Fig. 5. Skeletal muscle lymphatic flux of hyaluronan. Solid bars, hyaluronan measured using the radiometric assay; open bars, hyaluronan with a molecular weight  $>0.79 \times 10^6$ ; shaded bars, hyaluronan with a molecular weight  $>4 \times 10^6$ . Values are means  $\pm$  SE;  $n = 7$  control and 5 volume-expanded animals. \* $P < 0.01$  compared with control.

Figure 6 shows a summary of hyaluronan content in the gastrocnemius muscle. The values from the volume-expanded animals were not different from the corresponding values from the control animals. The amount of hyaluronan measured with the radiometric assay was not different from the amount of hyaluronan with a molecular weight  $>0.79 \times 10^6$  measured using gel electrophoresis. The fraction of hyaluronan with a molecular weight  $>4 \times 10^6$  was  $58 \pm 3\%$  and was not significantly different from the value for skin. The wet weight-to-dry weight ratio for the control was  $3.95 \pm 0.09$ . The value for the expanded group was  $4.29 \pm 0.06$  and was significantly greater than the control.

*Hyaluronidase activity in control tissue.* The hyaluronidase activity at pH 4.0 was determined in tissues

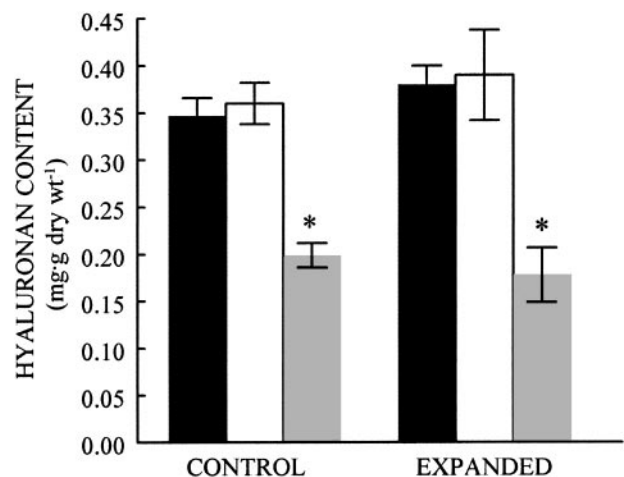


Fig. 6. Summary of hyaluronan content in gastrocnemius muscle. Solid bars, hyaluronan content measured using the radiometric assay; open bars, hyaluronan with a molecular weight  $>0.79 \times 10^6$ ; shaded bars, hyaluronan with a molecular weight  $>4 \times 10^6$ . Values are means  $\pm$  SE;  $n = 7$  control and 5 volume-expanded animals. \* $P < 0.01$  compared with content measured with the radiometric assay.



taken from control rabbits. As shown in Fig. 7, the activity in skin was 40% of the activity in the liver and more than six times the activity in skeletal muscle. The activity in plasma and lymph was measured from eight control rabbits. The activity in plasma was  $0.14 \pm 0.03$   $\mu\text{mol } N\text{-acetyl-glucosamine produced} \cdot \text{h}^{-1} \cdot \text{ml}^{-1}$ . The activity in skin lymph was  $0.04 \pm 0.01$   $\mu\text{mol } N\text{-acetyl-glucosamine produced} \cdot \text{h}^{-1} \cdot \text{ml}^{-1}$  and was significantly less than the value for plasma.

## DISCUSSION

The size of hyaluronan in lymph was considerably smaller than the size in the tissue. Increasing lymph flow with volume expansion produced a preferential increase in the lymphatic flux of low-molecular-weight hyaluronan. Because lymph was collected from prenodal lymphatics, the low-molecular-weight hyaluronan was not produced by degradation within lymph nodes. These results support in part our hypothesis that tissue contains two pools of hyaluronan with different sizes. Although the tissue measurements cannot distinguish between matrix associated and disassociated hyaluronan, the movement into lymph may be used to characterize a mobile pool within the tissue. This pool was predominately low-molecular-weight hyaluronan. Measurements in tissue did not show a significant amount of low-molecular-weight hyaluronan, as we originally proposed. The amount of low-molecular-weight hyaluronan in tissue may have been too small to be measured. The size of hyaluronan in tissue and lymph did not appear related to specific functions in skin because the results from skin and skeletal muscle were qualitatively similar.

We studied the size of hyaluronan using a combination of agarose gel electrophoresis and a radiometric assay. Both methods used radiolabeled HABP that were isolated from cartilage and specific for hyaluronan. Because hyaluronan has one negative charge on

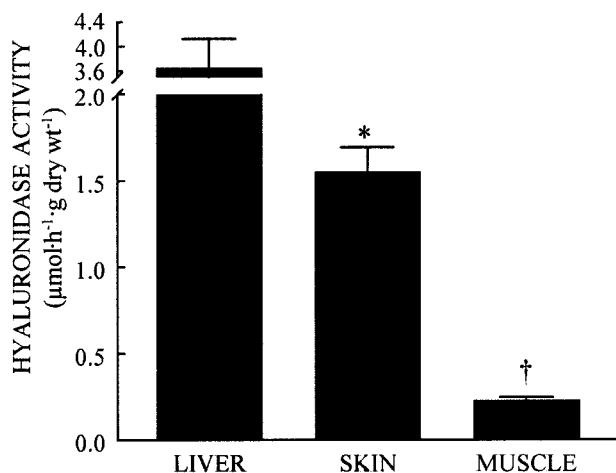


Fig. 7. Hyaluronidase activity at pH 4.0 in the liver ( $n = 5$ ), heel skin ( $n = 8$ ), and gastrocnemius muscle ( $n = 7$ ) from control rabbits. Activity represents the amount of *N*-acetyl-glucosamine at the reducing end of hyaluronan produced per hour. Values are means  $\pm$  SE. \* $P < 0.05$  compared with the liver; † $P < 0.05$  compared with skin.

each disaccharide, the electrophoretic mobility depended on the number of repeating disaccharides in the linear chain (10). Both concentration and molecular weight standards were applied to the agarose gel. The standards for concentration were purified high-molecular-weight hyaluronan. The concentration was measured with the radiometric assay so that measurements from the two methods could be directly compared. The concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$  or  $4 \times 10^6$  was measured in lymph or tissue digest using electrophoresis. The concentration of hyaluronan irrespective of size was measured using the radiometric assay. This assay measures a decasaccharide (2 kDa) or larger (1). The use of both assays avoids the problems associated with measuring low-molecular-weight hyaluronan using agarose gel electrophoresis (1). The concentration of low-molecular-weight hyaluronan was calculated as the difference between the concentration measured with the radiometric assay and the concentration of hyaluronan with a molecular weight  $>0.79 \times 10^6$ .

We found no evidence for low-molecular-weight hyaluronan in tissue from either skin or skeletal muscle. Values for the amount of hyaluronan with a molecular weight  $>0.79 \times 10^6$  were not different from the values measured using the radiometric assay. The absence of a significant amount of low-molecular-weight fragments in tissue was consistent with previous studies on noncancerous tissue showing no extracellular hyaluronidase activity at physiological pH (15). Our values for the hyaluronan content measured using the radiometric assay agreed with values reported previously for hind leg skin and skeletal muscle from the rat (34). Despite the large difference in hyaluronan content between skin and skeletal muscle, the electrophoretic patterns were similar. The pattern showed a single high-molecular-weight peak for both tissues. Lee and Cowman (10) obtained a very similar pattern for human synovial fluid using agarose gel electrophoresis and staining with Stains-All. They estimate that the peak for synovial fluid corresponded to a molecular weight of  $6 \times 10^6$  using a cross-linked derivative of hyaluronan as a high-molecular-weight standard. They emphasized that this is an approximation because cross-linked hyaluronan may not behave exactly like the linear polymer. The similarity between our electrophoretic patterns and theirs suggests that the peak for skin and skeletal muscle corresponds to a molecular weight of  $\sim 6 \times 10^6$ , similar to synovial fluid.

The fraction of hyaluronan with a molecular weight  $>4 \times 10^6$  was at least 57% for both skin and skeletal muscle. A similar value was found for synovial fluid (10). The similar fractions for high-molecular-weight hyaluronan among tissues may indicate a common synthetic pathway. Hyaluronan synthase, which has three isoenzymes, is located in the cell membrane of many cells (4). Li and Heldin (12) measured the size of hyaluronan produced by mesothelioma cells transfected with hyaluronan synthase-2 using gel filtration chromatography. Their chromatographic pattern was very similar to the electrophoretic pattern for skin

tissue shown in Fig. 1. Similar to our results, the majority of hyaluronan had a molecular weight  $>4 \times 10^6$ . The size of hyaluronan in skin and skeletal muscle may reflect the high-molecular-weight product of hyaluronan synthase with little extracellular depolymerization. Thus the molecular weight of hyaluronan in tissue may not be as polydisperse as generally proposed.

Previous reports on the size of hyaluronan in skin (17, 23) showed a much lower molecular weight. In contrast to synovial fluid, skin, or other solid tissues must be digested to release hyaluronan associated with the extracellular or pericellular matrix. Reed and colleagues (23) suggested that the digestion procedure may cause depolymerization. Although tissue hyaluronidase is not active at the pH used for digestion (5), the release of iron from cells during the process may cause depolymerization. Iron causes hyaluronan depolymerization by free radical attack of the glycosidic linkages that is inhibited by deferoxamine (13, 27). We found in preliminary experiments that omitting deferoxamine from the digestion buffer resulted in a time-dependent decrease in the molecular weight of hyaluronan. This decrease was consistent with the earlier observations by Reed and colleagues (23) and showed the need to include deferoxamine in the digestion buffer.

Lymph contained a much smaller fraction of high-molecular-weight hyaluronan than tissue. Only 7% of the hyaluronan in skeletal muscle lymph had a molecular weight  $>4 \times 10^6$ . Presumably, the high-molecular-weight hyaluronan in tissue was associated with pericellular and extracellular matrixes and could not easily move into lymphatics. Lymph, however, contained a significant fraction of low-molecular-weight hyaluronan that was not detected in tissue. The fraction of hyaluronan with a molecular weight  $<0.79 \times 10^6$  was 31% and 54% in lymph from skin and skeletal muscle, respectively. Further evidence for a small pool of dissociated low-molecular-weight hyaluronan within these tissues was shown when lymph flow was increased. Volume expansion caused an increase in lymph flow and lymphatic flux of hyaluronan similar to a previous observation on the dog hind leg (24). There was little or no increase in the flux of high-molecular-weight hyaluronan, whereas the flux of low-molecular-weight hyaluronan was five times control for skin and eight times control for skeletal muscle. These changes suggest that low-molecular-weight hyaluronan within tissue is more mobile than high-molecular-weight hyaluronan and can move into lymph. Although more complex mechanisms may be involved, the increase in lymphatic flux of hyaluronan after volume expansion may be due to a simple washout of low-molecular-weight hyaluronan that is not associated with matrixes. The amount of low-molecular-weight hyaluronan, however, appears to be small because we could not measure it in tissue.

Volume expansion did not produce a significant decrease in tissue hyaluronan content. The increases in lymphatic flux were small compared with the hyaluro-

nan content in the tissue. For skin, the increase in flux (expanded minus baseline) was equivalent to 17  $\mu\text{g/g}$  dry wt during the 4 h of expansion. This value represented 0.7% of the total hyaluronan content or 1.5% of the hyaluronan with a molecular weight  $<4 \times 10^6$ . Any decrease in tissue hyaluronan due to this increased lymphatic flux would be much smaller than the variation between animals or the error in the measurements. The calculations and conclusion for skeletal muscle were similar to skin. The absence of a change in tissue content was consistent with lymph draining only a small fraction of the total amount of hyaluronan in tissue.

The lymphatic fluxes of hyaluronan may be overestimated due to the method used to measure the weight of tissue drained by the lymphatic. The method compared the transvascular clearance of labeled albumin to the lymphatic clearance of endogenous albumin. Skin measurements have been reported previously (33), and the measurements for skeletal muscle were presented in Table 1. The values for both tissues are within the ranges estimated using local dye injections (18). The major assumption used in the method was that the transvascular clearance of labeled albumin, corrected for the loss in lymph, was equal to the lymphatic clearance of endogenous albumin. The transvascular clearance represented a unidirectional flux due to the negligible accumulation of labeled albumin in the extravascular space. The lymphatic flux represented a net flux because there may be diffusive movement across the microvascular wall from the extravascular into vascular space. The extent of this movement is not known and depends in part on the importance of convective transport across the microvascular wall (16). The calculated tissue weight represented a minimum weight because the unidirectional flux for labeled albumin may have been greater than the net flux for endogenous albumin. Thus the fraction of tissue hyaluronan removed by lymph was probably overestimated in the present study.

Although the catabolism is not well understood, hyaluronan can be removed from the extracellular space through lymphatic drainage or through endocytosis followed by lysosomal degradation. Our data showed that the daily removal of hyaluronan by lymph was small for both skin and skeletal muscle. The lymphatic flux from skin was  $0.6 \mu\text{g} \cdot \text{h}^{-1} \cdot \text{g dry wt}^{-1}$ , equivalent to  $14 \mu\text{g} \cdot 24 \text{ h}^{-1} \cdot \text{g dry wt}^{-1}$ . This value represented  $<0.6\%$  of the tissue content. For skeletal muscle, the daily flux was  $<0.4\%$  of the tissue content using the control value for lymphatic flux of  $0.05 \mu\text{g} \cdot \text{h}^{-1} \cdot \text{g dry wt}^{-1}$ . Studies using radiolabeled acetate as a precursor showed that the turnover of hyaluronan in rabbit skin was rapid with a half-life of  $\sim 2$  days (29). These observations suggest that lymphatic removal represents  $<3\%$  of the catabolic pathway. This value represents a maximum because our lymphatic fluxes may be overestimated. Studies using hyaluronan conjugated to cellobiose showed that less than one-half of the conjugate was removed through endocytosis, whereas the remaining fraction was removed by lymph (8). These



studies may have overestimated the importance of lymph due to the use of a low-molecular-weight conjugate. The small size may have permitted rapid diffusion into the lymphatic system without substantial contact with cellular receptors.

Because lymphatic flux of hyaluronan was too small to explain the rapid turnover of hyaluronan in skin and skeletal muscle, we measured the hyaluronidase activity at an acidic pH. This activity represented lysosomal activity. Our value for the liver, equivalent to  $0.9 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g wet wt}^{-1}$ , was similar to the value of  $0.8 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{g wet wt}^{-1}$  reported for the rat liver (2). Our value for skin was higher than the value Cashman and colleagues (3) reported for partially purified extracts from rat skin. They used a longer time to measure the production of *N*-acetyl-glucosamine at the reducing end of hyaluronan. Loss of activity during purification and the longer time may have underestimated the activity. The hyaluronidase activity in skin and skeletal muscle was 1.55 and  $0.23 \mu\text{mol N-acetyl-glucosamine produced}\cdot\text{h}^{-1}\cdot\text{g dry wt}^{-1}$ , respectively. This rate was equivalent to the hydrolysis rate of  $\beta$ -1,4 bonds. With the use of 419 g/mol for the molecular weight of a disaccharide, the minimum amount of hyaluronan that could be degraded was 0.65 and  $0.10 \text{ mg}\cdot\text{h}^{-1}\cdot\text{g dry wt}^{-1}$  for skin and skeletal muscle, respectively. These values are minimum estimates because the calculation assumed the maximal number of bonds hydrolyzed for a single hyaluronan chain. These rates were much greater than lymph flux and more than enough to account for a rapid turnover of hyaluronan in these tissues. Thus the limiting step in hyaluronan catabolism appears to be the rate of endocytosis.

Although the mechanism for internalization is not known, it may involve a small amount of fragmentation at the cell surface. These fragments would be released into the extracellular space and represent the small, mobile pool of low-molecular-weight hyaluronan that appears in lymph. Receptor-mediated endocytosis of hyaluronan has been studied in a variety of cultured systems (30, 31). In contrast to liver or lymphatic endothelial cells, internalization by keratinocytes or fibroblasts appears primarily through the CD44 receptor. It is not associated with clatherin-coated vesicles, caveolae, or pinocytosis. Tammi and colleagues (30, 31) showed that cultured keratinocytes internalized newly synthesized hyaluronan, which presumably was high-molecular-weight hyaluronan. They found, however, that most of the intracellular hyaluronan was located in early endosomes and had a low molecular weight, suggesting partial degradation before entering lysosomes. Hyal2, a type 1 hyaluronidase, has been found associated with cell membranes (11). Although the optimal activity is at a low pH, changes in the cell membrane during endocytosis may enhance the activity, producing a small amount of hyaluronan fragments. Irrespective of the mechanism that produces the low-molecular-weight fragments found in lymph, our data shows that the amount of low-molecular-weight hyaluronan in either lymph or tissue is very small compared with the amount of high-molecular-weight hyaluronan in tissue.

In summary, both skin and skeletal muscle contain predominately high-molecular-weight hyaluronan that was not removed by lymph after expansion. Lymph contained mostly low-molecular-weight hyaluronan, indicating that tissue had a pool of mobile low-molecular-weight hyaluronan. The size of this pool was small because it could not be measured in the tissue. The maximum daily removal of hyaluronan by lymph was <1% of the tissue content, indicating that lymphatic drainage is not significant in the normal catabolism of hyaluronan. The amount of lysosomal hyaluronidase activity in the tissues was more than enough to account for the rapid turnover of hyaluronan.

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