HYALURONAN METABOLISM IN RAT TAIL SKIN FOLLOWING BLOCKAGE OF THE LYMPHATIC CIRCULATION

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ABSTRACT

This study was undertaken to explore the effects of lymphatic blockage on the metabolism of hyaluronan in the skin. In initial experiments, [³H] hyaluronan was injected subcutaneously into the tail skin of rats that either had no surgical intervention (control) or into those that had their lymphatic drainage blocked two hours earlier (acute lymphedema) or after the lymphatics had been blocked for three months (chronic lymphedema). The removal of tritiated hyaluronan from the injection sites was determined by the appearance of [³H] in the plasma. The results showed that the clearance of injected hyaluronan was delayed in rats with lymphatic blockage. The half- life of injected hyaluronan in the controls was ~70-75 hr, compared with ~105-110 hr in the lymph blocking rats. The levels of radioactivity in the plasma from rats with both acute and chronically blocked lymphatics were lower than that of control rats during the entire follow up period. In addition, biochemical analysis revealed that there was a significant increased amount of hyaluronan in the tail skin three months after lymphatic blocking. These results suggest that lymph absorption is an important factor in the transport of hyaluronan from the interstitium. Blockage of regional draining lymphatics likely impairs the catabolism of hyaluronan, which stagnates in skin tissue.

It is known that lymphatic transport plays a major role in the metabolism of macromolecules of the interstitial matrix and, in particular, plasma proteins such as albumin. However, little is known about the role of lymphatics in the turnover of the polysaccharide components of the matrix, such as hyaluronan. The interstitium has been considered a static structure in which matrix component are catabolized locally. This view has been modified because at least one of its major components—hyaluronan, a large macromolecule (MW ~ 1 X 10⁶Kd) shares the same catabolic pathway as albumin, being drained away from tissues through lymphatic pathways (1).

Hyaluronan is partly metabolized in lymph nodes and enters the general circulation and is rapidly degraded in the liver. Because the lymph circulation is the main pathway for transport of hyaluronan from tissues, an impaired lymph circulation should not only promote stagnation of interstitial fluid, but also alter the metabolism of hyaluronan. In our previous study (2), the hyaluronan content was significantly increased in the tissue fluid of patients with peripheral lymphedema compared with tissue fluid in healthy limbs.

In order to clarify the influence of lymph flow on hyaluronan metabolism in the skin, we surgically blocked the lymphatic network of rat tail skin and then injected tritium

labeled hyaluronan subcutaneously either the same day or three months after operation. The specific radioactivity in blood was measured at specific time intervals after injection. The results showed for the first time that the turnover of hyaluronan was greatly impaired by damage to the lymphatic vasculature.

MATERIALS AND METHODS

Animal Model

Group 1 — Acute lymphatic blockage

Sprague-Dawley rats (~100-150 grams) were used in the study. To block lymph flow and create an edema model, a surgical procedure was followed according to that described by Slavin et al (3). Thus, rats were anesthetized with Metofane (Schering Plough Animal Health Corp., Union, NJ), the tail was shaved and cleaned with 75% ethanol and then 10 ul of 5% patent blue (Sigma, St. Louis, MO) in saline was injected into the tip of the tail. A circular incision was made at the base of the tail and a strip of skin approximately 1 cm wide was removed to the fascia membrane. Under the dissecting microscope, two deep lymphatic vessels stained with patent blue were identified and ligated with 5-0 sutures and divided. Electrocoagulation was applied to the divided end of the lymphatic vessels, as well as to the edges of the circular wound, with a resultant 1 cm gap between the edges of skin. The turnover of isotopic labeled hyaluronan injected into the tail skin was carried out 2 hours after the operation.

Group 2— Chronic lymphatic blockage (18 rats including 10 controls)

In another group of rats the same surgical procedure was applied and the tail wound was allowed to heal by secondary intent with development of "lymphedema." Three months later, tritiated hyaluronan was injected and traced as described below.

Tracer Injection and Sampling

Tritiated hyaluronan was prepared as described previously (4). In brief, rat fibrosarcoma cells that secrete large amounts of hyaluronan were cultured in the presence of [^{3}H] acetate for several days. The medium was collected, digested with protease and then dialyzed against distilled water. The labeled hyaluronan was isolated by precipitation with acetyl pyridium chloride, extracted with a high ionic strength buffer and precipitated with ethanol. The final precipitate was redissolved in saline, placed in a boiling water bath for 10 mm to inactivate any residual protease, filter sterilized and the concentration adjusted to 100 µg/ml. The final preparation had a specific activity of 4.5 X 10^5 DPM/ug and an average molecular weight of approximately 1 X 10^6 Kd. The [^{3}H] hyaluronan was injected subcutaneously into the distal part of the tail using a tuberculin syringe and 30G needle. The total injected volume (100 µl) was divided into smaller doses (20 µl) and injected separately to minimize leakage. Ten non-operated rats of the same age were used as control in each group and received a similar injection. After injection, 1 ml of blood sample was collected by puncturing the post-eyeball venous plexus in a heparinized vial at different times as indicated. The blood samples were centrifuged, and 100 µl of plasma were pipetted into glass scintillation vials. Thereafter, 1 ml of scintillation fluid was added and mixed and the radioactivity was determined in a scintillation counter.

Calculation of Hyaluronan Turnover Rate

The removal of [^{3}H] hyaluronan from the tissue was quantified according to the method described by Laurent et al (1). In brief, [^{3}H] hyaluronan leaves tissues primarily via the lymphatic network. Once it enters the bloodstream, it is rapidly taken up by liver endothelial cells where it is rapidly metabolized into [^{3}H] H$_2$O in which form it is found in the

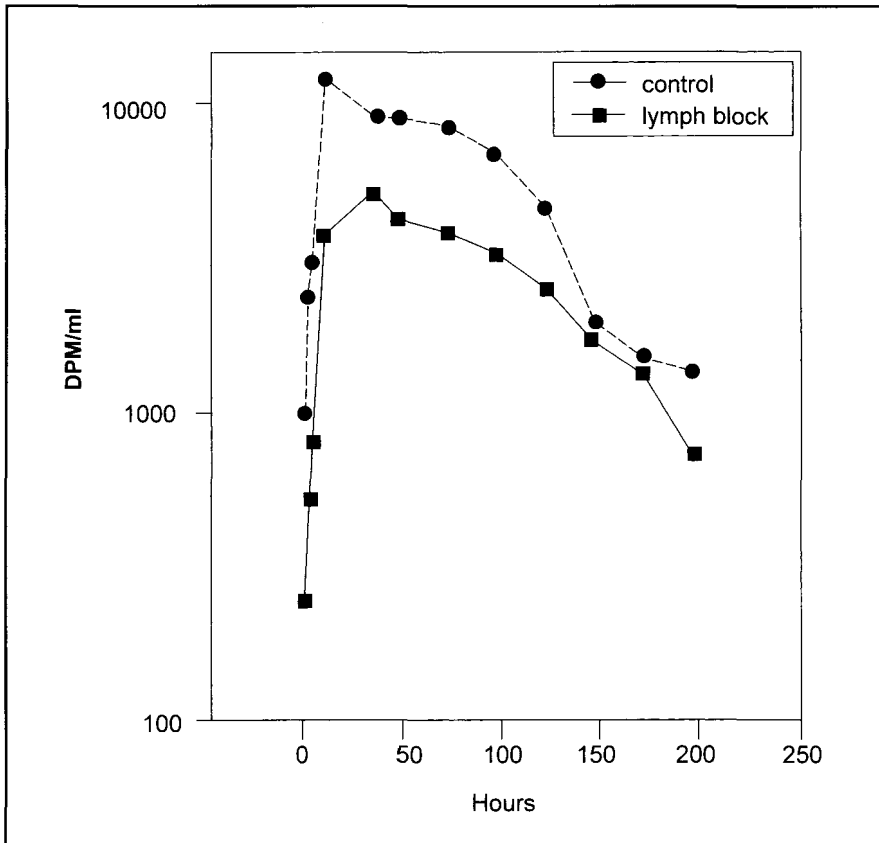*Fig. 1. The plasma concentration of tritium after injection of [³H] hyaluronan subcutaneously into the tail skin of rats with or without "acute" blockage of lymph transport.*

plasma (5). The catabolism of injected hyaluronan can therefore be followed by sequential determination of tritium in plasma (5). Tritiated water is lost from the tissue along the same pathways as non-radioactive water. Plasma radioactivity curve, therefore, initially increases as radiotracer leaves the injection sites, reaches a plateau when input of radioactivity from these sites equals elimination of [³H] H₂O from the body, and then declines as elimination of [³H] H₂O from the body exceeds the influx of tracer into the plasma compartment. The plasma levels of radioactivity were calculated using the formulations described by Laurent and colleagues (1). One first determines plasma radioactivity and obtains a curve as a function of time. A correction is then made on the curve to compensate for the turnover of [³H] H₂O from the body. Finally, the relative proportion of tritiated hyaluronan remaining at the injection sites is obtained by subtracting the corrected radioactivity in plasma at a given time.

Hyaluronan Concentration in Tissue

To quantify hyaluronan in the tissue, the skin specimens were cut into small pieces and minced, then homogenized by sonication in lysate buffer (20mM Tris- HCL, 137 mM NaCl, 2mM EDTA, 1% Triton X-100, 10% glycerol, 1mM PMSF). Hyaluronan content in skin was measured using a modified ELISA method. The key element of this assay was a biotinylated hyaluronan-binding complex
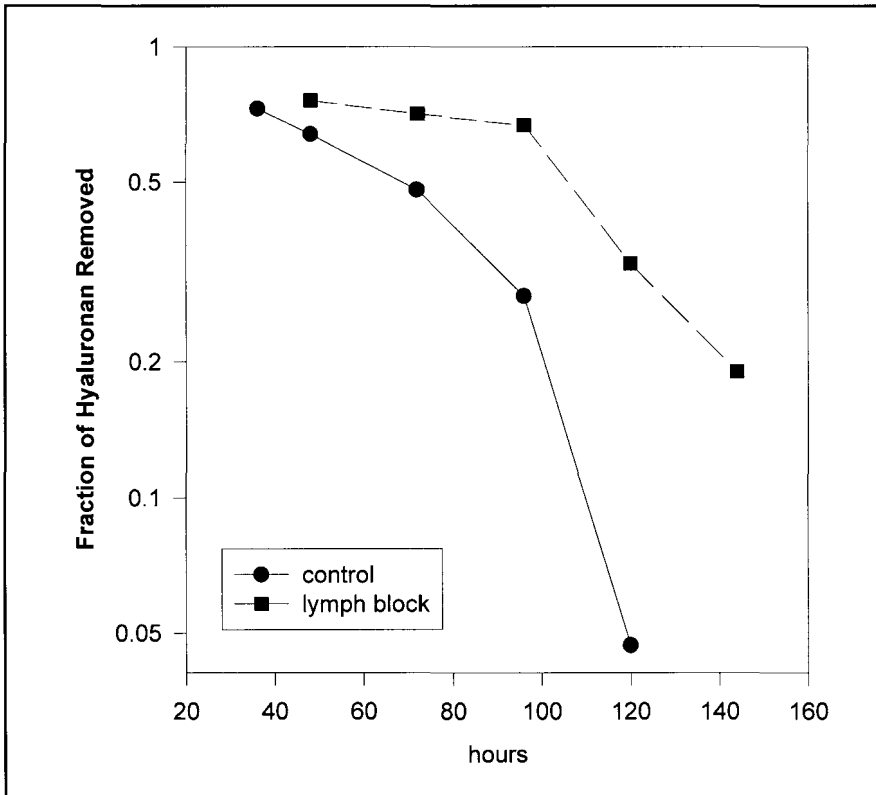
Fig. 2. The disappearance of [³H] hyaluronan from the tail skin after "acute" lymphatic blockage.

isolated from cartilage termed b-HABP. This complex was prepared by extracting bovine nasal cartilage with guanidine, digesting the resulting extract with trypsin to reduce its size, further reducing it with biotin and finally isolating the hyaluronan-binding fraction by affinity chromatography on hyaluronan coupled to Sepharose. This b-HABP was then used in an ELISA as follows. A high-bound ELISA plate (Falcon, BD Lab. Lincoln Park, NJ) was coated overnight with 100 ug/ml crude human umbilical cord hyaluronan (Sigma, St. Louis, MO) in PBS at room temperature and blocked with 10% calf serum, 90% PBS. The tissue lysate was normalized to equal protein concentration and then 25 μl samples were mixed with 100 μl of 10 μg/ml of b-HABP in a reaction plate at 37°C for 1 hour and then transferred to the ELISA plate. The unbound b-HABP remaining in the sample mixture could then bind to the hyaluronan on the coated plate and was detected by incubation with 0.5 μg/ml of peroxidase labeled streptavidin followed by 0.5 mg/ml of peroxidase substrate consisting of H_2O_2 and azinobis (3-ethyl-benzthiazoline sulfonic acid) in 0.1 M Na citrate buffer, pH 5.0. The plate was read at A_{405} with Emax ELISA reader (Molecular Devices, Sunnyvale, CA).

The concentration of HA in the samples was calculated from a standard curve performed simultaneously. The results were compared with the concentration in the "chronic" group before and after operation.

RESULTS

Group 1

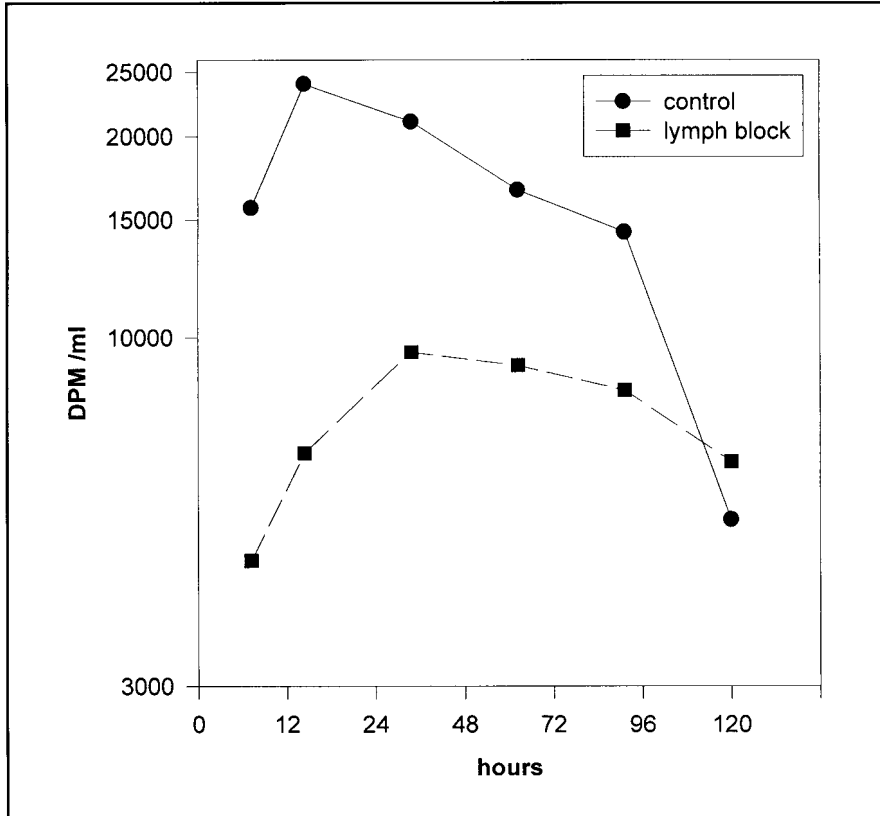The turnover of [³H] hyaluronan following subcutaneous injection is shown as

Fig. 3. The plasma concentration of tritium after injection of [^{3}H] into the tail skin of rats three months after blocking the lymph circulation.

Fig. 1. The tracing of tritium was started 2 hr after blockage of the lymph flow. The ^{3}H-content in blood was detectable ~4hr after injection in the tail skin. At each time point, the amount of tritium in the plasma was higher in the non-operated group than in that with acute lymphatic blockage. In the control group, the amount of tritium in the serum reached a maximum at 12 hr after injection, while in the group with lymphatic blockage, the maximum was reached at 36 hours. *Fig. 2* shows the turnover of hyaluronan at the injection sites as a result of calculation after correction for the turnover of water. In the control rats with intact lymphatic drainage, the half-life was ~ 70 hours, while in rats with an interrupted lymphatic circulation, the half-life was ~ 110 hours.

Group 2

Three months after interruption of lymphatic flow, the tails of the rats were examined for edema. The circumference of the operated tails (1 cm distal to the base) increased about 20-40% more than that of the non-operated rats of the same age. The increase in the circumference of the tails was due largely to the accumulation of free fluid in the interstitium, since this fluid was observed to flow out during harvesting of skin samples, a phenomenon not seen in tail skin under normal conditions.

The tracking of injected [^{3}H] hyaluronan was started 8 hr after injection. Compared with controls, the tritium present in the plasma was much lower in the rats with
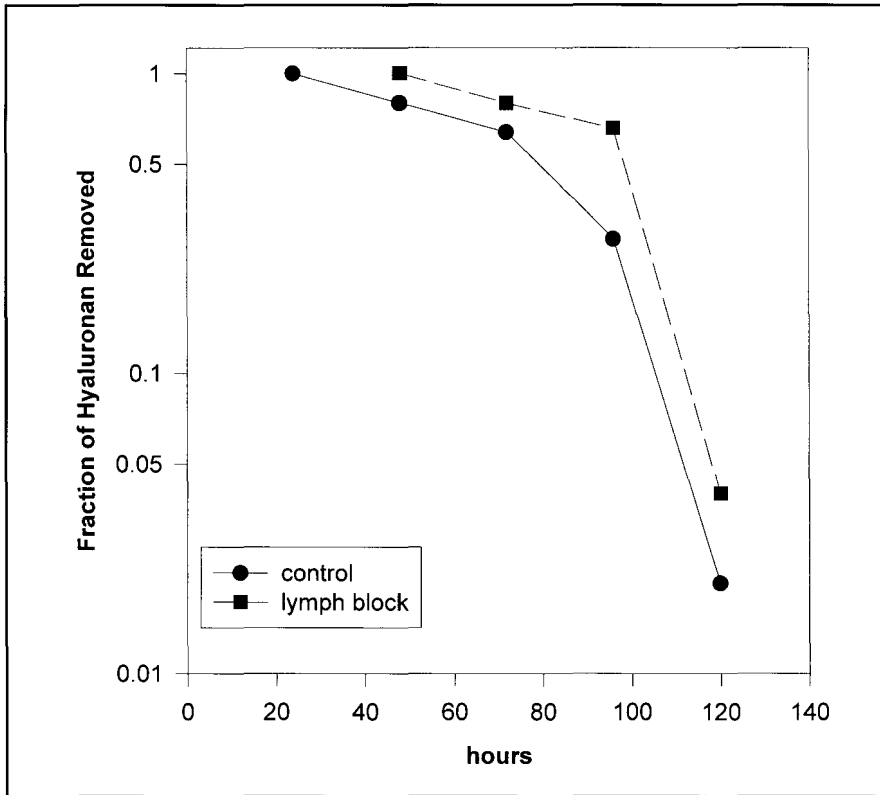
Fig. 4. The removal of injected hyaluronan from the tail skin three months after blocking the lymphatic circulation.

chronic lymphatic blockage during the entire observation period *(Fig. 3)*. The clearance of labeled hyaluronan from the injection sites is shown in *Fig. 4*. The half-life of [^{3}H] hyaluronan in the tissue with lymph flow interrupted was ~105 hr compared with ~75 hr in the controls.

In the group of rats with "chronic edema," the hyaluronan concentration in the skin tissue was measured using a modified ELISA. As a self-comparison the hyaluronan concentration was 2,634 ng/mg protein before operation and 50,492 ng/mg protein three months after operation *(Fig. 5)* (p<0.001).

DISCUSSION

Lymphedema has been termed a high protein edema due to impaired lymphatic transport with accumulation of plasma protein in the tissues. Indeed, the removal rate of protein injected into the interstitium is reduced in patients with peripheral lymphedema (3). Other factors such as capillary permeability, plasma colloid osmotic pressure and capillary hydrostatic pressure may also influence the concentration of protein in the interstitium. Some studies have shown, however, that the protein concentration in lymphedematous tissue is not increased and may, in fact, be lower than that in non-edematous tissue (2,7). Accordingly, a rise of capillary pressure and an increased micro-vascular filtration rate as well as altered interstitial fluid pressure, has been implicated in arm swelling and lowered protein content of edema fluid in patients treated for breast cancer (8).

Previous studies of [^{3}H] hyaluronan has demonstrated that the interstitial turnover of
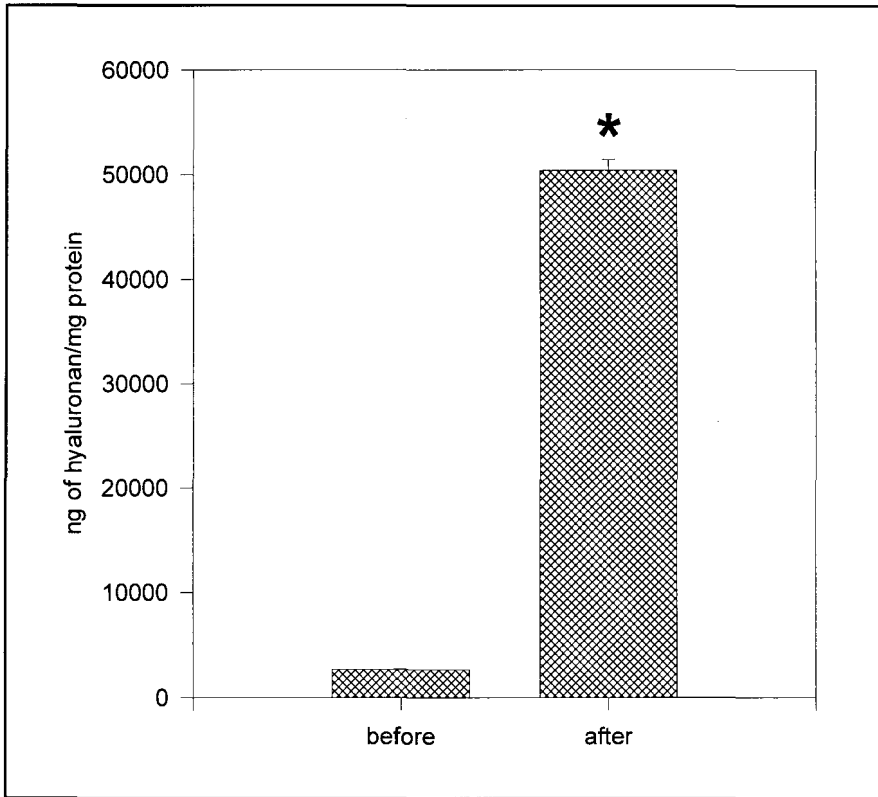
*Fig. 5. Changes of hyaluronan content in the skin of tail three months after blockage of lymphatics. *p<0.001.*

hyaluronan is similar to that of albumin (9). But the relationship of hyaluronan to lymphedema remains unclear. In contrast to the plasma proteins, hyaluronan is one of the main components of the intercellular matrix and is synthesized by tissue fibroblasts. Thus, microvascular dynamics or the "input factor" has less effect on the content of hyaluronan in the interstitium. Whereas the plasma protein concentration in draining lymph decreases when transcapillary and interstitial flux are increased, the hyaluronan concentration remains unchanged with increased venous pressure (6). Because the concentration of hyaluronan is determined entirely by the properties of the interstitium, its behavior in tissue can supply more accurate information about the functional properties of lymphatic and the interstitial dynamics. In a previous study, we found that there was a significant increase in the hyaluronan content of the interstitial fluid from the swollen limbs of patients with lymphedema. This observation probably relates directly to impaired lymphatic transport of hyaluronan produced in the tissue since ~75% of hyaluronan in the interstitium is carried away via lymphatics. Another possible mechanism for the heightened concentration of hyaluronan in these patients is an imbalance between tissue metabolism of hyaluronan and its transport from the interstitium. Thus far, information regarding catabolism of hyaluronan in the interstitium, especially with lymphedema, is lacking. An imbalance with increased hyaluronan production or reduced degradation in skin may also explain increased hyaluronan concentration in lymphedematous tissue.

In the present study, we used tritiated

hyaluronan as a tracer to follow the metabolism of the "free pool" of hyaluronan tested in rat tail skin tissue immediately ("acute") or three months ("chronic") after lymphatic blockage. The findings demonstrated that after injection of high molecular weight hyaluronan into the tail skin, there was a significant delay in the clearance of the tracer from tissues in both acute and chronic lymphatic blockage. The half-life of the tritiated hyaluronan injected into the "edematous" tissue was prolonged over that of control tissue. Moreover, the rate at which [^{3}H] hyaluronan was turned over from the injection site in rats with blocked lymph flow was lower than that in control rats during the whole observation period as reflected by the levels of radioactivity in the plasma. Finally, the concentration of hyaluronan in the tissue with impaired lymph drainage was significantly higher than that in the controls, a result consistent with tissue findings in patients with lymphedema. Taken together, the findings favor that blockage of lymph outflow directly influences the capacity of local tissue to dispose of hyaluronan and therefore alters the catabolism of this macromolecule.

The functional importance of hyaluronan trafficking is unclear, as is the true significance of a greater content of hyaluronan on effecting the homeostasis within the interstitium. Hyaluronan has unique macromolecular properties that are thought important for maintaining the fluid balance of tissues (11). Accordingly, further studies directed at the biological significance of increased concentration of hyaluronan in regional tissues is warranted to understand better the pathophysiology of lymphedema and its accompanying trophic skin changes. If increased tissue hyaluronan concentration also contributes to fluid imbalance in the extracellular space, then regulation and metabolism of tissue hyaluronan (by either reducing its production or increasing its degradation), may also prove useful in the management of peripheral lymphedema.

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