CHANGES OF TISSUE FLUID HYALURONAN (HYALURONIC ACID) IN PERIPHERAL LYMPHEDEMA

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ABSTRACT

Hyaluronan (hyaluronic acid or HA) is an important component of the extracellular matrix which is synthesized in the tissue, transported in lymph and catabolized mainly in lymph nodes and the liver. In 39 patients with chronic peripheral lymphedema, the HA content in lymphedematous interstitial fluid was measured using radioimmunoassay. For comparison, the concentration of HA in serum and normal tissue fluid were also determined. These samples were also tested for protein concentration. The results showed that the HA concentration in interstitial fluid of a lymphedema limb was $22x10^3 \pm 10^3$ (aspiration) and $30x10^3 \pm 4x10^3$ (wick) ng/ml which were significantly higher than that in interstitial fluid, serum and lymph of normal limbs (control) and interstitial fluid of limbs with venous edema (p<0.001). The protein concentration in these fluids did not show significant differences between lymphedema and those with normal limbs. The findings suggest that HA stagnates in the limb with impaired lymph drainage which may exert a deleterious effect on the interstitium.

The pathogenesis of the trophic changes that accompany peripheral lymphedema remains unclear. Although lymphedema is characterized by surplus tissue fluid, high in protein content (1,2), it is typically lower than plasma but similar to that of the contralateral normal limb (3). Accordingly, other factors may be responsible for the persistence and accumulation of fluid in the interstitium and trophic changes that accompany peripheral lymphedema. For many years, it has been believed that the interstitium is a static structure in which most of the ground substances are entirely synthesized and catabolized locally. However, a large amount of newer information indicates that hyaluronan (hyaluronic acid or HA), a major macromolecule responsible for the interstitial gel matrix, is absorbed from the interstitium by lymph and degraded in lymph nodes. Another component is transported to the bloodstream and is degraded in the liver (4,5). Synthesized and extruded through plasma membranes of fibroblasts, HA is present in all tissue fluid but its concentration varies widely. Structurally, HA has an extended random coil configuration with high molecular weight (10^6 and 10^7) and exhibits several important physical properties. Solutions of HA are extremely viscous and inhibit free solute diffusion and restrict the bulk flow of water (6). It has been suggested that HA plays a key role in regulating microvascular transport processes, interstitial hydration and plasma protein exclusion and the amount of HA is presumably important for maintaining interstitial fluid volume. The interstitial turnover of free HA is similar to the turnover of albumin (7,8) and is removed from tissues at a rate determined by lymph flow (9). It is

reasonable to hypothesize, therefore, that when the pathway for HA drainage is impaired and macromolecules stagnate in the tissues, accumulation of HA may influence local biologic function and homeostasis which in turn leads to the clinical trophic changes or the clinical manifestations in the skin and subcutaneous tissue.

The present study investigated changes of HA and protein content in lymphedema tissue and its implications were examined in terms of clarifying the pathogenesis of lymphedema.

MATERIALS AND METHODS

Clinical Studies

Thirty-nine patients with chronic lymphedema of an extremity were studied. Five had unilateral arm edema after mastectomy for breast cancer. The other 34 patients had unilateral edema of the legs. Of these, 10 had primary lymphedema and 24 secondary lymphedema. The longest duration of limb swelling was 29 years and the shortest was 2 years. The circumference differences between the lymphedema limb and normal varied from 2 to 15 cm. For purposes of comparison, 5 patients with leg edema secondary to deep venous thrombosis were included. In addition, lymph of the thoracic duct was collected for analysis from 4 patients with malignant tumors of the head and neck.

Collection of Interstitial Fluid

Two methods were employed. First— after puncture of the ventral forearm or calf with a fine needle, clear, yellow fluid from edematous limbs flowed through the needle and collected in a syringe. Sometimes gentle massage of the limb facilitated tissue fluid collection. Any samples contaminated with blood were discarded. Second—to compare findings between the edematous and nonedematous contralateral limb, tissue fluid was sampled using the soaked wick method

(3) in 10 patients. In brief, after anesthetizing the skin with lidocaine intradermally at the point of needle puncture in the ventral forearm or calf, a wick (multifilamentous nylon thread of 7 cm) was drawn through the subcutaneous tissue by needle. Five wicks were used in each patient. After 90 min, the wicks were removed and placed into mineral oil. Then the wicks were centrifuged in a two-compartment centrifuge tube at 3000 rpm for 10 min. The colorless wick fluid was stored at -20°C until analysis.

Analysis of HA

HA concentration was measured using a competitive radioimmunological binding assay kit (Naval Medical Research Institute, Shanghai). Pure HA of known solutions containing 50, 100, 200, 400, 800 ng/ml were used as standards. Samples of tissue fluid and lymph were diluted 50 times before testing. The assay was performed according to a five-step procedure. 50 µl of samples and standard solutions of HA were pipetted into test tubes. Then, 100 µl of protein with affinity for HA-HABP (HA binding protein) were added. The contents of the tubes were mixed and incubated at 37°C for 3 hrs. Then 100 µl of ^{125}I-HA was added, the contents were remixed, and the tubes were further incubated for 30 mins at 37°C. To each tube was added 100 µl of rabbit anti-HABP antibody and incubated for 30 min. A second antibody of sheep anti-rabbit IgG was added, the contents mixed, and then incubated for 30 min. The tubes were then centrifuged at 3500 rpm for 15 min. The supernatants were poured off and the tubes containing radioactive precipitate were counted in sequence in an automatic multigamma II counter (LKB1260, SWIDEN). The data were processed by computer analysis. Total protein and albumin concentration were determined in a Synchron Clinical System (CX4 Beckman).

Statistical Analysis

Fig. 1 HA concentration in aspirated interstitial fluid, lymph and serum
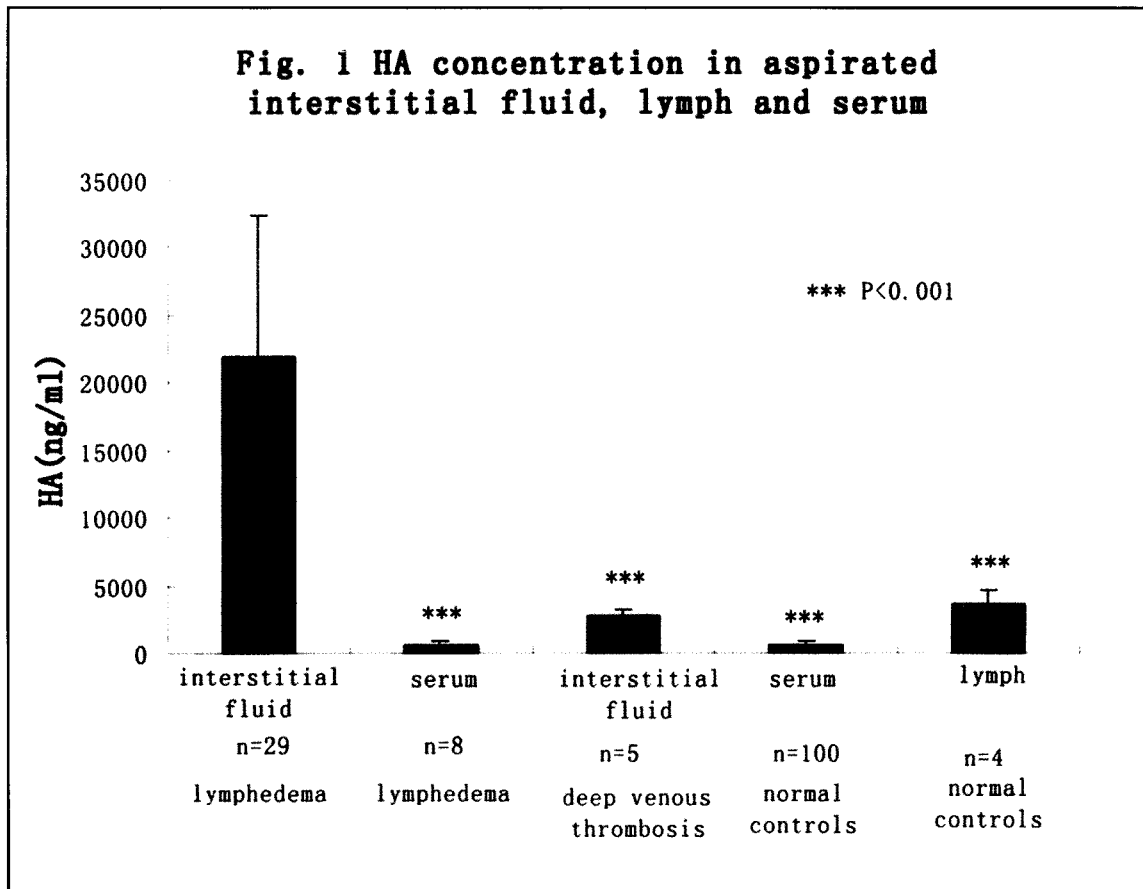

Fig. 1. HA concentration in aspirated interstitial fluid, lymph and serum.

Mean values of subgroups (aspirated tissue fluid, serum, lymph) were compared with analysis of variance (Dunnet test). The statistical comparison of the "wick" fluid between edematous and nonedematous limbs was done using a student t-test.

RESULTS

HA Content

Concentration of HA in aspirated interstitial fluid in limbs with lymphedema or venous edema and serum and thoracic duct lymph are shown in *Fig. 1*. The mean ± S.D. value of HA in lymphedematous fluid was $21x10^3 ± 10x10^3$ ng/ml, or about 330x that in serum ($65±20$), 8x that in venous edema fluid ($2744±463$ ng/ml; $p<0.001$) and 7x the level in thoracic duct lymph ($3612±1013$ ng/ml; $p<0.001$). There was no notable difference of HA content of serum of patients with lymphedema compared with normals ($57±27$ ng/ml). A comparison of HA and interstitial fluid obtained by the "wick" method showed a significant difference between the lymphedematous limb ($30x10^3 ± 4x10^3$ ng/ml) and the nonedematous contralateral limb ($8x10^3 ± 0.8x10^3$ ng/ml; $p<0.001$) *(Fig. 2)*.

Protein Content

The mean ± S.D. level of protein concentration in aspirated lymphedema fluid was

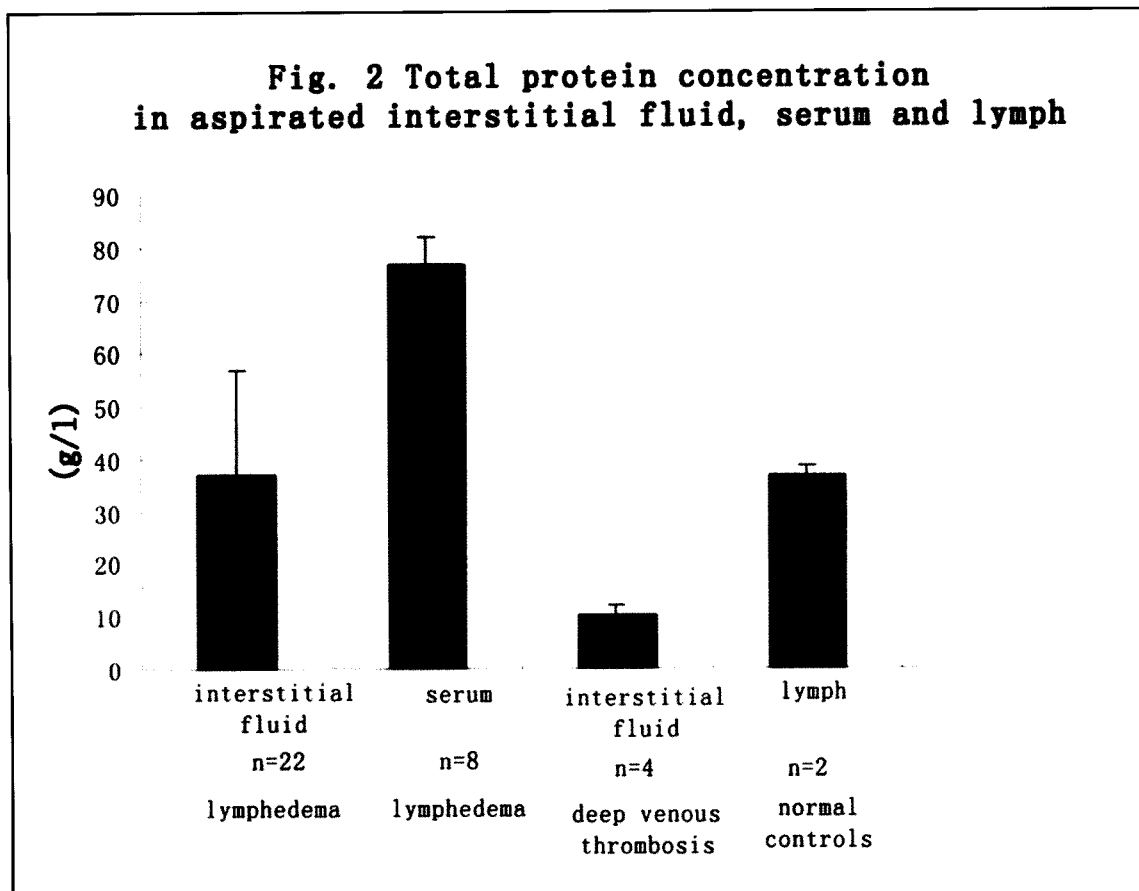## Fig. 2 Total protein concentration in aspirated interstitial fluid, serum and lymph

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36.8 ± 20 g/l or 48% that of serum (76.6 ± 5.0 g/l) *(Fig. 3)*. The protein concentration in venous edema fluid was 10.2 ± 1.6 g/l, whereas in normal lymph it was 36.5 ± 2 g/l. The total protein content in "wick" fluid was 34 g/l in the lymphedematous limb or 44% of the level in serum and 25 g/l in the contralateral limb. The difference of protein content in the interstitium, however, between lymphedematous and nonedematous normal limbs was not significant (p=0.39) *(Fig. 4)*. Albumin represented 56% of the total protein in the aspirated lymphedema fluid.

In lymphedema fluid, the HA concentration did not correlate with the protein concentration (correlation coefficient r=0.37, p=0.14).

DISCUSSION

For a long time the interstitium was considered a static structure in which matrix components were entirely catabolized locally. This view has had to be changed, however, in light of the finding that HA, especially HA from skin, is drained away from tissue in significant amounts through lymph. There seems to be two interstitial pools of HA (8). HA is initially attached to the pericellular membranes of fibroblasts and subsequently released into the free pool to be drained by lymph. Engstrom-Laurent et al (10) showed that skin contains about one-half of total body HA. A rapid turnover (half-life of 15-20 h) of HA in skin is a major determinant
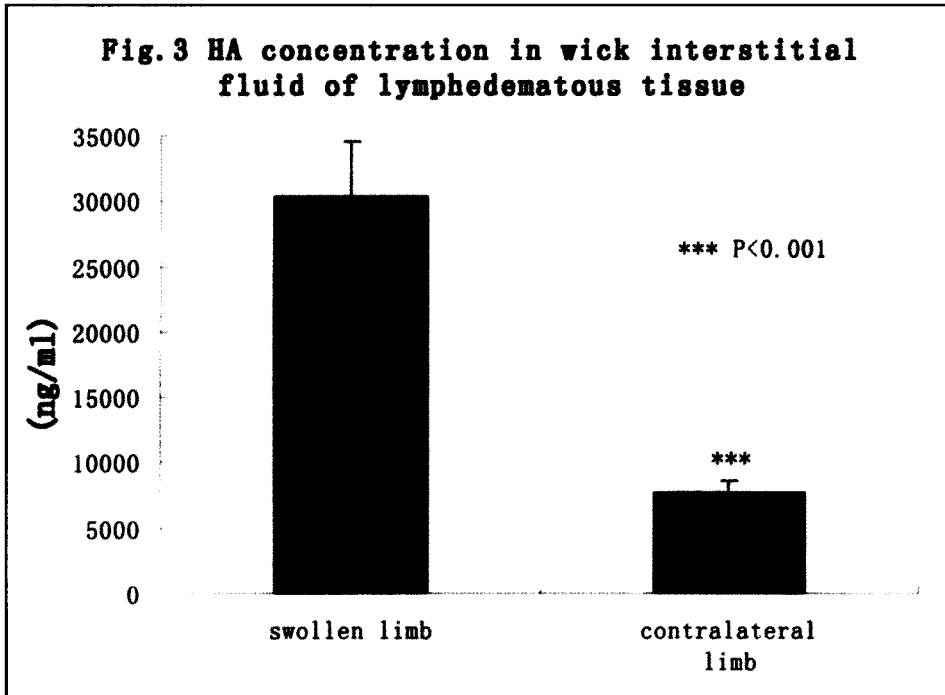
Fig. 3 HA concentration in wick interstitial fluid of lymphedematous tissue

Fig. 3. HA concentration in wick interstitial fluid of lymphedematous tissue.

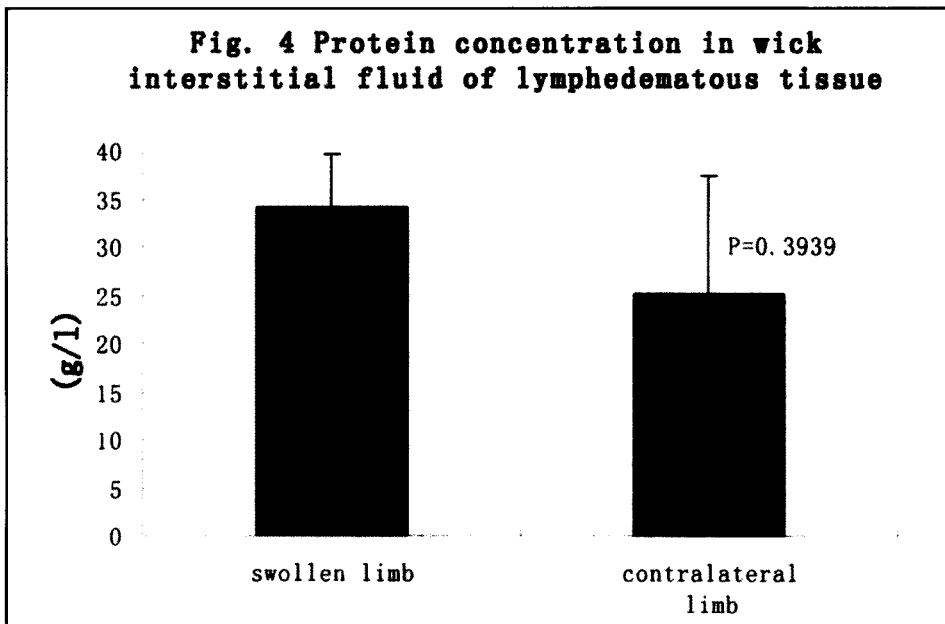## Fig. 4 Protein concentration in wick interstitial fluid of lymphedematous tissue

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of the turnover of HA in the whole body, and is at least 150 mg/day based on thoracic duct lymph collection emphasizing the importance of lymphatic drainage and the turnover of HA. HA concentration in skin apparently varies in different species being 1.6 mg/g in rat skin (9) and 200 mg/l in human dermis (11). Tissue fluid has a lower HA content which represents a free pool and amounts to about 25% of interstitial HA (8). Our study demonstrated that the HA content in normal human skin tissue fluid was $7.6 \times 10^2 \pm 8.4 \times 10^2$ ng/ml. No other similar findings have as yet been reported. In lymphedema fluid of the arm, it was 0.28 mg/ml as reported by Bates et al (3) using an Alcian Blue staining method. In our study, the HA content in lymphedema fluid was $22\text{-}30 \times 10^3$ ng/ml using a radioimmunological binding assay. Whereas disparity may relate to methodological differences, the significantly higher HA in the interstitial fluid of a lymphedematous limb compared with the nonedematous contralateral limb supports that impaired lymph drainage led to stagnation of HA in the interstitium.

That an accumulation of HA contributes to edema has been documented in the lung and infarcted myocardial tissue (12,13), although direct influence of increased HA content on lymphedematous tissue has not as yet been shown. Bates et al (3) showed that the viscosity of lymphedema fluid did not correlate well with tissue protein concentration suggesting that other factors may be influencing increased viscosity. The HA content in lymphedema fluid was very high and may have even been higher as HA exerts an osmotic force attracting water. Compared with pure HA solution used *in vitro* study (14) of viscosity, the concentration of HA in tissue fluid seen in lymphedema is much lower. Nonetheless, the *in vivo* influence of altered HA concentration on tissue fluid viscosity and its effect on tissue biologic responses still needs study. Because the HA content in lymphedema tissue was much higher than that in the normal limb, stagnated HA may

contribute to changes of the extracellular components including the cellular environment in a lymphedematous limb.

Lymphedema has long been thought of as a high protein edema due to stagnation of plasma proteins in the tissues from impaired washout of macromolecules with lymphatic "failure." Yet some report the protein concentrations are not high (2) or even slightly lower than normal (3). The present study supports that in most patients, the content of total protein in the interstitial fluid is similar to normal or about half that in serum. Two possible explanations exist for this finding. One is that Starling's microvascular forces concept that "elevated levels of tissue fluid proteins caused attraction of capillary water and lowered the protein concentration to physiologic values" (15). Another possible mechanism is the exclusion effect of HA and collagen. Thus, a polysaccharide network tends to exclude stoichometrically other macromolecules from the compartment that it occupies (11). Experiments have shown that collagen is responsible for two-thirds and HA for one-third of albumin exclusion in rat skin (16). Under normal conditions, the protein concentrations in lymph and interstitial fluid are nearly identical, which suggests that lymph is derived from the protein available space of the interstitium (8). Because the lymph transport of skin interstitial protein is similar to that of HA, it suggests that both plasma protein and HA are transported away from the same space. Increased concentration of HA and increased collagen content characteristic of lymphedema may act to exclude thereby plasma protein from the tissue space, although this effect still needs quantification.

The metabolism of HA in lymphedema tissue is unknown. Studies have shown that at most 25% of the HA in skin is catabolized locally, the remaining being broken down in regional lymph nodes and the liver (17). Although detailed reports are not available, injection of hyaluronidase or similar agents to

loosen the extracellular matrix in lymphedema has been discouraged (18). Intravenous infusion of testicular hyaluronidase, however, has reduced HA content in edema in infarcted myocardial tissue in man and lowered HA content in rabbit lung by 40%, with a resultant fall in lung water of 14% (8). The different outcomes, however, of using hyaluronidase to reduce edema from impaired lymphatic drainage as opposed to an imbalance in microvascular forces or permeability is unknown.

The present study demonstrated that in lymphedema, HA accumulates in the tissues and is significantly higher than HA levels in venous edema fluid and normal tissue fluid. Besides plasma proteins, other macromolecules such as HA are stagnating in the interstitium with lymphedema and along with impaired cell trafficking and cytokine release probably contribute to the imbalance in tissue homeostasis and the resulting trophic changes characteristic of chronic lymphedema.

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