Hyaluronic Acid Content of the Interstitial Fluid of Walker Carcinoma 256[1] (34525)

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(Introduced by W. E. Heston)

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Since a competitive inhibitor for hyaluronidase was found in the serum of tumor-bearing patients (1) it was considered important to ascertain whether the tumor was the source of low molecular weight hyaluronic acid which could act as inhibitor in the hyaluronidase assay. The cellular, vascular, and interstitial spaces of Walker carcinoma 256 contain respectively 54, 7, and 39% of the total tumor water (2). The interstitial space is occupied by collagen fibers (7 mg/g wet tumor) (3) and by the tumor interstitial fluid (IF). This fluid has a composition only partially known (4, 5) but it is expected to contain the mucopolysaccharides (MPS) of the tumor. In this work IF of Walker carcinoma and the subcutaneous area, where the tumor grew, were compared, and it was found that: (1) hyaluronic acid was the major component of the MPS fraction in both IF; (2) different regions of the subcutaneous tissue of the same animal had a different content of hyaluronic acid in IF, and (3) in Walker carcinomas the concentration of hyaluronic acid per milliliter of IF was about one half that of the subcutaneous area where the tumor was transplanted, and the total content, calculated from the size of the tumor interstitial space, was 6.4 μg/g wet tissue.

Materials and Methods. Male Sprague-Dawley rats, 3 months old were used. Walker carcinoma 256 was transplanted in the form of small fragments placed in a pouch of the subcutaneous tissue with a micropore chamber. This chamber is constituted by Millipore[3] filters and was used to sample the interstitial fluid of the tumor or the subcutaneous tissue as described (3). The sampling is based on the observation that neoplastic cells grow to cover the chamber in contact with the filter, without interposition of granulation tissue. The pore size of the filter (0.45 μ) precludes the penetration of the neoplastic cells into the chamber; however, the fluid surrounding these cells *in vivo* fills the chamber and can be sampled. The large size of the pores makes unlikely any "molecular sieving" by the filter; micropore chambers dipped into a solution of hyaluronic acid,[4] which is supposed to have about the same molecular weight as MPS of IF, showed equal concentrations inside and outside the chamber. Three groups of animals were used. In one, two sampling chambers were inserted into the subcutaneous tissue, one in the scapular, and the other in the lumbar region. In the second group, two chambers were also inserted, one in the normal subcutaneous tissue of the scapular region, the second in the lumbar region with addition of tumor fragments. The third group received only one chamber in the scapular region, and the interstitial fluid was sampled first when the chamber was in the normal subcutaneous tissue and then after the chamber had been engulfed by the tumor transplanted after the first sampling. The interstitial fluid from all areas was sampled 7–9 days after the chamber was inserted; the size of the tumors was

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[3] Millipore Filter Corporation, Bedford, Massachusetts.

[4] Grade I umbilical cord, Sigma Chemical Company, St. Louis, Missouri.

5–10 g. The animals were starved overnight and the sampling was done in the morning.

Sterility was tested by inoculating 2 drops of IF into 10 ml of Bacto-NIH thioglycollate broth (B 257 Difco Manual). The cultures were observed for 10 days. Infection was exceptional, and the contaminated samples were discarded.

The isolation and identification of MPS was done on a sample of about 2.5 ml interstitial fluid pooled from about 12 animals. The sample was digested with papain,[5] and the MPS were recovered by cetylpyridinium chloride (CPC) precipitation and then fractionated by a quantitative CPC-cellulose microcolumn technique (6). The concentration of MPS in each fraction was estimated by the content of uronic acids determined by the carbazole reaction (7). The fractionation procedure showed that the interstitial fluid was very rich in hyaluronic acid while the other MPS, if present, were in amounts not sufficient to be measured. The IF was therefore analyzed for hyaluronic acid content by a modified turbidimetric method. Turbidity of the sample was compared before and after depolymerization of hyaluronic acid by hyaluronidase. From the same sample of fluid two 50-μl aliquots were taken. To one aliquot 50 μl of buffer, pH 6.0, were added (CH_3COOH 0.25 M-KH_2PO_4 0.05 M–EDTA $2.68 \times 10^{-3} M$). To the second aliquot, 50 μl of a solution of 800 units/ml of bovine testicular hyaluronidase[5] in the same buffer, pH 6.0, were added. The mixture was incubated at 37° for 30 min and cooled for 10 min at 4°. To 1 vol of this mixture 4 vol of 0.5 M acetate buffer, pH 4.2, were added and left at room temperature for 30 min. The turbidity was read at 570 mμ, a wavelength best suited to avoid interference of the yellow color due to the IF. The hyaluronic acid content was measured by the difference between the two readings. The standard curve was done at concentrations between 5 and 100 μg/ml. Each animal yielded at least 200 μl of IF, and all determinations were done in duplicate.

Results. The fractionation of the interstitial fluid of normal subcutaneous tissue and tumors showed that hyaluronic acid was the only MPS present in both fluids in sufficient amount to be measured. The volume of fluid used for the fractionation varied from 1.5 to

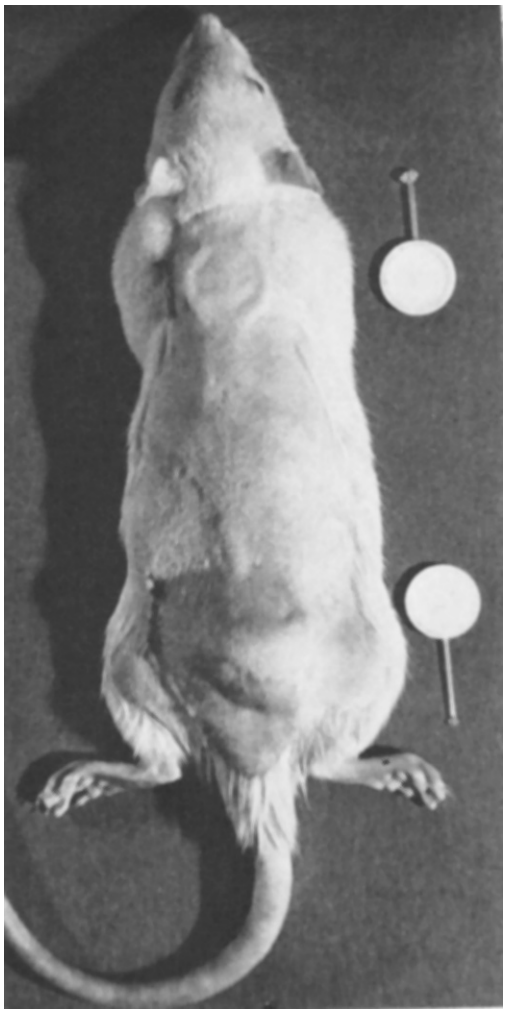

FIG. 1. Animal with one micropore chamber in the scapular region for the sampling of subcutaneous interstitial fluid and a second chamber incorporated by the tumor growing in the sacral region. Two micropore chambers are located beside the animal to show their position in the subcutaneous tissue of the rat. Note the polyethylene catheter heat-sealed at one extremity. The interstitial fluid passed through the filters (white discs) and was sampled by opening the catheter and collecting the amount accumulated in the chamber.

[5] Sigma Chemical Company, St. Louis, Missouri.

TABLE I. Hyaluronic Acid Content (μg/ml) of Subcutaneous Interstitial Fluid.

Rat no.	Interstitial fluid		Scapular/lumbar ratios
	Scapular	Lumbar	
1	42.4	27.2	1.56
2	64.0	52.0	1.23
3	78.4	59.2	1.32
4	70.4	44.0	1.60
5	71.2	44.8	1.58
6	75.2	42.4	1.77
7	26.4	19.2	1.37
8	40.0	27.2	1.47
9	40.0	27.2	1.47
10	40.0	28.8	1.39
11	33.5	21.6	1.55
Mean	53.52	37.16	1.45

3.5 ml, and the total amount of MPS isolated for each fractionation varied from 55 to 130 μg. A total of seven specimens were analyzed. They consisted of samples pooled from several rats, and the source of the samples was either the normal subcutaneous or the tumor interstitial space. The SIF and TIF samples, compared in order to detect possible differences in composition, were collected either from separate animals or from the scapular and sacral region, respectively, of the same rat. Traces of heparin sulfate were found in only one TIF specimen, but further work is needed to be certain of its presence.

With the turbidimetric method, large amounts of hyaluronic acid were found in the interstitial fluid of the subcutaneous tissue (Table I). In the same animal the concentration was not uniform; the scapular region constantly had about 45% more hyaluronic acid than the lumbar region. There was a relatively large variation from one animal to another, but there was very little difference in the ratio between the two regions of the same animal (Table I).

The hyaluronic acid content of the interstitial fluid of the tumor growing in the lumbar region was 20.1 $\pm$ 1.1 μg/ml (Table II). This value is about 45% lower than the initial concentration of hyaluronic acid in the lumbar region where the tumor was transplanted, and remained rather constant in different animals. Since the total tumor water is 82%, and 39% of this water is in the interstitial space (2), 1 g of tumor has about 0.32 ml of interstitial fluid and 6.4 μg of hyaluronic acid.

The presence of the tumor appeared to influence the hyaluronic acid concentration of distant areas of the host's derma. When the tumor was transplanted into the lumbar region, the scapular region showed a 26% reduction of the hyaluronic acid content only 1 week after transplantation, before any clinical sign of cachexia was appreciable (Table II). Hyaluronic acid was absent in the serum of normal and tumor-bearing animals as measured by the turbidimetric method.

Discussion. Since the neoplastic cells of Walker carcinoma are of epithelial origin, they are not expected to produce hyaluronic acid (8, 9). The tumor, however, has a hyaluronic acid concentration in the IF which is constantly lower than in the area where it was transplanted. Therefore, the neoplastic cell population in some unknown way does influence the hyaluronic acid production of the host's cells forming the stroma of the tumor. Moreover, the presence of the tumor in the host is sufficient to reduce the hyaluronic acid content of the subcutaneous IF in areas distant from the neoplastic mass.

TABLE II. Hyaluronic Acid Content (μg/ml) of the Interstitial Fluid in the Scapular and Lumbar Regions of Normal and Tumor-Bearing Rats.[a]

	Normal rat (13)	Rat with tumor in lumbar region (13)	% Decrease in hyaluronic acid concentration
Scapular region	51.7 $\pm$ 5.4	38.4 $\pm$ 2.0	25.7
Lumbar region	36.6 $\pm$ 3.3	20.1 $\pm$ 1.1	45.1
Ratio scapular/lumbar	1.41	1.91	

[a] Number of determinations in parentheses.

Since the hyaluronidase activity of the IF of Walker carcinoma was found to be twice as high as the activity of the fluid in the subcutaneous area where the tumor was transplanted (10), one could speculate that the tumor is actually responsible for a more rapid degradation of the hyaluronic acid of the host. This more rapid turnover could liberate into the blood serum a larger number of small molecular-weight frgaments of hyaluronic acid which may act as competitors in a test of hyaluronidase activity. As mentioned before, this activity was found reduced with tumor-bearing patients (1).

The 45% difference in the hyaluronic acid content of the subcutaneous IF between the scapular and lumbar region of intact animals was an unexpected finding. Since the concentration in both regions maintained a surprisingly constant ratio despite a large variation of the total hyaluronic acid content among different animals, it is probable that the observed difference has physiological importance. Whether the higher degree of mobility of the scapular region is responsible for this difference is a conjecture which cannot be substantiated at this time.

Summary. The interstitial fluid of Walker carcinoma transplanted into Sprague-Dawley rats was sampled *in vivo* and compared with the interstitial fluid of the subcutaneous area where the tumor was transplanted. Hyaluronic acid was the only mucopolysaccharide present in both fluids. In the normal subcutaneous tissue the fluid of the scapular region had 45% more hyaluronic acid than the fluid of the lumbar region. Development of the tumor depressed the hyaluronic acid content about 45% in the lumbar region where it grew, and about 25% in the scapular region which was distant from the tumor mass.

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