Lysosomal Hyaluronidase Activity in Normal Rat Liver and in Chemically Induced Hepatomas

B. Fiszer-Szafarz and D. Szafarz
Fondation Curie, Institut du Radium, Bâtiment 111, 91405-Orsay, France

SUMMARY

It has been demonstrated that solid hepatoma lysosomes lack hyaluronidase activity, whereas normal rat liver lysosomes contain a high level of this enzyme. This result was also found with lysosomes from an ascites hepatoma, Walker carcinoma 256, and virally transformed BHK cells.

In contrast, $\beta$-N-acetylglucosaminidase activity was shown to be present in lysosomes from tumor cells, although its level was decreased by about 75%, compared with that of controls. As it is known that hyaluronidase and $\beta$-N-acetylglucosaminidase activities are increased in the extracellular space of tumors, we suggest that the lack of hyaluronidase and the important decrease of $\beta$-N-acetylglucosaminidase in tumor lysosomes are due to a perturbation of the control of enzyme distribution from the Golgi apparatus to the lysosomes and the extracellular space.

INTRODUCTION

Invasiveness is a characteristic property of most cancer cells. The destruction of their surrounding connective tissue could be brought about by a series of hydrolytic enzymes (19), the activity of which, although playing a role in normal tissue physiology, is perturbed by carcinogenesis. Since the ground substance of the connective tissue is essentially composed of hyaluronic acid, we have studied the activity of lysosomal hyaluronidase in normal rat liver and in some chemically induced hepatomas.

In previous experiments, we observed that the activity of hyaluronidase, which is an acid hydrolase normally present in lysosomes (1) and in the extracellular space, is doubled in the latter fraction of tumors (12). Consequently, the concentration of hyaluronic acid in the extracellular space is reduced to about one-half its normal value (11), and its degradation products appear in the circulating blood (10).

In order to explain this observation, we undertook the study of hyaluronidase activity in lysosomes, which are considered to be the specific organelles that contain all acid hydrolases. Our results show that hyaluronidase activity is absent from tumor cell lysosomes, whereas in normal cell lysosomes this activity is fairly high.

This difference was also found in some other tumors, such as the Walker carcinoma 256, the Zajdela ascites hepatoma, and BHK21 cells transformed by the hamster sarcoma virus and Rous sarcoma virus (SR-RSV).

MATERIALS AND METHODS

Animals and Tumors. Six- to 12-month-old male Wistar or Sprague-Dawley rats were used. The animals were maintained on a standard pellet diet and water ad libitum. All food was withheld 16 hr prior to sacrifice.

Hepatomas were induced in Wistar rats with diethyl nitrosamine and in Sprague-Dawley rats with p-dimethylaminoazobenzene.

Walker carcinoma 256-bearing rats were given to us by Dr. P. M. Gullino (NIH). Zajdela ascites hepatoma cells were obtained from Dr. F. Zajdela. BHK21 cells transformed by the hamster sarcoma virus were obtained from Dr. L. Montagnier, and the same cells transformed by the Rous sarcoma virus (SR-RSV) from Dr. P. Vigier.

Chemicals. All reagents were purchased from the Sigma Chemical Co. (St. Louis, Mo.) except hyaluronic acid, which was prepared in our laboratory, by the method of Mathews (14).

Preparation of Lysosomal Extracts. The crude lysosomal fraction was prepared by the method of Aronson and Davidson (1) from normal liver and excised nonnecrotic hepatoma. The fractions studied were checked by electron microscopy for the relative abundance of lysosomes.

We obtained the water extract of the lysosomal pellets by extracting them with distilled water for 3 hr at 0° and clarified the solution by centrifuging it for 30 min at 98,600 $\times$ g.

Cell sap was obtained by centrifugation of the supernatant of the lysosomal fraction for 2 hr at 105,000 $\times$ g.

Assay of Hyaluronidase (EC 3.2.1.35). Hyaluronidase was assayed in the water extracts by incubation of the following mixture at 37°: 0.15 ml of extract and 0.50 ml of hyaluronic acid (1 mg/ml) in 0.05 M formate buffer, pH 3.5, containing 0.3 M NaCl. Aliquots (0.05 ml) were assayed for liberated GlcNAc after 0, 15, 30, 60, 120, and 240 min of incubation. Enzyme activity was expressed in nmoles of GlcNAc liberated per hr per ml of extract. For comparative purposes, the activity was also calculated per g of fresh tissue, per mg of tissue DNA (indicative of the activity per cell), and per mg of protein present in the lysosomal extract.

Assay of $\beta$-N-Acetylglucosaminidase (EC 3.2.1.30). This
enzyme was assayed under the same conditions as was hyaluronidase, with 5 mm p-nitrophenyl-N-acetyl-β-D-glucosaminide. To 0.5 ml of substrate solution was added 0.15 ml of lysosomal extract appropriately diluted with 0.1% aqueous bovine serum albumin. One-tenth-ml aliquots were taken every 3 min up to 12 min. The reaction was stopped by the addition of 0.6 ml of an alkaline buffer solution (133 mm glycine, 83 mm Na₂CO₃, and 67 mm NaCl, adjusted to pH 10.7) (23), and the amount of released p-nitrophenol was measured by its absorbance at 400 nm.

Chemical Determinations. GlcNAc was estimated by the method of Reissig et al. (20). DNA was estimated in the initial tissue homogenate by the diphenylamine method of Burton (4). Protein was estimated in the extracts by a modification of the method of Lowry et al. (as described in Ref. 9).

Dialysis of the Extracts. For the experiments with dialyzed extracts, 1 ml of extract was dialyzed twice against 2 liters of distilled water for 16 hr at 4°C.

RESULTS

Hyaluronidase Activity in Lysosomes of Normal Liver and Hepatoma. Lysosomal extracts, prepared in the standard manner as described in “Materials and Methods,” had a protein concentration of about 3 mg/ml for the liver extracts and about 2 mg/ml for the hepatoma extracts. Comparable values were obtained when these concentrations were related to the DNA content and to the tissue fresh weight. Table 1 shows the average values thus obtained. The protein/DNA and protein/tissue weight ratio for tumors are not changed significantly, compared with those of normal cells. Table 2 shows the hyaluronidase activity related to different parameters of the tissues. Normal liver lysosomes show significant activity, which, as can be seen from the low standard errors, seems constant, especially when expressed per mg liver DNA. Hepatoma lysosomes display no activity.

Hyaluronidase Activity in Fractions Other Than Lysosomes. An explanation for the lack of hyaluronidase activity in tumor lysosomes is the possibility of a different distribution among the various cell fractions. However, only in the soluble cell sap was an activity found, which was probably due to the presence of the extracellular fluid in that fraction.

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ratio, mg protein/mg tissue DNA</th>
<th>Ratio, mg protein/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver (10)</td>
<td>1.30 ± 0.11*</td>
<td>2.41 ± 0.12</td>
</tr>
<tr>
<td>Hepatoma DAB (8)</td>
<td>0.96 ± 0.15*</td>
<td>1.96 ± 0.19</td>
</tr>
<tr>
<td>Hepatoma DEMA (2)</td>
<td>0.86 ± 0.32*</td>
<td>2.25 ± 0.50</td>
</tr>
<tr>
<td>Walker carcinoma 256 (4)</td>
<td>0.75 ± 0.14*</td>
<td>2.36 ± 0.33</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, numbers of different lysosomal extracts.

Effect of Enzyme Concentration on Hyaluronidase Activity. We thought that the latter result might be due to the inactivation of the enzyme in the hepatoma extracts by too high a dilution of the proteins in these extracts. Although the protein concentration of the tumor cell extracts was shown to be on an average 30% lower than in normal extracts (due to the technical procedure of extraction), it was, nevertheless, relatively high. Even by increasing the relative proportion of the tumor extract in the reaction mixture, we could detect no hyaluronidase activity.

As a further check on a possible inactivation of the enzyme by dilution, crystalline bovine serum albumin, a protein stabilizing agent, was added to the tumor extracts. Various concentrations of added albumin failed to induce any activity in these extracts.

Following the same line of thought, we examined the effect on hyaluronidase activity of diluting the normal extract. The results (Chart 1, Curve a) demonstrate a linear relationship between enzyme activity and protein concentration. At rather low total protein concentration (much lower than that of the hepatoma extracts) there was still a quite easily detectable enzyme activity.

It can thus be concluded that the lack of activity in the hepatoma extracts is not due to excessive dilution.

Effect of Mixing Normal Liver Lysosomal Extracts with Hepatoma Lysosomal Extracts. The missing activity in hepatoma extracts, as shown in Table 2, might be explained either by the disappearance of lysosomal hyaluronidase in hepatoma or by the presence of an inhibitor. To test these possibilities, we studied the activity resulting from mixing the active normal extract with the inactive hepatoma extract.

On mixing a normal extract with a hepatoma extract, we found that the mixture had in fact a higher activity than the sum of the activities of each extract. This implied that there was no inhibitor involved. Therefore, we decided to study the activity of mixtures in which 1 of the components was kept constant.
When the concentration of normal extract was varied and that of hepatoma extract was kept constant (Chart 1, Curve b), the activity of the mixtures as a function of the protein concentration of the normal extract varied in a way parallel to that of the normal extract. This implied that the increase in activity due to the hepatoma was constant for all the mixtures studied.

When the concentration of normal extract was kept constant and that of the hepatoma was varied (Chart 2), the resulting activity remained equal to that of the normal extract alone until a concentration of about 1 mg of hepatoma extract per ml was reached; at higher concentrations it increased linearly with the protein concentration. Thus, again, the increase in activity depended on the hepatoma extract.

Closer examination of this effect showed that activation was detectable only if the ratio of tumor extract protein to normal extract protein was larger than 1; merely increasing the concentration of the components of the mixture was not enough to facilitate detection of activation.

When different normal extracts were mixed with the same hepatoma extract, all of the activations were equal. Conversely, when different tumor extracts were mixed with the same normal extract, all activations were different.

All of these results showed that the activation that resulted from mixing depended directly upon the hepatoma extract, although the latter did not display any activity by itself. This led to the hypothesis that the tumor extract might contain an inactive enzyme that was activated by some factor present in the normal extract.

Effect of Dialysis of the Extracts on Hyaluronidase Activity. To test this hypothesis, we dialyzed the aqueous extracts, both normal and tumor, against water for 16 hr at 4°. Then the various dialyzed and nondialyzed extracts were mixed in all possible combinations, and the resulting activities were measured. Dialysis removed hyaluronidase activity in the normal extract as well as the activity of the hypothetical activator.

Effect of Heating the Extracts. The effect of heating the extracts for 15 min at 100°, centrifuging the insolubilized material, and using the supernatants in the manner described above again showed that both hyaluronidase and activator had been inactivated.

Kinetics of the Activation Produced on Mixing Normal and Tumoral Extracts. The preceding results showed that the activator was probably a protein, and possibly an enzyme; therefore we decided to study the kinetics of the activation. Chart 3 shows the result of deducting from the activity of a mixture the activity of the normal component alone. The curve thus obtained is a typical enzymatic reaction curve.

This very important result led us to abandon the hypothesis of an "activator" and to turn our attention toward the possibility that a multienzyme system acts upon hyaluronic acid. There exist only 2 methods of estimating hyaluronidase activity, namely, turbidimetric determination of non-hydrolyzed hyaluronic acid (10) and colorimetric determination of the GlcNAc (20). The former method is very
cumbersome and aleatory. We therefore used the latter method.

Aronson and De Duve (2) demonstrated that the 3 enzymes necessary for the complete hydrolysis of hyaluronic acid are present in normal liver lysosomes. These enzymes are (a) hyaluronidase (EC 3.2.1.35), which hydrolyzes hyaluronic acid to tetrasaccharides; (b) β-N-acetylglucosaminidase (EC 3.2.1.30); and (c) β-glucuronidase (EC 3.2.1.31), which act alternately and in a coupled way on these tetrasaccharides releasing GlcNAc and glucuronic acid. Thus it appears that both hyaluronidase and β-N-glucosaminidase release GlcNAc groups, which are then estimated by the colorimetric method we had to use.

This fact led us to conclude that the liver lysosomal extracts contained all of the 3 above-mentioned enzymes, whereas the hepatoma extracts contained only the latter two. This would explain all our preceding results, namely, (a) the activity observed in normal extracts; (b) the absence of activity in hepatoma extracts, as highly polymerized hyaluronic acid cannot act as substrate for enzymes b (β-N-acetylglucosaminidase) and c (β-glucuronidase); (c) the fact that, when the extracts are mixed, the products of the normal hyaluronidase become available as substrates for both liver and hepatoma enzymes b and c; and (d) the effects of dialysis and heating.

We thus had to check the presence of β-N-acetylglucosaminidase in the lysosomal extracts.

β-N-Acetylglucosaminidase Activity in Lysosomal Extracts. β-N-Acetylglucosaminidase activity was determined in liver and hepatoma lysosomal extracts. The results (Chart 4) show the presence of a high activity in liver lysosomal extracts, a much lower activity in hepatoma lysosomal extracts, and a simple additive effect when both types of extract were mixed.

It was further confirmed that this activity was destroyed upon dialyzation of the extracts.

Hyaluronidase Activity in Tumors Other Than Primary Hepatoma. Lysosomal hyaluronidase was also studied in Zajdela ascites hepatoma, in Walker carcinoma 256, and in hamster sarcoma virus- and Rous sarcoma virus-transformed BHK21 cells. Lysosomal extracts were prepared as for the preceding experiments. Estimation of hyaluronidase activity showed that this enzyme was absent from all 4 types of tumors; β-N-acetylglucosaminidase, on the contrary, was present.

In addition, hyaluronidase activity was detected in lysosomal extracts from normal untransformed BHK21 cells. When any tumor extract was mixed with liver extract, an increase in GlcNAc-releasing activity was also observed.

Stability of Hyaluronidase in the Extract of Normal Liver Lysosomes. Activity measurements of the hyaluronidase in extracts of normal liver lysosomes showed that the enzyme was stable at least 40 days when kept at 4°. However, after several days at 4°, the solutions became turbid. When the insoluble material was pelleted, all the hyaluronidase activity was recovered in the supernatant. If the solutions were kept frozen, no inactivation was observed, even after several months.

However, acidified solutions lost their activity in 24 hr.

Nevertheless, it could be demonstrated that a short treatment of the normal extract at pH 4 (acidifying with HCl) precipitated 20% of the proteins but left all of the hyaluronidase activity intact. This could be a useful step for the purification of the enzyme.

Electron Microscopy of the Lysosomal Fractions. On checking the various lysosomal preparations used for preparing the extracts by electron microscopy, we observed that the tumor fractions contained larger lysosomes than the normal ones and, also, that they seemed emptier. This agrees with the observations of Wattiaux et al. (24).

DISCUSSION

In previous experiments, one of us had shown that in the extracellular fluid of tumor there was a doubling of hyaluronidase activity, compared with that of normal homologous tissue. The presence of other acid hydrolases, such as glycosidases and acid phosphatase (3, 16), cathepsin (21), β-glucuronidase, and others (18) has been demonstrated in tumor extracellular fluid, in which all these lysosomal enzymes would be active in changing the properties of the tumor cell surface, the pericellular region, and even of the adjacent normal cells, thus facilitating invasive-ness and metastasis. The lack of ionic communication between tumor cells and the alteration of communication in normal cells adjacent to tumor cells, as observed by Loewenstein and Kanno (13), could be due to these enzyme activities.

These enzymes are stocked mainly in the lysosomes. Therefore, we have assayed for hyaluronidase activity in these organelles, comparing mainly hepatoma cells with liver cells. Our experiments gave the striking result that no hyaluronidase activity could be detected in tumor lysosomes. This was true for 2 types of solid, chemically induced hepatomas and for an ascites hepatoma, as well as for the
Walker carcinoma 256 (a transplantable mammary carcinoma) and for virally transformed tissue culture BHK21 cells.

Taking into account the increased presence of this enzyme in the extracellular space, we propose the following hypothesis to explain its absence from the lysosomes. In normal cells, synthesis of hyaluronidase, which is a glycoprotein, is completed in the Golgi apparatus. From there it is distributed to the lysosomes, probably through the small vesicular bodies, as suggested by Novikoff (Ref. 15; see also Ref. 8), and to the extracellular space, probably in the same way as are other secreted glycoproteins. There may exist a controlled equilibrium between the synthesis of the enzyme and its distribution to these 2 different sites. In tumor cells, this equilibrium is apparently perturbed in such a way that no enzyme gets into the lysosomes and all the enzyme assembled in the Golgi apparatus is secreted outward. This phenomenon might also affect some other enzymes, such as collagenase. This increase in peripheral hydrolytic activity upon macromolecules would explain the structural degradation of extracellular components, facilitating the spreading of tumor cells.

However, we observed that the 2 other enzymes, $\beta$-$N$-acetylglucosaminidase and $\beta$-glucuronidase, which terminate the hydrolysis of hyaluronic acid, are still present in tumor lysosomes. These biochemical results can be related to the morphological observations by electron microscopy which show that in tumor cells the lysosomes are scarcer (17), are enlarged, and look emptier. It has already been shown, mainly by histochemical techniques, that different tumors contain very little or no activity of some lysosomal enzymes, such as acid phosphatase (5, 16), nonspecific esterase (5), acetylglucosaminidase and $\beta$-glucuronidase, which terminate the digestion of extracellular components. Therefore, it is clear that an equilibrium is apparently perturbed in such a way that no enzyme gets into the lysosomes and all the enzyme assembled in the Golgi apparatus is secreted outward. This phenomenon might also affect some other enzymes, such as collagenase. This increase in peripheral hydrolytic activity upon macromolecules would explain the structural degradation of extracellular components, facilitating the spreading of tumor cells.

The literature on lysosomal enzymes in tumors is full of apparently conflicting results. We feel that the results obtained are affected by the way the enzymes studied are isolated. Many workers call “lysosomal enzymes” those obtained by homogenization and extraction of the tissue, which mixes truly lysosomal enzymes with extracellular ones that have the same activity. The latter, however, are not lysosomal in origin and may vary independently in the case of tumors.

ACKNOWLEDGMENTS

We would like to express our appreciation to O. Pierre for performing the electron microscope examinations.

REFERENCES

Lysosomal Hyaluronidase Activity in Normal Rat Liver and in Chemically Induced Hepatomas

B. Fiszer-Szafarz and D. Szafarz