Comparison of Hepatic Cathepsin D Activity in Response to Tumor Growth and to Caloric Restriction in Mice

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ABSTRACT

In malignant disease, the caloric intake is often reduced. We have studied the role of this nutritional deprivation in increased lysosomal enzyme activities in liver and muscle tissue in tumor-bearing mice.

Three study groups were used: Group 1, sarcoma-bearing mice (MCG 101); Group 2, starved mice; Group 3, mice pair fed according to the ad libitum intake of the tumor-bearing animals.

Cathepsin D activity (V_{max}) was increased in the homogenates of liver tissue in all study groups. 

β-Glucuronidase activity was increased in liver homogenates from tumor-bearing mice and starved mice. In muscle tissue homogenates, only the tumor-bearing mice showed increased activities of these enzymes. Gel filtration of the crude cathepsin D fraction prepared from liver tissue showed only one main peak of enzyme activity in the study groups and in controls. This argues against the possibility that increased cathepsin D activity in liver tissue was due to migrating cells of lymphoid origin.

The characteristics of the purified cathepsin D activity from liver homogenates differed among the groups. In starved animals, the partially purified enzyme showed significantly higher V_{max} and K_{m} than in tumor-bearing mice and controls. The apparent increased activity of the enzyme in liver homogenates from tumor-bearing animals disappeared after purification.

It is concluded that the increased activities of lysosomal enzymes in liver and skeletal muscle tissue from tumor hosts are not due to poor nutrition only.

INTRODUCTION

Several studies have shown increased lysosomal enzyme activities in various tissues from tumor-bearing animals and cancer patients. Lysosomal enzymes have been considered important for degradation of proteins and thus for the normal regulation of protein turnover and also may be involved in the development of cancer cachexia. These suggestions are partly based on the finding that these enzyme activities are controlled by nutritional substrates and are activated in starvation and malnutrition.

Reduced caloric intake is a common feature in cancer disease. However, the possible connections between nutrition and metabolic tumor-host reactions are not clarified. Hence, it is not known whether the increased cathepsin D activity in liver tissue and in skeletal muscle tissue in cancer disease is secondary to poor nutrition of the host or due to a more specific effect of the tumor.

This study was designed to compare the effects of tumor growth, starvation, and reduced caloric intake on cathepsin D activity in liver tissue and skeletal muscle tissue in mice.

MATERIALS AND METHODS

Reagents

All reagents for tissue preparation were of analytical grade and from Sigma Chemical Co. Hemoglobin was Sigma bovine type II. 2-Naphthyl-β-D-glucuronide was from Koch-Light Laboratories Ltd. Sephadex, Sephacryl, and cyanoargentim bromide-activated Sepharose were from Pharmacia, Uppsala, Sweden.

Experimental Protocol

Male and female C57BL/6J mice were used. All experiments were performed in 3-month-old mice. They were randomly allocated to study groups and corresponding control groups at birth. All the mice were kept in metabolism cages, one per cage, during the study. Body weight was recorded daily in all groups.

Group 1. These animals were inoculated with a methylcholanthrene-induced sarcoma (MCG 101), which has been passed in vivo in our laboratory for more than 3 years. The tumor was implanted s.c. in the flank of the animals under aseptic conditions. This tumor does not metastasize or penetrate the abdominal cavity, and it leads to metastatic host reactions similar to those found in human cancer disease. The animals had free access to food and tap water (Purina maintenance diet, 13 KJ/g; Astra, Södertälje, Sweden). The animals were killed by cervical dislocation 12 to 13 days after tumor implantation, when the tumor comprised 15 to 20% of the preinoculation body weight. The controls were sham inoculated and then handled in the same way as the tumor animals.

Group 2. These animals were deprived of all food but had free access to tap water the last 72 hr before they were killed (Chart 1). The controls had free access to food and water. The starved group was considered representative for restricted caloric intake in some experiments in which the pair-fed group was not studied.

Group 3. These animals were supplied with food equivalent to the amount spontaneously eaten by the tumor mice. This means a gradual decrease in the energy intake from the fifth to sixth day after the start of the experiment. The intake was reduced to 50 to 60% of the intake of the controls at the 13th day (Chart 1). The food was supplied at 8 a.m., 3 p.m., and 10 p.m.

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2 To whom requests for reprints should be addressed.

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p.m. each day. These animals will be referred to as pair-fed animals. The controls had free access to food and water.

**Preparation of Animal Tissues and Enzyme Determinations**

Liver and skeletal muscle tissue was immediately excised and trimmed from fat and connective tissue at 4°. The tissue was cut into small pieces and rinsed of blood and debris. The skeletal muscle tissue was always taken by excision of the muscles between the hip and the knee joint of the hind leg. Skeletal muscle and liver tissue were homogenized (10%, w/v) as previously described (8). Homogenization of liver tissue was performed by hand in an all-glass homogenizer with a loosely and a tightly fitted pestle as described previously (9). Tissue homogenates or tissue fractions were always incubated in the presence of Triton X-100 (final concentration, 0.2%) at 4° for 60 min to solubilize the enzymes. The homogenates were centrifuged at 20,000 \( \times \) g for 15 min, and the enzyme activities were determined in the supernatant. This procedure solubilized approximately 95% of the total cathepsin D (EC 3.4.23.5) and \( \beta \)-glucuronidase (EC 3.2.1.31) activity in liver and muscle tissue from controls and the study groups. In some control assays of enzyme activities, Triton X-100 was removed from the enzyme fraction by Sephadex G-25 gel filtration. The activities of cathepsin D and \( \beta \)-glucuronidase were not influenced by the presence of Triton X-100. Triton X-100 was thus not used throughout the various steps of enzyme purification. Cathepsin D activity was determined following incubation in 0.2 M sodium acetate, pH 3.8, at 37°, using bovine hemoglobin as the substrate, as described by Anson (1). The enzyme activity was determined as tyrosine equivalent (nmol-min\(^{-1} \cdot \)mg protein\(^{-1} \)). \( \beta \)-Glucuronidase was determined as described by Vaughan et al. (23) and given as nmol-min\(^{-1} \cdot \)mg protein\(^{-1} \). Cathepsin D and \( \beta \)-glucuronidase were measured in the presence of substrates giving the maximum reaction velocity (V\( _{max} \)) if not otherwise stated. Enzyme activities were referred to the protein content of the enzyme fraction. Protein was determined according to the method of Lowry et al. (7), using bovine albumin as a standard. The possible interference of Triton X-100 on protein determination was not compensated for, but concentrations of Triton X-100 below 1% produce low blank values (24).

**Subcellular Fractionation of Liver Tissue**

Liver tissue was homogenized by hand in ice-cold 0.25 M sucrose with 0.001 M sodium EDTA (10%, w/v) as described above. The homogenate was filtered through 4 layers of nylon cloth and then centrifuged at 300 \( \times \) g for 10 min to eliminate unbroken cells and tissue fragments, which were discarded. The remaining tissue homogenate was then fractionated according to the description for human liver tissue (3).

**Gel Filtration and Enzyme Purification**

Liver tissue was homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl solution (30%, w/v). Triton X-100 was added to a final concentration of 0.2%, and the homogenate was incubated at 4° for 60 min. The homogenate was centrifuged at 20,000 \( \times \) g for 20 min, and the pellet was discarded. The supernatant was prepared by 2 centrifugations at 105,000 \( \times \) g for 2 hr for gel filtration of solubilized enzyme activities. The clear supernatant (5 ml) was filtered on a Sephadex G-100 superfine column (1.6 x 90 cm) and equilibrated in the homogenizing buffer, and fractions of 3 ml each were collected for enzyme determination according to routine procedures. \( \beta \)-Glucuronidase activity was fractionated on a Sephacryl 200 superfine column (1.6 x 90 cm) as described above. Effluent fractions were then analyzed for enzyme activities.

Cathepsin D was purified as described by Smith and Turk (21) after a slight modification to make the method more suitable for smaller amounts of tissue material. Fifteen g of the liver tissue were homogenized in 0.25 M sucrose with 0.001 M EDTA and Triton X-100 (final concentration, 0.2%). The homogenate was incubated at 4° for 60 min. A supernatant was prepared by centrifugation at 105,000 \( \times \) g for 2 hr. This was then dialyzed at 4° overnight against 0.25 M sodium acetate, pH 3.8, containing 1 M NaCl. From this step of purification on, Triton X-100 was not present. Denatured proteins were eliminated by centrifugation. Cathepsin D activity in the supernatant was bound to a hemoglobin-Sepharose matrix column (1.6 x 15 cm) prepared according to the manufacturer’s recommendation (Pharmacia). The enzyme was eluted with 0.1 M Tris-HCl, pH 8.6, containing 1 M NaCl. The enzyme fraction was further purified by gel filtration on a Sephadex G-100 superfine column (2.5 x 40 cm). After dialysis against 0.25 M sodium acetate, pH 3.8, containing 1 M NaCl, the enzyme reaction showed proportionality to time and amount of enzyme protein.

**Statistics**

The nonparametric Mann-Whitney U test was used to evaluate the statistical significance between independent samples. Spearman rank correlation coefficient was calculated to measure the relationship between paired variables, and the covariance is given by the regression lines (19).

**RESULTS**

Food intake decreased spontaneously in the tumor-bearing mice from the fifth day after tumor implantation (Chart 1A). The intake of the pair-fed animals was somewhat higher between the seventh and tenth days but was not significantly different from the intake of the tumor-bearing mice at the end of the experiment. The body weight decreased significantly only in the starved animals (Chart 1B). Cathepsin D activity (determined as V\( _{max} \)) was increased in homogenates of liver tissue from the animals in all study groups (Table 1). Tumor-bearing and starved mice also showed increased activity of \( \beta \)-glucuronidase in liver homogenates. In muscle tissue homogenates, only the tumor-bearing mice showed significantly increased activities of cathepsin D and \( \beta \)-glucuronidase. The total protein content per g, wet weight, was not determined in the present study but was found not to be significantly decreased in our previous work. The protein content in the enzyme fraction of the various animal groups is shown in Table 1. The significantly increased protein content in the hepatic and muscle enzyme fraction from pair-fed and starved animals was probably due to

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significantly decreased tissue water content. Tumor-bearing animals had 3 to 5% more water in liver and muscle tissue as compared to controls. Statistically significant correlations were found between cathepsin D and β-glucuronidase activities in liver and muscle tissue from tumor-bearing mice, starved mice, and controls (Table 2). Here, calculations were not done in the pair-fed group, because there were too few measurements made of the β-glucuronidase activity. These correlations were different in tumor-bearing and starved mice. These findings agree in essence with our previous results in cancer patients (8).

The recovery of cathepsin D and β-glucuronidase activities in the subfractions of liver homogenates from study groups and controls varied from 85 to 95% and 60 to 65%, respectively. The reason for the lower recovery of β-glucuronidase activity is not known. The specific activities of cathepsin D were higher in all subfractions of liver homogenates from tumor-bearing and starved mice (Chart 2). These increases were due both to an increase in enzyme activities and to a decrease in protein content in the nuclear, mitochondrial, and lysosomal fractions from tumor-bearing and from starved mice. The specific activity of β-glucuronidase did not show higher values in the mitochondrial fraction from the starved animals (Chart 2).

Gel filtration of liver homogenates from study groups and controls showed only one main peak of cathepsin D activity in the chromatograms (Chart 3). To analyze possible differences in hepatic cathepsin D activities between tumor-bearing mice and starved mice, the enzyme was partially purified as described in "Materials and Methods." The purified enzyme fraction from all groups hydrolyzed hemoglobin at a constant rate for at least 40 min, and the activity was proportional to the amount of incubated enzyme fraction. The pH optimum of the activity (pH 3.8) did not change after purification. The Kₙₐ₅ (μM) of cathepsin D purified from liver homogenates was 10.9 ± 0.6 (S.E.) for tumor-bearing mice, 16.0 ± 0.5 for starved mice, and 8.0 ± 0.6 for controls (Chart 4). These values were all statistically significantly different from each other (p < 0.05; p < 0.01). The apparent Vₘₐₓ (nmol-min⁻¹-mg protein⁻¹) differed significantly in starved mice only (p < 0.01) and was 220 ± 7 for tumor-bearing mice, 361 ± 11 for starved mice, and 217 ± 7 for controls. The specific activity of cathepsin D throughout the purification for the control experiment was (nmol-min⁻¹-mg protein⁻¹): crude tissue homogenate, 3.67; supernatant of the homogenate, 5.21; supernatant after dialysis, 3.89; after

<table>
<thead>
<tr>
<th>Enzyme activities were determined in the supernatant from detergent-treated homogenates centrifuged at 20,000 × g for 15 min.</th>
<th>Liver tissue</th>
<th>Skeletal muscle tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cathepsin D</td>
<td>β-Glucuronidase</td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-bearing mice (15)</td>
<td>6.24 ± 0.39</td>
<td>4.34 ± 0.30</td>
</tr>
<tr>
<td>Controls (15)</td>
<td>5.02 ± 0.40</td>
<td>3.53 ± 0.29</td>
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<tr>
<td>Group 2</td>
<td></td>
<td></td>
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<tr>
<td>Starved mice (12)</td>
<td>6.96 ± 0.47</td>
<td>4.47 ± 0.31</td>
</tr>
<tr>
<td>Controls (12)</td>
<td>4.86 ± 0.26</td>
<td>3.49 ± 0.12</td>
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<tr>
<td>Group 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed mice (9)</td>
<td>6.47 ± 0.44</td>
<td>3.42 ± 0.15 (6)</td>
</tr>
<tr>
<td>Controls (9)</td>
<td>5.03 ± 0.57</td>
<td>3.35 ± 0.23 (6)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of animals.
** Mean ± S.E.
*** p < 0.025 (Mann-Whitney U test).
**** p < 0.05 (Mann-Whitney U test).
Table 2
Covariance between cathepsin D and β-glucuronidase activity in liver and skeletal muscle tissue from tumor-bearing, starved, and control mice

| Enzyme activities were determined as $V_{\max}$ (nmol·min$^{-1}$·mg protein$^{-1}$) as described in “Materials and Methods.” The starved animals were deprived of food for 72 hr with ad libitum access to tap water. Controls include the control groups from both tumor-bearing and starved animals. |

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor-bearing mice (15a)</th>
<th>Starved mice (12)</th>
<th>Controls (27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman rank correlation coefficient</td>
<td>0.63 ($p &lt; 0.025$)</td>
<td>0.63 ($p &lt; 0.025$)</td>
<td>NS</td>
</tr>
<tr>
<td>Regression coefficient</td>
<td>0.58</td>
<td>0.61</td>
<td>NS</td>
</tr>
<tr>
<td>Regression line</td>
<td>$Y = 6.573 - 0.3874X$</td>
<td>$Y = 2.154 + 0.344X$</td>
<td></td>
</tr>
<tr>
<td><em>NS</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor-bearing mice</th>
<th>Starved mice</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman rank correlation coefficient</td>
<td>0.89 ($p &lt; 0.0005$)</td>
<td>0.60 ($p &lt; 0.01$)</td>
<td>0.60 ($p &lt; 0.01$)</td>
</tr>
<tr>
<td>Regression coefficient</td>
<td>0.83</td>
<td>0.50</td>
<td>NS</td>
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<tr>
<td>Regression line</td>
<td>$Y = 0.1117 - 0.038X$</td>
<td>$Y = 0.0229 + 0.0809X$</td>
<td></td>
</tr>
<tr>
<td><em>NS</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses, number of animals.

NS, not significant.

The striking similarity among the changes in cathepsin D activity in liver homogenates from the mice in all study groups suggests a common trigger mechanism. However, purification of the enzyme fraction by affinity chromatography and gel filtration revealed different characteristics of the enzyme(s). In the starved animals, both $K_m$ and $V_{\max}$ were considerably different from those of controls and tumor animals, suggesting other mechanisms behind this induction of enzyme activity. The apparent difference between the enzyme capacity in homogenates from the tumor hosts and controls was extinguished after purification of the enzyme. The reason for this is not known. A possible explanation is that the increased hepatic cathepsin D found in homogenates from tumor-influenced liver tissues...
Chart 3. Distribution of hepatic cathepsin D activity in relation to molecular size of hepatic proteins. Liver tissue from tumor-bearing mice (X), starved mice (O), and controls (•) was homogenized in 50 mM Tris-HCl, pH 7.4, containing 0.15 mM NaCl plus Triton X-100 (final concentration, 0.2%). The enzyme fraction, prepared as described in "Materials and Methods," was filtered on a Sephadex G-100 superfine column (1.6 x 90 cm). Fractions of 3 ml each were collected for determination of enzyme activities as described in "Materials and Methods." Protein content in the various fractions was registered by light absorbance at 280 nm (---). The distribution of albumin in the chromatogram was determined by means of immunoelectrophoresis.

Chart 4. Activity of purified cathepsin D from liver tissue. The purification procedure and enzyme assay were as described in "Materials and Methods." Lineweaver-Burk plot is shown (inset). X, tumor-bearing mice ($Y = 0.58X + 5.274$; $r = 0.99$); O, starved mice ($Y = 0.44X + 2.773$; $r = 1.00$); •, controls ($Y = 0.037X + 4.611$; $r = 0.99$).
was to some extent based on significantly decreased protein content in the enzyme fraction. The differences between tumor animals and controls would then be decreased when the enzyme is purified. Such an explanation implies that the absolute controls was dependent on a relative lack of this inhibitor. The possibility that the increased cathepsin D activity in liver and muscle homogenates from the tumor host as compared with that of controls was dependent on a relative lack of this inhibitor. During purification of the enzyme, the inhibitor is removed, as indicated by the results of Smith and Turk (21). The possibility that the increased lysosomal enzyme activity in liver and muscle homogenates of tumor hosts originated from migrating cells must also be considered. The fact that the activity of cathepsin D appeared in one main peak in the gel filtration of homogenates argues against this possibility. It has been shown that cathepsin D activity of lymphoid origin is composed of 2 molecular fractions corresponding to 95,000 and 45,000 daltons (4). Moreover, the DNA content in the tumor-influenced livers was not different from that of control livers, and, further, the histological appearance of tumor-influenced livers seemed to be normal (10).

In summary, our results indicate that the increase in lysosomal enzyme activities in liver and muscle tissue in response to tumor growth cannot be ascribed to poor nutrition only.

REFERENCES


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