Host Cathepsin D Response to Tumor in the Normal and Pepstatin-treated Mouse

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ABSTRACT

In view of the postulated role of cathepsin D in cachexia, investigations have been pursued on the host tissue response of cathepsin D activity in DBA/2 mice inoculated with $5 \times 10^6$ L1210 tumor cells. The results confirmed previous investigators' findings of the increase in cathepsin D activity (specific activity) in liver and muscle of tumor bearers. In addition, it was found that this increase was a general response of the host since heart, kidney, lung, and spleen cathepsin D specific activity were also enhanced in tumor bearers. These increases ranged from an average of 10% for spleen to 100% for gastrocnemius muscle. This effect was age related in heart and kidney. As a working hypothesis, we propose the concept that tumor bearers release protease-enhancing factor(s) which trigger increase or enhancement of cathepsin D activity in host tissues by yet unknown mechanisms.

Pepstatin (60 mg/kg), a known inhibitor of cathepsin D in vitro, was shown to provide long-lasting inhibition (3 to 6 days) of cathepsin D in vivo in non-tumor bearers particularly in spleen, liver, kidney, lung, and heart. Evidence is provided from assays of cell fractions that this inhibition takes place at or in the lysosome. The duration of the effectiveness of pepstatin was altered in tumor bearers that cathepsin D activity of heart, lung, and spleen had returned to near normal values in 48 hr following pepstatin injection. However, in muscle, liver, and kidney, significant inhibition (90%) still persisted in tumor bearers as it did in non-tumor bearers.

Pepstatin or related antiproteases may prove useful as "anti-cachexia" agents by decreasing proteolysis in muscle and other tissues.
with water to bring the enzyme activity within the linear range of the assay.

**Cathepsin D Activity.** The assay was described previously by Roffman and Greenbaum (11). Extract (20 μl) was incubated with 20 μl of tritiated hemoglobin (approximately 300,000 cpm) and 20 μl of 0.1 M acetate buffer at pH 4.0 for 30 min at 37°. At 30 min, 150 μl of cold hemoglobin (3%) were added followed by 100 μl of cold 10% TCA. Following centrifugation, 100 μl of the supernatant was added to 3.0 ml of scintillation cocktail (Ready-Solv HP; Beckman Corp.). Zero time substrate (without enzyme) blanks were prepared at the same time, and all samples were counted in a scintillation counter. One unit of cathepsin D activity was defined as that amount of enzyme that liberates into the TCA-soluble supernatant 1% of the radioactivity of the substrate under zero order kinetics and the conditions of the assay. Activity of the enzyme solution was expressed as units of enzyme per ml. This was calculated as follows:

\[
\text{cpm of sample} - \text{cpm zero time blank} \times 100 \times \text{dilution factors.}
\]

Specific activity was defined as units of enzyme per mg of protein. For each new tritiated hemoglobin substrate prepared, a new standard curve to determine the zero order kinetics was run. It should be noted that on "aging," the tritiated substrate TCA blank increases, resulting in reduced hydrolysis of the substrate by cathepsin D. Consequently, when blank values reach counts of 750, the substrate is dialyzed overnight against buffer. Care was also taken when comparisons between groups were to be made to assay the cathepsin D of the tissue extracts of all groups with the same substrate.

Protein. This was estimated by an automated procedure of Lowry et al. (8) and expressed as mg of protein per ml of extract.

**RESULTS**

**Tumor-induced Increases in Host-Tissue Cathepsin D.** Studies were carried out to observe the influence of the presence of the L1210 tumor on cathepsin D activity levels in a variety of tissues in DBA/2 mice. Preliminary studies (15) had suggested that, as tumor growth had progressed, host-tissue cathepsin D activity levels had also increased. The age of the host mice appeared to also have an influence on the tissue response.

Three groups of 3 mice were inoculated i.p. with \(5 \times 10^5\) L1210 tumor cells. The ages of the groups at inoculation were 30, 45, and 75 days, respectively. On the ninth day following tumor inoculation, each of these groups was sacrificed by cervical dislocation along with 3 age-matched non-tumor-bearers. Brain, heart, lung, liver, spleen, kidney, and muscle were removed, homogenized, and assayed for cathepsin D activity.

Table 1 demonstrates that the specific activity of cathepsin D of all tissues in the tumor-bearing mice were increased over non-tumor bearers with the exception of the brain. A 2-way analysis of variance (18) showed that an increase in response to tumor with age of the animal for heart and kidney is significant at the \(p < 0.05\) level.

**Effect of Pepstatin on Cathepsin D Levels of Non-Tumor Bearers.** Studies on the effect of pepstatin on non-tumor bearers were carried out to determine (a) the duration of cathepsin D inhibition by pepstatin and (b) the location of the inhibition in the cell.

Twenty-four age-matched (70-day-old) non-tumor-bearing mice were given injections daily for 4 days of pepstatin (60 mg/kg) in 10% glycerol-formal. Groups of 6 animals were sacrificed on Days 1, 2, 3, and 6 following the fourth injection of pepstatin. A group of 3 animals served as controls. These were given injections of appropriate volumes of 10% glycerol-formal daily for 4 days and sacrificed 6 days after the fourth glycerol-formal injection.

In Chart 1, it may be clearly noted that pepstatin dramatically inhibits cathepsin D activity *in vivo* in spleen, liver, kidney, and lungs over a prolonged period of time. In the case of spleen, liver, and kidney cathepsin D, significant inhibition occurred over the entire 6 days. Lung cathepsin D remained significantly inhibited for the first 3 days but returned to near normal levels by the sixth day. Inhibition of brain cathepsin D was not statistically significant. Heart cathepsin D was markedly inhibited on the first day following injection; the cathepsin D activity returned to normal by the sixth day.

Studies were carried out to determine the location within the liver of the inhibition of cathepsin D by pepstatin. Twelve age-matched (70-day-old) DBA/2 male mice were given injections of pepstatin (60 mg/kg) in 10% glycerol-formal. Three mice of the same age were given injections of 10% glycerol-formal to serve as controls. Groups of 3 mice were sacrificed on Days 1, 2, 3, and 6 following the injection. The livers were removed, and a portion of each liver was homogenized as described previously for assay of the "total" cathepsin D activity while the rest of the liver was treated by the method to isolate lysosomes using 0.25 M sucrose (see "Materials and Methods"). All fractions (the cell debris, the cytosol fraction, the lysosomal pellet, and the lysosomal and mitochondrial membrane pellet) were assayed for cathepsin D activity.

**Table 1**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age (days)</th>
<th>Non-tumor bearer (units/mg protein)</th>
<th>Tumor bearer (units/mg protein)</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>30</td>
<td>190 ± 15^a</td>
<td>400 ± 10</td>
<td>+107%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>230 ± 20</td>
<td>440 ± 30</td>
<td>+65%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>230 ± 15</td>
<td>430 ± 15</td>
<td>+65%</td>
</tr>
<tr>
<td>Liver</td>
<td>30</td>
<td>1035 ± 35</td>
<td>1200 ± 60</td>
<td>+24%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>1020 ± 20</td>
<td>1580 ± 65</td>
<td>+54%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>895 ± 40</td>
<td>1410 ± 120</td>
<td>+58%</td>
</tr>
<tr>
<td>Kidney</td>
<td>30</td>
<td>8970 ± 10</td>
<td>1100 ± 60</td>
<td>+27%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>940 ± 15</td>
<td>1300 ± 40</td>
<td>+38%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>820 ± 30</td>
<td>1270 ± 30</td>
<td>+55%</td>
</tr>
<tr>
<td>Lung</td>
<td>30</td>
<td>862 ± 12</td>
<td>1050 ± 50</td>
<td>+20%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>720 ± 7</td>
<td>1020 ± 30</td>
<td>+42%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>715 ± 25</td>
<td>1250 ± 190</td>
<td>+75%</td>
</tr>
<tr>
<td>Heart</td>
<td>30</td>
<td>769 ± 24</td>
<td>830 ± 46</td>
<td>+8%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>650 ± 17</td>
<td>900 ± 60</td>
<td>+38%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>810 ± 60</td>
<td>1190 ± 75</td>
<td>+47%</td>
</tr>
<tr>
<td>Spleen</td>
<td>30</td>
<td>3790 ± 30</td>
<td>2960 ± 70</td>
<td>-20%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>3560 ± 190</td>
<td>4000 ± 220</td>
<td>+12%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>3230 ± 200</td>
<td>3420 ± 100</td>
<td>+40%</td>
</tr>
<tr>
<td>Brain</td>
<td>30</td>
<td>595 ± 27</td>
<td>588 ± 11</td>
<td>-1%</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>645 ± 14</td>
<td>738 ± 21</td>
<td>+14%</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>639 ± 22</td>
<td>643 ± 27</td>
<td>+1%</td>
</tr>
</tbody>
</table>

* Gastrocnemius.

\(\text{a} \times \text{Mean} \pm \text{S.E.}\)

\(\text{p} < 0.001.\)

\(\text{p} < 0.02.\)

\(\text{* Tissues which show age-related increases in host cathepsin D specific activity at } p < 0.05.\)
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Table 2 demonstrates a significant reduction in cathepsin D activity in all fractions of the tissues from animals receiving pepstatin. It also shows that at least 50% of the activity recovered in the fractionation procedure is in the pellet that contains the isolated lysosomes. It also demonstrates that the degree of inhibition of cathepsin D by pepstatin is approximately the same for all fractions within the experimental error of the methods used. The sum of the cathepsin D activity of the several fractions ranged from 93 to 140% of the activity measured in the sample of the liver assayed for "total" cathepsin D.

Effect of Pepstatin on Cathepsin D Levels of Tumor Bearers. In view of the ability of parenterally administered pepstatin to inhibit cathepsin D in vivo in a variety of tissues, experiments were carried out to determine if pepstatin treatment could alter (reduce) the usual increases in host tissue cathepsin D seen in tumor bearers. Three age-matched (75-day-old) DBA/2 mice were inoculated with $5 \times 10^5$ tumor cells. Four days following the inoculation, pepstatin was administered i.p. daily for 4 days at a dose of 60 mg/kg/day. The mice were sacrificed 48 hr later. The tissues were assayed for cathepsin D activity and compared to controls which had been treated with the vehicle alone. The results, as seen in Table 3, demonstrate that in the tumor bearers, pepstatin can inhibit cathepsin D in liver, kidney, and muscle and thus prevent the usual increase in activity seen in these host tissues following tumor inoculation. Lung, spleen, and heart recovered a good part of their cathepsin D activity in 48 hr, unlike the non-tumor bearers.

**DISCUSSION**

Increases in the number of lysosomes and cathepsin D have been noted in the liver of human and animal tumor bearers. Such observations have been made in rats bearing transplanted tu-

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**Table 2**

Comparison of cathepsin D activity in livers from pepstatin-injected and vehicle-injected male DBA/2 mice. One portion of each liver was homogenized and assayed for "total" activity. A second portion was fractionated as seen below. Pepstatin was injected in 4 daily doses of 60 mg/kg, and the groups were sacrificed 1, 2, 3, and 6 days following the last injection. For all groups, $n = 3$.

<table>
<thead>
<tr>
<th>Cell debris pellet</th>
<th>Cytosol supernatant</th>
<th>Lysosomal pellet</th>
<th>Membrane pellet</th>
<th>Total nonfractionated</th>
</tr>
</thead>
<tbody>
<tr>
<td>(600 x g)</td>
<td>(25,000 x g)</td>
<td>(25,000 x g)</td>
<td>(50,000 x g)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control (Mean ± S.E.)</th>
<th>1 day (Mean ± S.E.)</th>
<th>2 day (Mean ± S.E.)</th>
<th>3 day (Mean ± S.E.)</th>
<th>6 day (Mean ± S.E.)</th>
<th>4 days (Mean ± S.E.)</th>
<th>7 days (Mean ± S.E.)</th>
<th>10 days (Mean ± S.E.)</th>
<th>14 days (Mean ± S.E.)</th>
<th>21 days (Mean ± S.E.)</th>
<th>28 days (Mean ± S.E.)</th>
</tr>
</thead>
</table>

*Mean ± S.E.*

**Table 3**

Effectiveness of pepstatin inhibition on cathepsin D in tumor bearers

Two groups of 3 male DBA/2 mice were inoculated i.p. with $5 \times 10^5$ L1210 tumor cells. To one group (treated), pepstatin in 10% glycerol-formal was administered in daily i.p. injections on the fourth, fifth, sixth, and seventh days following tumor inoculation. The second group (controls) were injected i.p. with 10% glycerol-formal alone on the same schedule. All animals were sacrificed 48 hr after the last pepstatin or vehicle injection. Values in units/mg protein.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Treated</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>1250 ± 190*</td>
<td>930 ± 75</td>
<td>26</td>
</tr>
<tr>
<td>Spleen</td>
<td>3420 ± 100</td>
<td>3210 ± 130</td>
<td>6</td>
</tr>
<tr>
<td>Heart</td>
<td>1190 ± 75</td>
<td>1045 ± 75</td>
<td>12</td>
</tr>
<tr>
<td>Liver</td>
<td>1410 ± 120</td>
<td>143 ± 12</td>
<td>90*</td>
</tr>
<tr>
<td>Kidney</td>
<td>1270 ± 30</td>
<td>81 ± 10</td>
<td>94*</td>
</tr>
<tr>
<td>Muscle</td>
<td>430 ± 15</td>
<td>43 ± 4</td>
<td>90*</td>
</tr>
</tbody>
</table>

*% of inhibition = Control-treated / Control x 100

# Mean ± S.E.

*p < 0.001.
mers such as the Walker tumor, Guerin tumor, squamous cell carcinoma, hepatomas, and various sarcomas (4, 14, 16). In mice, Butterworth (1) found an increase in the number of hepato-
cellular lysosomes adjacent to hepatocellular sarcomas in-
culated into the liver. This also occurred in the ascites form of the
tumor. Ghadially et al. (6) have also reported that sarcoma and
Ehrlich tumors in either solid or ascites form will cause a signifi-
cant increase in mouse liver lysosomal enzymes. Similarly, in-
creases in the number of lysosomes have been found in the liver
of patients with Hodgkin’s disease (3). Increase in muscle cathe-
psin D specific activity is also well known to occur in tumor-
bearing animals and humans (9, 10) and has led to the hypothesis
that cathepsin D may be the cause of enhanced proteolysis, one
of the metabolic derangements of cachexia.

In the current study, the increase in liver and muscle cathepsin
D specific activity following L1210 tumor cell inoculation in mice
confirms the above observations. In addition, our study clearly
demonstrates that other host tissues including heart, kidney,
lung, and spleen also respond to the tumor by increases in
specific activity of cathepsin D to about the same extent as that
which occurs in liver. Compared to other tissues, increases in
muscle cathepsin D in tumor bearers would appear to be the
most dramatic. In 2 tissues, heart and kidney, this increase was
age related. Since increases in cathepsin D occurred in many of
the organs studied, the possibility exists that the enhanced
proteolysis could result in injury to these organs in tumor bearers.
Furthermore, the enhanced proteolysis may enlarge the free
amino acid pool resulting in added nutrition for the tumor cells
(7). In view of these possibilities, we investigated the potential
of pepstatin for its ability to inhibit cathepsin D in vivo in tumor-
bearing animals. Studies on non-tumor bearers were conducted
to contrast the effects of the tumor on this inhibition.

Our results as indicated in Chart 1 demonstrate that pepstatin
treatment of non-tumor bearers inhibits cathepsin D of all tissues
assayed except the brain for prolonged periods of up to or more
than 6 days (the last period of assay). Several lines of evidence
indicated that pepstatin was bound to cathepsin D in situ in or
at the lysosomes and did not combine with cathepsin D during
homogenization. First, the activities of the “cell debris” and
“cytosol” fractions show the same percentage of the total
activity for the pepstatin group as these fractions showed for the
noninjected group (Table 2). If free pepstatin was present, it
would be expected that there would be very little, if any, cathe-
psin D activity in these 2 fractions since this activity would be
most readily available for inhibition. Secondly, the “lysosomal”
fraction has been washed by 2 changes of sucrose solution.
However, the degree of inhibition is approximately the same as
that of the “total” cathepsin D from the same tissue. If free
pepstatin was present, it would have been markedly diluted by
the wash fluid, and the degree of inhibition would have been
much less than in the “total” sample. Thus, our conclusion is
that pepstatin penetrates into the lysosomal component of the
cell.

When pepstatin was administered to tumor bearers, it was
found (Table 3) that in tissues such as spleen, heart, and lung
the inhibition of cathepsin D was considerably shortened (to less
than 48 hr). Since pepstatin-treated non-tumor bearers showed
a much longer inhibition of cathepsin D in spleen and lung, it is
reasonable to assume that the shortened effect in tumor bearers
is due to increased cathepsin D synthesis. These results, in
conjunction with other evidence cited above, lead us to propose
the concept that in tumor bearers PEF is released which causes
an increased cathepsin D activity of tissues. Whether this en-
hancement occurs by induction of new enzyme or removal of an
inhibitor must await further experimentation. Whether PEF is
blood borne or arises in the host cell itself must also await further
experimentation. The elucidation of the nature of PEF and its
properties would be of significant importance in finding agents
which could prevent increased proteolysis in tumor bearers and
thus potentially ameliorate cachexia and organ injury.

The ability of pepstatin to inhibit cathepsin D in tumor bearers
in situ now allows for testing of the hypothesis that cathepsin D
is a prime enzyme in cachexia and related injury to the host.
Such studies are now being pursued.

ACKNOWLEDGMENTS

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