Inhibition of Experimental Blood-borne Lung Metastasis by Protease Inhibitors

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ABSTRACT

The inhibitory effects of protease inhibitors on blood-borne metastasis in male Donryu rat lung were studied. Injection i.v. of $10^6$ Yoshida ascites hepatoma AH7974 cells induced about 118 ± 92 (S.D.) metastatic foci in rat lung after 3 weeks. Leupeptin (50 mg/kg body weight twice a day), injected i.p. from 2 days before to 4 days after the inoculation of tumor cells, reduced the number of metastatic foci to about 49 ± 45 ($p < 0.005$). Leupeptin also suppressed the formation of metastatic foci of Yoshida ascites hepatoma AH100B cells ($p < 0.001$). Elastatinal (100 mg/kg body weight twice a day) and chymostatin (100 mg/kg body weight once a day) did not inhibit formation of metastatic foci of AH7974 cells. Injection i.v. of $10^6$ AH7974 cells induced pulmonary thrombi within 1 hr. Leupeptin (50 mg/kg body weight twice a day) reduced the number of thrombi from 1298 ± 395 to 646 ± 218, when injected i.p. for 2 days before the inoculation of the cells ($p < 0.005$). Chymostatin and elastatinal did not significantly change the number of pulmonary thrombi. These results indicate that leupeptin inhibited metastasis formation and suggest that this effect may be due to the inhibition of thrombus formation after the arrest of circulating tumor cells.

INTRODUCTION

Several protease inhibitors of low molecular weight have been isolated from Streptomyces (1). They all have peptide-like structures and specific patterns of protease inhibition. For example, leupeptin inhibits thrombokinase, plasmin, kallikrein, cathepsin B, trypsin, and papain; chymostatin inhibits chymotrypsin and cathepsins A, B, and D; and elastatinal inhibits elastase. Leupeptin was reported to inhibit mouse skin tumorigenesis induced by treatment with 7,12-dimethylbenz(a)-anthracene and croton oil (8). Moreover, leupeptin partially suppressed tumor formation in the colon, esophagus, and mammary gland of rats (12). Furthermore, recently, Little et al. (9) reported that leupeptin and antipain suppressed radiation-induced malignant transformation in vitro.

The development of hematogenous tumor metastasis involves several steps: (a) invasion of cells from the primary tumor into the surrounding tissue with penetration of the blood vessels; (b) detachment or release of single or multiple tumor cell emboli into the circulation; (c) arrest of circulating emboli in small vascular beds of organs; (d) invasion of tumor cell emboli into the wall of the arresting vessel, their infiltration into adjacent tissue, and their multiplication; and (e) growth of a vascularized host tumor, as described by Fidler (5). Fidler (4) reported that most circulating tumor cells fail to survive to give rise to metastases. Therefore, it is likely that lodgment or growth of tumorigenic cells at new sites plays a key role in metastasis formation. We studied the inhibitory effect of protease inhibitors on lung metastasis in the rat induced by i.v. inoculation of tumor cells, and we suggest that the effect is due to the inhibition of thrombokinase activity, which is related to thrombus formation, by leupeptin.

MATERIALS AND METHODS

Animals. Male Donryu rats were obtained from the Nippon Rat Co. Ltd. They were 6 weeks old and weighed about 120 g at the beginning of the experiment. They had free access to CE-2 diet (CLEA Japan Inc., Tokyo, Japan) and water.

Tumor Cell Lines. Yoshida ascites hepatoma AH7974 and AH100B cells were supplied by Dr. H. Sato, Sasaki Institute, Tokyo, Japan. They were maintained by serial i.p. implantation in syngeneic Donryu rats in our laboratory.

Protease Inhibitors. Leupeptin, chymostatin, and elastatinal were obtained through the Research Resources Program for Cancer Research, the Ministry of Education, Science and Culture. Leupeptin was dissolved in PBS and was injected i.p. at a dose of 50 mg/kg body weight twice a day. Chymostatin was dissolved in 0.5% CMC solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and was injected i.p. at a dose of 100 mg/kg body weight once a day. Elastatinal was dissolved in 0.9% NaCl solution and was injected twice a day at a dose of 100 mg/kg body weight. Protease inhibitors were injected from 2 days before to 4 days after inoculation of tumor cells. Control rats were treated in the same way with the solvents only for 6 days.

Assay of Lung Foci. Rats were given injections of $10^6$ AH7974 cells or $10^5$ AH100B cells in 1 ml of 0.9% NaCl solution via the tail vein; 3 weeks (AH7974) or 2 weeks (AH100B) later, the animals were killed and their lungs were removed. The number of metastatic foci on the lung surface was counted by infiltration of the lungs with India ink (17). Before inoculation, the viability of the tumor cells was examined microscopically by the 0.4% erythrosin exclusion method.

Assay of Pulmonary Thrombi. Seven groups of rats were treated as follows. All rats in Groups 1 to 6 were given injections of $10^6$ AH7974 cells in the tail vein. Rats in Groups 1, 3, and 5 were treated with i.p. injections of PBS, 0.5% CMC solution, and 0.9% NaCl solution, respectively, for 2 days before inoculation of AH7974 cells. Rats in Groups 2, 4, and 6 were given...
D. Saito et al.

i.p. injections of leupeptin (50 mg/kg body weight twice a day), chymostatin (100 mg/kg body weight once a day), and elastatinal (100 mg/kg body weight twice a day), respectively, for chymostatin (100 mg/kg body weight once a day), and elastatinal dissolved in 0.9% NaCl solution and injected i.p. at a dose of 100 mg/kg body weight twice a day. Chymostatin was dissolved in 0.5% CMC solution and injected i.p. and injected i.p. of PBS for 2 days and then i.v. injections of 1 ml of 0.9% NaCl solution without tumor cells.

One hr after injection of tumor cells or 0.9% NaCl solution, the lungs of each rat were examined histologically. For this, the lungs were fixed with Carnoy’s solution for 30 min, sectioned at the largest cross-dimension passing through the pulmonary hilus of the left lung, and stained with hematoxylin and eosin. For identification of fibrin, the sections were stained with the Mallory method. Localized thrombi were stained red to show fibrin deposition. Then, blind counts were made by light microscopy of the number of pulmonary thrombi with and without embolic tumor cells.

RESULTS

Table 1 shows the effect of protease inhibitors on blood-borne lung metastasis of AH7974 cells. The average number of lung metastatic foci per rat was 118 ± 92 (S.D.) (25 rats) in controls and 49 ± 45 (23 rats) in leupeptin-treated rats, as shown in Table 1. The difference in the numbers of foci in these 2 groups was statistically significant by Student’s t test ($p < 0.005$). Chymostatin and elastatinal did not inhibit metastatic foci formation of AH7974 cells.

Table 2 shows that leupeptin also suppressed formation of metastatic foci by AH100B cells. The average numbers of foci per rat was 192 ± 88 (24 rats) in controls and 120 ± 56 (27 rats) in leupeptin-treated rats ($p < 0.001$).

Histological and quantitative studies were made on thrombi in the lungs of rats in the 7 groups described in “Materials and Methods.” One hr after inoculation of AH7974 cells, thrombi were observed in the capillaries of the lung (Fig. 1). Some of these thrombi were embolized tumor cells. As shown in Table 3, the average numbers of pulmonary thrombi per 100 sq mm of lung were 22 ± 22 in Group 7, 1298 ± 395 in Group 1, and 646 ± 218 in Group 2, and the difference between the numbers in Groups 1 and 2 was significant ($p < 0.005$). Thus, leupeptin reduced formation of pulmonary thrombi by AH7974 cells. Chymostatin and elastatinal did not reduce the number of pulmonary thrombi. The number of thrombi with CMC solution or 0.9% NaCl solution alone was the same as that with PBS in Group 7.

DISCUSSION

In most patients with clinical cancer, metastases have already occurred by the time the diagnosis is made (6, 15). Therefore, it goes without saying that the prevention of metastasis is essential for complete treatment of cancer. The tumor cell arrest in the capillaries of the distant organ is an important step of formation of metastasis.

Leupeptin inhibited experimental blood-borne metastasis of AH7974 and AH100B cells. The finding that leupeptin does not have a specific effect on only one tumor cell line indicates that proteases that can be inhibited by leupeptin are probably involved in blood-borne metastasis. The leupeptin used in this study (100 mg/kg/day) did not affect the growth of rats, although leupeptin (200 mg/kg/day i.p.) caused a decrease in the body weight of the rats. The blood level of leupeptin was 110 µg/ml 30 min after i.p. injection of leupeptin (100 mg/kg body weight), and then it decreased.4 The growth of AH7974 cells in culture was not inhibited by leupeptin (500 µg/ml). Therefore, decrease of metastatic foci is not due to the cytotoxic effect of leupeptin.

Leupeptin reduced the number of pulmonary thrombi. This effect may be due to inhibition of thrombus formation after the arrest of circulating tumor cells, since thrombus formation is a prerequisite for the formation of metastasis (3). The reason control rats given injections of 0.9% NaCl solution without tumor cell (Group 7) developed a few thrombi may be that i.v. injection of a large volume of 0.9% NaCl solution may cause thrombosis in pulmonary capillaries.

It is reported that patients with advanced cancer often show...
Inhibition of Metastasis by Protease Inhibitors

Fig. 1. Formation of experimental pulmonary thrombi. A rat was sacrificed 1 hr after i.v. injection of Yoshida ascites hepatoma AH7974 cells, and the lungs were fixed and serially sectioned. Short arrows, tumor cells; long arrow, a thrombus. H & E, x 400.

Table 3

<table>
<thead>
<tr>
<th>Group No./rat</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (9)</td>
<td>748, 1010, 1060, 1120, 1140, 1340, 1500, 1790, 1980</td>
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<tr>
<td>2 (9)</td>
<td>400, 449, 465, 586, 586, 610, 757, 943, 1020</td>
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<tr>
<td>3 (8)</td>
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<tr>
<td>4 (8)</td>
<td>698, 750, 849, 1151, 1198, 1292, 1294, 1345</td>
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<tr>
<td>5 (8)</td>
<td>750, 900, 1148, 1150, 1246, 1347, 1446, 1597</td>
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<tr>
<td>6 (9)</td>
<td>852, 1051, 1098, 1146, 1150, 1246, 1267, 1297, 1596</td>
</tr>
<tr>
<td>7 (10)</td>
<td>0, 3, 4, 8, 18, 23, 23, 31, 36, 72</td>
</tr>
</tbody>
</table>

Numbers in parentheses, number of animals in each group. *p < 0.005.

Inhibition by protease inhibitors of pulmonary thrombi formation after i.v. inoculation of 10⁶ AH7974 cells

In Groups 1, 3, and 5, rats were given i.p. injections of PBS, CMC solution, and 0.9% NaCl solution, respectively, for 2 days, and then 10⁶ AH7974 cells were inoculated i.v. In Groups 2, 4, and 6, rats were given i.p. injections of leupeptin (50 mg/kg body weight twice a day), chymostatin (100 mg/kg body weight once a day), and elastatinal (100 mg/kg body weight twice a day), respectively, for 2 days and then inoculated i.v. with tumor cells. In Group 7, rats were treated with PBS and were given i.v. injections of 1 ml of 0.9% NaCl solution. One hr after inoculation of tumor cells, the rats were killed for histological and quantitative observation of pulmonary thrombi.

Pulmonary thrombi

not inhibit metastasis. It is reported that leupeptin but not chymostatin or elastatin inhibited thromboplastin activity (1). Therefore, it is likely that thromboplastin activity is involved in the formation of metastatic foci. However, thromboplastin is present in both the host tissue and tumor cells (10, 11), and it is still unknown whether leupeptin acts on host thromboplastin, tumor cell thromboplastin, or both. We are now examining this problem using tumor cells treated with leupeptin in vitro.

Leupeptin is used clinically for treatment of pancreatitis and burns (18). It has been found to inhibit tumorigenesis (8, 12) and transformation (9) as well as blood-borne metastasis (18). Therefore, clinical use of leupeptin for treatment of neoplastic disease will be expected.

ACKNOWLEDGMENT

The authors wish to thank Dr. I. Hirono, Institute of Medical Science, University of Tokyo, Tokyo, Japan, for valuable suggestions.

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D. Saito et al.


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