Antitumor Activity of Some Bacterial Proteases: Eradication of Solid Tumors in Mice by Intratumor Injection

Hiroshi Maeda, Yasuhiro Matsumura, and Akhteruzzaman Molla
Department of Microbiology, Kumamoto University Medical School, Kumamoto 860, Japan

ABSTRACT
A protease isolated from the culture filtrate of a Gram-negative bacterium, Serratia marcescens kums 3958, showed very potent antitumor activity when injected into Meth-A or RL51 tumors in BALB/c mice at 30 µg per tumor or more. This and certain other proteases, which are resistant to many protease inhibitors in plasma, appear to be new candidate drugs for regional treatment of solid tumors.

INTRODUCTION
Cancerous tissue is unique in that it has (a) hypervasculature because of active angiogenesis, (b) enhanced permeability, and (c) a poorly developed lymphatic system (1-6). These characteristics are clearly in contrast to normal tissue, but they have not been fully exploited to utilize cancer chemotherapy. These basic properties, however, comprise diagnostic bases in angiography (7) and tumor scintigraphy (8), and the recent development of nuclear magnetic resonance computerized tomography may also be traced to them (9).

In contrast to low-molecular-weight substances that diffuse easily and traverse blood capillaries more freely, thus reaching equilibrium among various tissues and organs, macromolecular and lipid-solubilized drugs leak out more in cancer tissues than in normal tissues (5,6). They thus accumulate more and remain for a prolonged period in tumor tissues because of the physiological, anatomical, and pathological differences noted above.

For the above reasons we have envisaged the possible use of proteolytic enzymes to solubilize or necrotize tumor tissues. In this paper we describe the potent antitumor effect of serratial M, 56,000 protease when injected into solid tumors in mice.

MATERIALS AND METHODS
Proteases. Serratial M, 56,000 protease (hereafter referred to as serratia protease) was purified from the culture filtrate of Serratia marcescens kums 3958 as described previously (10). This enzyme has an approximate molecular weight of 56,000 and possesses one zinc atom per mol. Its substrate specificities are highest for Z-Phe-Arg-MCA (methylcoumarine amide), followed by Pro-Phe-Arg-MCA (11), and it activates Hageman factor and subsequent bradykinin generating cascade process (11).

Protease Inhibitors. Human plasma α-1 protease inhibitor, human α-2-macroglobulin, and chick ovomacroglobulin were generous gifts from Dr. C. B. Glaser, Institute for Medical Sciences, San Francisco, CA; M. Okada, Institute for Chemoserotherapy, Kumamoto, Japan; and T. Mizuta, Japan Immunoresearch Inc., Takasaki, Japan, respectively. All these preparations are purified by chromatography, and purity was more than 90 to 95% as determined after polyacrylamide gel electrophoresis.

RESULTS
As shown in Figs. 1 and 2, all doses (from 30 to 300 µg/tumor) were effective as either single or multiple injections of serratia protease. The tumor became a blackish scab at these doses (Fig. 1C). After several days the scabs were mostly detached, and the tumor site could not be distinguished from surrounding tissue (Fig. 1D). Pathological evaluation of resected sites showed little evidence of tumor. Results with RL51 were very similar to Meth-A tumor (not shown).

A single dose of 300 µg in 100 µl injected into the tumor was almost as effective as double or multiple injections of a smaller dose. Extensive testings revealed that the volume of enzyme injected appears to be similarly important as the total amount of enzyme above a certain limit. For instance, 50 µg of protease need 50 µl or more of volume. Access of the enzyme to the entire tumor tissue appeared to be required and was achieved usually by more than 100 µl for the tumor size used in this experiment.

Mitomycin C as a representative low-molecular-weight anticancer agent was tested similarly to the above at a dose of 1/5 the 50% lethal dose (i.v.) as a control of local injection. As shown in Fig. 2 mitomycin C was found to be effective against this tumor, although much more moderately.

The effect of two major plasma protease inhibitors, α-1-protease inhibitor and α-2-macroglobulin, on serratia protease was examined. The result showed that they have no appreciable inhibitory effect on serratia protease. On the contrary ovomacroglobulin from chicken egg white inhibited serratia protease almost completely (Table 1). Thus, we tested serratia protease after inactivating with ovomacroglobulin as to whether this protease activity parallels the antitumor activity. The result showed that there was much less antitumor activity in ovomacroglobulin-inactivated protease (Fig. 2).

Fig. 3 shows the effect of serratia protease on the ascitic Meth-A tumor administered i.p. The 80% cure rate was obtained at a dose of 3 mg/kg during 60 days of observation. The 50% lethal dose of the serratia protease was 14.0 mg/kg i.v. and 19.5 mg/kg i.p. Intradermal injection of the protease was well tolerated at a dose of 1 mg per mouse, about 40-mg/
ANTITUMOR EFFECT OF MICROBIAL PROTEASE

Fig. 1. Antitumor effect of serratal M, 56,000 protease on Meth-A tumor. Representative Meth-A tumor in BALB/c mouse at Day 7 (A) and Day 21 (B) after tumor inoculation without treatment (control). Dashed circle indicates tumor mass in A. C, same tumor on Day 9 that had received intratumor injections (100 μg) of serratal protease on Days 7 and 8, one injection per day: C became D on Day 21; no trace of tumor was observed. Multiple injections with a total of approximately 0.1-ml volume into a tumor mass were usually done with a 1-ml syringe fitted with a 27/32 gauge needle. Tumor masses subjected to treatment were 0.8 to 1.0 cm in diameter.

Table 1 Effect of plasma protease inhibitor on Serratia M, 56,000 protease activity in vitro

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Residual protease activity with or without inhibitors at the following E/I ratios*</th>
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<tbody>
<tr>
<td></td>
<td>α₁-Protease inhibitor</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
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* Assays were made by the fluorescence polarization method using fluorescein-labeled gelatin as substrate in 0.05 M Tris-HCl buffer, pH 8.0.

DISCUSSION

As shown in Figs. 1 to 3, serratal protease exhibited a pronounced antitumor activity when given into the tumor at a relatively low dose. This effect was more than that of mitomycin C given at an equivalent toxic dose to the protease. A complete eradication of tumor was possible by the protease (Figs. 1 and 2).

More than 95% inhibition of serratal protease activity was achieved with ovomacroglobulin obtained from chicken egg white at an E/I² ratio of 1.4/1 (mol/mol) (15), whereas neither human plasma α₁-protease inhibitor nor plasma α₂-macroglobulin inhibited the enzyme activity by more than 90% at an E/I ratio of 1.5/1. The major toxic effect was tissue necrosis when the enzyme solution leaked out into the tail tissue upon injection. Death in the mice which received a lethal dose (i.v.) was caused by the hemorrhagic damage in the lung, kidney, liver, and alimentary tract.

The abbreviation used is: E/I, enzyme/inhibitor ratio (w/w).
Protease activity was thus examined using ovomacroglobulin-protease activity. The association of antitumor activity and plasma inhibitors were degraded by the protease as revealed by polyacrylamide gel electrophoresis (15). This fact may explain that sustained antitumor effect of protease in vivo requires protease activity. The association of antitumor activity and protease activity was thus examined using ovomacroglobulin-inactivated serrattia protease. The result showed only a little tumor suppression, indicating proteolytic activity is a major mechanism of antitumor activity. The reason for marginal tumor suppression of this E/I complex is, however, unclear although a residual (5%) enzyme activity might be responsible.

Prolonged retention of the protease molecule in the tumor tissue when injected into tumor can be envisaged in view of the poorly developed lymphatic recovery system in the solid tumor (5, 16–18) and its large molecular size, which prevents its movement into the blood capillaries (6, 16–18).

We have examined different proteases and other solid tumors, such as Sarcoma 180 of the mouse (ddY). Subtilisin and thermolysin were similarly effective to Meth-A and Sarcoma 180 in mice (19). On the contrary other proteases, such as trypsin or papain, enhanced tumor growth and exhibited no suppressive effect (19).

In separate experiments we found that activity of proteases, which exhibited no antitumor effect, was inactivated by serum. This fact and decreased antitumor activity of serrattia protease with ovomacroglobulin indicate that persistent protease activity appears essential for antitumor activity against tumor cells. Detailed study in vitro is under way. However, involvement of plasmin or its activator is not clear at this point.

It is interesting in the present context that recent findings in the mechanism of killer cells of the immune system are now being considered to involve the action of proteases (20). On the other hand, the indirect immunological effect as an antitumor mechanism of the present protease was eliminated. Namely, an experimental tumor was inoculated at the two sites on the back of mouse skin, and serrattia protease was given to only one side. The result showed that the tumor which received protease was eradicated completely, while the other without protease did not exhibit any effect (not shown). There may still be, however, a possibility of local activation of tumoricidal cells such as macrophages, although it remains to be clarified.

We have found that Serratia protease becomes a complex with α2-macroglobulin and that the enzyme activity is suppressed to about 30 to 40% transitorily. This inhibitory state, however, becomes reversed, and the enzyme activity is regenerated after 4 h (15). This inactive E/I complex seems to be internalized into cells via α2-macroglobulin receptor by endocytosis and then exerts cytotoxicity (21). Rat hepatocytes seem to have a lesser amount of this receptor than fibroblasts and, thus, little cytotoxicity (21). The target of the protease action at the subcellular level is not clear at this stage.

We have been working on a macromolecular antitumor agent, smancs M₀₁, 5,000 to 18,000 and its lipid-solubilized form (smancs/Lipiodol), and we found that the accumulations of aqueous smancs and smancs/Lipiodol were remarkably high in tumor tissue after i.v. injection (aqueous smancs) or after injection into the tumor-feeding artery (smancs/Lipiodol) (5, 6, 16, 17). The concentration of smancs/Lipiodol in the tumor tissue was more than 1200-fold higher than that in the plasma (5). This unique and tumor-specific accumulation may be explained by the macromolecular (6, 17, 18), or oily, nature of the drug (5).

Inasmuch as the proteolytic action is the major mechanism of antitumor effect seen here, resistance of this protease to various protease inhibitors in the plasma, such as α₁-protease inhibitor or α₂-macroglobulin, appears to be important. This protease actually degrades these inhibitors and remains active in their presence (Table 1). The above results indicate that the serrattia protease and other microbial proteases such as thermolysin may have therapeutic value when given locally similar to known antitumor agents of low molecular weight. Recently, local application of anticancer agents is getting more attention and appears more promising than the systemic administration (22), not to mention interferons, tumor necrosis factor, and others.

We have previously examined the effect of this protease on the cornea of rabbit and guinea pig and the tissue damaging associated with active protease but not with inactive enzyme nor heat-killed serratal whole cells (23, 24). Therefore, this circumstantial evidence supports the idea that the degenerating effect of tumor tissue is most likely to result from proteolytic activity.

Protease has a unique mechanism of action as an anticancer agent as compared with many other antineoplastic agents; it does not affect the nucleic acid metabolism or DNA or RNA, nor modify them, as do many other anticancer agents. Thus, the data provided here show that certain proteases alone can be an effective anticancer agent. The therapeutic use of this and other proteases appears applicable to any visible solid tumors. Thus, these proteases warrant further exploration as a new group of antitumor agents as regional therapy.

REFERENCES

ANTITUMOR EFFECT OF MICROBIAL PROTEASE


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