In Vivo and in Vitro Growth-inhibitory Effect of Bovine Seminal Ribonuclease on a System of Rat Thyroid Epithelial Transformed Cells and Tumors

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ABSTRACT

We investigated the antitumoral effect of bovine seminal RNase (BS-RNase) in vitro and in vitro on a model system of epithelial tumor- and metastasis-derived cells as well as on epithelial tumors derived from the same system. We found that while BS-RNase significantly inhibited the growth in vitro of the epithelial tumor-derived cells, its inhibitory effect was even more dramatic on the growth of metastasis-derived cells. BS-RNase exerted no appreciable growth inhibition on normal thyroid epithelial cells. When administered in vivo to rats bearing solid carcinomas, having the same thyroid origin, BS-RNase induced a drastic reduction in the tumor weight, with no detectable toxic effects on the treated animals. These data show, for the first time on a system of neoplastically transformed epithelial cells, that BS-RNase has a potent specific antitumoral activity.

INTRODUCTION

There are several factors which render BS-RNase\(^\text{1}\) an uncommon RNase: its dimeric structure, atypical for a RNase; its unusual enzymic properties, including allosteric regulation and the ability to cleave effectively both single- and double-stranded RNA; and its peculiar biological actions on tumor cells, on activated T cells, and on the male germ cell line (1).

The antitumoral action of BS-RNase has been studied in vitro on several cell lines, including mouse leukemic cells (2), HeLa and human embryo lung cells (3), mouse neuroblastoma cells and human fibroblasts (4), and mouse plasmacytoma (5) cell lines. Only the growth of the tumor cells was severely inhibited by the enzyme. The selectivity of the cytotoxic action of BS-RNase toward tumor cells was also verified with a self-controlled experimental system, consisting of virus-transformed cell lines and of their nontransformed counterparts: transformation rendered the cell sensitive to the action of the enzyme (4, 6). Furthermore, the monomeric derivative of the protein and homologous RNase A from bovine pancreas, more than 80% identical with the seminal enzyme, did not display any appreciable growth-inhibitory effect (4) when used at the same concentrations of BS-RNase.

As for in vivo studies, Matousek (7) reported an antitumor action of BS-RNase on Crocker tumors in mice and on Walker carcinosarcoma in rats (8). These experiments were carried out with partially purified preparations of the enzyme, and a high mortality rate was observed for the treated animals (7, 8). No further in vivo experiments have been carried out since.

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\(^2\) To whom requests for reprints should be addressed.

\(^3\) The abbreviations used are: BS-RNase, bovine seminal RNase; CPD, cumulated population doublings.

availability of highly purified preparations of BS-RNase (9) and recent reports on a newly discovered RNase with antitumor activity in vitro and in vivo (10) convinced us to further investigate the in vivo antitumor action of homogeneous preparations of the enzyme. Furthermore, to date no experiments have been performed to test the growth-inhibitory effect of BS-RNase on a model system of epithelial neoplastically transformed cells or on epithelial derived tumors.

Here we report the effects of BS-RNase on two neoplastic epithelial cell lines. These lines have been obtained in our laboratory: (a) from a rat thyroid follicular carcinoma (line TK-6), induced by direct injection into the thyroid gland of the Kirsten murine sarcoma virus, a retrovirus carrying the v-ki-ras oncogene; (b) from its lung metastases (line MPTK-6) (11). The latter cell line displays a more malignant phenotype (11). The effects on these two cell lines were compared with those obtained on a normal syngeneic rat thyroid differentiated cell line in continuous culture (line FRTL-5) (12). We also compared the effects of BS-RNase in vitro on solid carcinomas obtained by injecting the tumor-derived cells (line TK-6) into syngeneic animals. BS-RNase was found to inhibit the growth in vitro of the epithelial tumor-derived cells, with a more dramatic effect on metastasis-derived cells, whereas it showed no significant growth inhibition on normal cells. When administered in vivo to rats bearing solid carcinomas, BS-RNase induced a drastic reduction in tumor weight, with no detectable toxic effects on the treated animals. This is the first report showing that BS-RNase has a potent specific inhibitory effect on the growth of neoplastically transformed epithelial cells.

MATERIALS AND METHODS

Cell Culture. The cell lines used throughout these studies were: Fischer rat thyroid cells (FRTL-5) in continuous culture (12); Fischer rat thyroid tumor cells (TK-6) derived from a rat thyroid follicular carcinoma obtained by injecting directly in the thyroid gland the Kirsten murine sarcoma virus, as previously described (11); and a cell line derived from Fischer rat lung metastases of the TK-6 tumor (MPTK-6) (11). Conditions of cell growth and the characteristics of the cell lines and of the solid tumors were described previously (11, 12).

Inhibition of Cell Growth. Cells (8 \(\times\) 10\(^6\)) were plated on 60-mm dishes. BS-RNase or RNase A were added 24 h later. After 96 h, the medium was removed and replaced with fresh medium containing the same concentration of enzyme. The results as reported represent the average from two experiments.

Tumor Induction and Treatment of the Animals with BS-RNase and RNase A. Two- to 3-month old Fischer rats were shaved on the dorsal right side and injected (day 0) s.c. with a suspension of 0.5 ml containing 5 \(\times\) 10\(^6\) viable cells from the tumor-derived cell line (line TK-6), as described previously (11). The site of injection was marked. At day 1 BS-RNase and RNase A dissolved in 0.5 ml of sterile saline solution (0.9% NaCl) were injected s.c. at the previous injection site. The saline solution was used for injection into the control animals. The injections were repeated as indicated in Table 2. At day 30 all animals were
sacrificed and the tumors were excised, weighed, fixed, and stained for histological examination.

**Purification of BS-RNase.** BS-RNase was purified from bovine seminal vesicles according to the method of Tamburrini et al. (9). Purity of each preparation was checked by electrophoresis on polyacrylamide gel in sodium dodecyl sulfate and by analytical high-performance ion-exchange chromatography (9) on prepacked Mono-S 5/5 columns (Pharmacia). RNase A (type IIIA) was purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade.

**Calculation of Cumulated Population Doublings.** CPD were calculated by the formula \( \log_2 \frac{y}{x} \) (where \( y \) represents the number of cells counted at day 7 and \( x \) represents the number of cells counted at plating).

**RESULTS**

**Growth-inhibitory Effect of BS-RNase on in Vitro-cultured Cells.** The effect of different concentrations of BS-RNase on FRTL-5, TK-6, and MPTK-6 cell lines is shown in Fig. 1. Fig. 1A depicts the growth-inhibitory effect exerted by the enzyme upon the FRTL-5 cell line. The maximal inhibition was reached with a dose of 50 \( \mu \)g/ml added 24 h after seeding. The extent of inhibition did not increase significantly when an additional dose of 50 \( \mu \)g/ml of enzyme was added. At the same BS-RNase concentrations the growth inhibition of TK-6 (Fig. 1B) and MPTK-6 (Fig. 1C) cell lines was much more pronounced. Fig. 1B shows the growth-inhibitory effect of BS-RNase on the TK-6 cell line. The growth inhibition observed with the dose added 24 h after seeding was increased with an additional dose of 50 \( \mu \)g/ml added 72 h after the first dose. Fig. 1C shows that the growth-inhibitory effect of the enzyme on MPTK-6 cell line was striking also at concentrations of 10 or 5 \( \mu \)g/ml. No dead cells were visualized in all BS-RNase-treated populations by the trypan blue exclusion test. The data therefore indicate that the effect of BS-RNase is due to a cytostatic activity. We have calculated from Fig. 1 the CPD of the three cell lines treated with BS-RNase. The results are shown in Table 1.

**Table 1 CPD and percentage of growth inhibition (GI) of the three cell lines in the absence or in the presence of different concentrations of BS-RNase**

<table>
<thead>
<tr>
<th>BS-RNase (( \mu )g/ml)</th>
<th>FRTL-5</th>
<th>TK-6</th>
<th>MPTK-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPD</td>
<td>GI</td>
<td>CPD</td>
</tr>
<tr>
<td>0</td>
<td>4.12</td>
<td>0</td>
<td>5.05</td>
</tr>
<tr>
<td>5</td>
<td>4.02</td>
<td>2.6</td>
<td>4.78</td>
</tr>
<tr>
<td>10</td>
<td>3.90</td>
<td>5.4</td>
<td>4.64</td>
</tr>
<tr>
<td>50</td>
<td>3.78</td>
<td>8.5</td>
<td>4.02</td>
</tr>
</tbody>
</table>

A dramatic change in cumulated population doublings of the metastasis-derived cell lines was obtained with a BS-RNase concentration of 50 \( \mu \)g/ml. However, even concentrations of 5 and 10 \( \mu \)g/ml were able to decrease the cumulated population doublings of the metastasis-derived cell line. In contrast, the CPD of the tumor-derived cell line were affected only by a BS-RNase concentration of 50 \( \mu \)g/ml. No significant differences in the CPD of the normal FRTL-5 cell line were observed in the presence of all BS-RNase concentrations tested. The percentage of growth inhibition obtained comparing the cumulated population doublings is shown in Table 1. We also tested the effect of pancreatic RNase (RNase A) on the three cell lines under the same experimental conditions. RNase A (Fig. 2) showed a slight growth-stimulatory effect on all three cell lines tested.

**Inhibitory Effect of BS-RNase on Epithelial Tumor Growth in Vivo.** The ability of BS-RNase to inhibit the neoplastic growth in vivo was evaluated in male Fischer rats bearing carcinomas induced by a s.c. injection of cells of tumoral origin (line TK-6). The effect of BS-RNase at different concentrations was compared with the effects observed on the animals bearing the same tumor, either untreated or treated with a saline solution. As shown in Table 2, the most effective dosage was 6
mg/rat in three different administrations, which induced a tumor growth inhibition of about 50%.

A larger group of rats was then treated with the most effective dose and compared with three groups of tumor-bearing animals, either untreated (control group), or treated with monomeric RNase A or saline (Table 3A). The group of animals treated with BS-RNase showed a tumor growth inhibition of about 60% compared to the control animals or to the rats treated with saline, whereas for the animals treated with pancreatic RNase A, a slight tumor growth stimulatory effect was detected (Table 3).

In order to verify if a prolonged treatment with BS-RNase at an increased total dosage could enhance the growth-inhibitory effect, the animals were treated with seven injections of the enzyme (Table 3B) to reach a total dosage of 14 mg/rat. As shown in Table 3B, no differences were detected between the results obtained with the two types of treatment. Fig. 3 shows the dramatic decrease of tumor size observed with the treated versus untreated tumors. Also in this case a slight, although statistically insignificant, increase in tumor weight was noted when the animals were treated with RNase A (Fig. 3; Table 3).

Since previous reports on the effects of BS-RNase on tumor-bearing animals suggested that the protein could be generally toxic (7, 8), blood samples from all the animals treated with the enzyme protein were tested for the main hematological parameters. No sign of anemia, leukopenia, or thrombopenia were detected in any of the treated, compared with untreated, animals (data not shown). Furthermore, no changes in the physical appearance, body weight or behavior of the treated animals were observed. No deaths resulted as a consequence of BS-RNase administration. Carcinomas excised from rats belonging to the control groups (treated with monomeric RNase A or with saline solution) and those removed from treated animals were not distinguishable upon macroscopic and histological examinations (data not shown).

Table 2. Dosage-dependent effect of BS-RNase on tumor growth in vivo

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Protein</th>
<th>Treatment</th>
<th>Tumor wt (g) ± SEM</th>
<th>Growth inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>None</td>
<td>None</td>
<td>2.51 ± 0.06</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Saline</td>
<td>0.5 ml x 3</td>
<td>2.48 ± 0.16</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>BS-RNase</td>
<td>0.5 mg x 3</td>
<td>1.99 ± 0.12</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>BS-RNase</td>
<td>1.0 mg x 3</td>
<td>1.83 ± 0.14</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>BS-RNase</td>
<td>2.0 mg x 3</td>
<td>1.31 ± 0.16</td>
<td>48</td>
</tr>
</tbody>
</table>

a The animals were given s.c. injections of 5 x 10⁶ cells of the TK-6 cell line and 24 h later received injections of saline solution or different amounts of BS-RNase dissolved in 0.5 ml of saline solution. The treatment was repeated twice at 72-h intervals (x 3). After 30 days the animals were sacrificed and the tumors were excised and analyzed. Standard error of the mean of the tumor weight was evaluated as

\[
SEM = \frac{s}{\sqrt{n}}
\]

where s represents the standard error and n the number of animals.

Table 3. Comparison between two treatment schedules of BS-RNase on tumor growth in vivo

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Protein</th>
<th>Treatment</th>
<th>Tumor wt (g) ± SEM</th>
<th>Growth inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 25</td>
<td>None</td>
<td>2mg x 3</td>
<td>2.63 ± 0.15</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>Saline</td>
<td>2mg x 3</td>
<td>2.58 ± 0.12</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>BS-RNase</td>
<td>2mg x 3</td>
<td>1.03 ± 0.05</td>
<td>62</td>
</tr>
<tr>
<td>25</td>
<td>RNase A</td>
<td>2mg x 3</td>
<td>2.81 ± 0.26</td>
<td>0</td>
</tr>
<tr>
<td>B. 25</td>
<td>None</td>
<td>2mg x 7</td>
<td>2.42 ± 0.20</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>Saline</td>
<td>2mg x 7</td>
<td>2.46 ± 0.22</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>BS-RNase</td>
<td>2mg x 7</td>
<td>1.01 ± 0.08</td>
<td>58</td>
</tr>
<tr>
<td>25</td>
<td>RNase A</td>
<td>2mg x 7</td>
<td>2.66 ± 0.24</td>
<td>0</td>
</tr>
</tbody>
</table>

a The treatment protocol was the same as that outlined in Table 2, Footnote a except that in B the enzyme was administered seven times at 72-h intervals.

DISCUSSION

The results reported in this paper demonstrate, for the first time, that BS-RNase has a definite growth-inhibitory effect, both in vitro and in vivo, on a well characterized homogeneous...
Fig. 3. Carcinomas obtained by s.c. injections of the cells of tumoral origin (line TK-6), excised after 30 days from untreated control rats (A), control rats treated with saline solution (B), rats treated with BS-RNase (C), and rats treated with RNase A (D).

experimental system of thyroid epithelial transformed cells. It should be emphasized that epithelial-derived tumor models may bear particular importance since the large majority of human neoplasias is indeed of epithelial origin (13).

Our data show that the growth-inhibitory effect of BS-RNase in vivo is clearly evident only on thyroid tumor-derived cells, whereas normal fully differentiated thyroid cells (line FRTL-5) are poorly affected. The most pronounced growth-inhibitory effect of BS-RNase was observed on thyroid cells of metastatic origin (line MPTK-6). These display a more malignant phenotype and express higher levels of the Ki-ras oncoprotein with respect to the tumor-derived cell line (11). These data lead to a totally novel and interesting conclusion that a molecule with antitumoral activity is more effective on a more malignant line than on primary, tumor-derived, less malignant cells. The same substance shows only a small growth-inhibitory effect on normal differentiated cells, belonging to the same histotype.

These data are of great interest when compared to the action of conventional antineoplastic drugs, such as anthracyclins, tested on the same cell system. These molecules, indeed, have a higher growth-inhibitory activity on normal FRTL-5 cells compared to tumor-derived cells and, conversely, a higher inhibitory effect on tumor-derived cells (TK-6) than on metastasis-derived cells (MPTK-6).4

Early observations suggested that native monomeric RNase A at high concentrations inhibits tumor growth and interferes with ascites cell multiplication in culture (14). We observed instead that RNase A, tested on the same experimental system, and at the same concentrations used for dimeric BS-RNase, has a slight growth-stimulatory effect, both in vitro and in vivo, although in vivo the observed effect does not appear to be statistically significant. This demonstrates that the growth-inhibitory effect in our thyroid epithelial experimental model is specific for dimeric BS-RNase. These results are in line with previous results obtained with naturally dimeric BS-RNase (2, 6) or synthetic dimers of RNase A (15) tested on several malignant cell lines of mesenchymal origin. The only data in the literature concerning an in vivo antitumoral effect of BS-RNase were reported several years ago (7, 8). Such data, however, obtained with partially purified enzyme preparations, have not been confirmed since. Furthermore, they were obtained with neoplastic models of uncertain histological origin, such as the Walker carcinosarcoma, the Sajdel hepatoma, and the murine Crocker tumor (7, 8). In fact, the data reported on Walker carcinosarcoma were obtained with too low a number of animals to reach any significant conclusion, whereas no differences in survival time between treated and control animals were obtained when BS-RNase was administered to rats transplanted with the ascites cells from Sajdel hepatoma (8). Finally, the mice bearing Crocker tumors (7) treated with BS-RNase presented a high mortality rate, probably due to the administration of a partially purified enzyme. In this respect, it should be noted that in the experiments reported here no side effects were observed in treated animals.

Recently, it has been reported that an RNase from the early embryos of Rana pipiens (protein P30), belonging to the RNase superfamily, with 30% identity with RNase A and 27% identity with BS-RNase, also exerts growth-inhibitory activity on neoplastic cell lines (10, 16–18). These recent data enlarge and confirm both our present and previous observations that proteins belonging to the RNase superfamily show a tumor

growth-inhibitory activity. The molecular mechanisms by which BS-RNase exerts its antineoplastic activity remain to be elucidated. Preliminary results obtained with the cell system described in this report seem to indicate that BS-RNase binds to FRTL-5, TK-6, and MPTK-6 cells with a relatively low affinity with a \( K_d \) of about \( 10^{-7} \) M (data not shown).

These data may suggest that one possible mechanism of action of BS-RNase is that of competitively displacing essential growth factors with which it shares common surface receptors. This factor(s) would be of greater importance for the tumor-derived cells than for normal cells.

Finally, the data described here show that BS-RNase, active on an epithelial ras-transformed model, belongs to a new class of antineoplastic drugs, the main property of which, not yet described for an antitumor substance, is that of being, at least \( \text{in vitro} \), more active against metastasis-derived cells that express a more malignant phenotype than against primary tumor-derived cells, and also to be more active against tumor cells than against the corresponding normal differentiated cells.

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