Antitumor Effects of SMANCS on Rat Mammary Tumor Induced by 7,12-Dimethylbenz[a]anthracene

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ABSTRACT

We previously found that a high-molecular-weight anticancer agent, polystyrene-co-maleic acid conjugated neocarzinostatin (SMANCS), in which two chains of styrene/maleic acid copolymer are conjugated to the anticancer protein neocarzinostatin (NCS), accumulated more selectively in tumor tissue than in normal tissue and was more stable than NCS in blood. These results indicate that SMANCS should have less systemic toxicity and a better therapeutic effect than NCS. In this study, the antitumor activity and adverse effects of SMANCS were compared with those of NCS by using rat mammary tumor induced by 7,12-dimethylbenz[a]anthracene. When tumors of rats, that had received 7,12-dimethylbenz[a]anthracene (20 mg/kg, one dose, p.o. in oily formulation), became palpable usually after 4–20 weeks, SMANCS treatment was initiated. Thirty days after i.v. administration of SMANCS (0.1 mg/kg 3 times and 0.3 mg/kg 3 times), tumors had shrunk in 35 of 37 rats (a mean weight was about 10% of control value; or decreased to about 30% of the value of before treatment in tumor weight); tumor size had not changed in 1 rat, and in the remaining 1 rat the tumor had enlarged. Thirty days after i.v. administration of NCS, tumors had shrunk in 8 of 14 rats, but the tumor size was unchanged in 1 rat and was enlarged in 5. In the control group, all tumors had enlarged. Development of new tumors was completely prevented by the administration of SMANCS. Histological examination of sequential slices of tumor revealed clear finding of degeneration and tumor encapsulation at 30 days after initial administration of SMANCS, with an accompanying fatty degeneration, but these effects were not observed for tumors treated with NCS. Although red blood cell counts and hemoglobin amounts decreased significantly in rats receiving NCS, no such effects were apparent in the SMANCS group.

INTRODUCTION

Regional cancer chemotherapy has been advanced and has been used to achieve substantial progress against visible or identifiable tumors. For instance, we have achieved a remarkable response with the use of the anticancer agent SMANCS in Lipiodol given i.a. (1–7). However, to cure cancer completely it is necessary to deliver drugs with a potent antitumor effect to tumors from the general circulation; thus one can eradicate all systemic and unidentified metastases to distant tissues. It is also important to maintain the drug concentration in the tumor at sufficiently high levels and for a long period to destroy cancer cells effectively.

It has been suggested that high-molecular-weight substances can leak out more selectively from blood vessels in the tumor compared with blood vessels in normal tissue (6, 8–10). In our previous studies we demonstrated the unique vascular properties of EPR effect in solid tumor tissue (6, 8–10). Namely, high-molecular-weight anticancer agents accumulate more selectively in tumor tissue as described above, even when administered by i.v. injection, because of the EPR effect. In this report we examine differences in antitumor activity and side effects of NCS and SMANCS by using DMBA-induced rat mammary tumors.

We previously demonstrated that SMANCS (M, 15,000) binds with plasma albumin and behaves like macromolecules of a size near 80 kDa (11). The parental compound NCS does not bind with albumin; thus, it is excreted very rapidly into the urine and consequently the in vivo half-life of SMANCS is 10 times longer than that of NCS (6, 8–10). Therefore, SMANCS exhibits an EPR effect, whereas NCS does not (6). Accordingly, the intratumor concentration of SMANCS has been known to be much higher than that of NCS in a grafted tumor system (6, 8, 9). However, no study has been reported concerning the antitumor effect of SMANCS on a chemically induced tumor, as well as hematological or other toxic effects in vivo when it was given i.v. This lack is in contrast to its application using i.a. SMANCS/Lipiodol, which has been most extensively studied (1–4, 6, 10). In vitro susceptibility of tumor cells and normal cells to SMANCS and NCS was reported; normal cells were more resistant, and DNA was the target of degradation (12).

MATERIALS AND METHODS

Drug. NCS, a proteinaceous antitumor antibiotic with a molecular mass of 11.7 kDa was obtained from Kayaku Antibiotics Research Laboratories Co., Ltd., Tokyo, Japan. SMANCS was prepared by reaction of NCS with a synthetic copolymer of styrene-maleic acid/anhydride (M, ~1500), as described previously (13, 14) and SMANCS contains one molecule of NCS, which was obtained from Kuraray Co. Ltd., Osaka, Japan. The resulting preparation had a molecular mass of about 15 kDa. Both NCS and SMANCS were dissolved in physiological saline solution at 1 mg/ml for injection.

Animals and Induction of Mammary Tumor. Seventy-six female Sprague-Dawley rats, 50 days old, were obtained from Japan SLC, Hamamatsu, Japan. A single dose of 20 mg DMBA/rat, obtained from Sigma Chemical Co., St. Louis, MO, was given by using an intragastric tube. Four to 20 weeks after administration of DMBA, palpable mammary tumors were incisionally biopsied for histological examination, and all tumors were identified by microscopic examination as papillary or tubular carcinoma. Only rats with a solitary single tumor were used; 6 of 76 rats had multiple tumors.

Experimental Groups and Treatment Protocol. Rats were divided into seven groups. Each drug or physiological saline solution was administered i.v. via the tail vein. A tumor weight of about 500–600 mg was found to be adequate for experimental use in each group.

For evaluation of the histological effect of the drug on the tumor during the early stage after the administration of the drug, three groups of rats were used: group 1, in which three rats received SMANCS at a dose of 0.9 mg/kg in 0.9 ml; group 2, in which three rats received NCS at a dose of 0.9 mg/kg in 0.9 ml; group 3, in which three rats received physiological saline solution of a dose of 0.9 ml/kg. Forty-eight h after administration of each drug, in these three groups were killed, and tumors were resected for histological examination. Resected tumors were then fixed in 10% neutral formalin solution. Sequential slices of tumors were stained with hematoxylin and eosin for light microscopy. For electron micrographs, resected tumors were fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight. The fixed tissues were then washed overnight in the same buffer and postfixed in 1% osmium tetroxide in the same buffer for 2 h. The tissues were then dehydrated and embedded in Epon.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: SMANCS, polystyrene-co-maleic acid-conjugated neocarzinostatin; NCS, neocarzinostatin; DMBA, 7,12-dimethylbenz[a]anthracene; EPR, enhanced permeability and retention; i.a., intraarterially.
For evaluation of antitumor activity and side effects of SMANCS and NCS, four groups of rats were used: group 4, in which 28 rats received SMANCS, a total of 0.9 mg/kg (0.3 mg/kg 3 times, once every 5 days); group 5, in which 9 rats received SMANCS, a total of 0.3 mg/kg (0.1 mg/kg 3 times, once every 5 days); group 6, in which 14 rats received NCS, a total of 0.9 mg/kg (0.3 mg/kg 3 times, every 5 days); group 7, in which 10 rats received physiological saline solution, a total of 0.9 ml/kg (0.3 ml 3 times, once every 5 days). Thirty days after the initial i.v. administration of each drug, rats in these four groups were killed, and tumors, livers, and kidneys were resected for histological examination. The anticancer effect of each drug on DMBA-induced mammary tumor was evaluated based on changes in tumor weight and on histological findings. Measurements of tumor weight were carried out on the day of initiation of the administration of drugs (day 0) and 30 days after this initial administration (day 30). Tumor weight \( (T_w) \) (mg) was calculated as:

\[
T_w = 0.5 \times W^2 \times L
\]

where \( W \) is width and \( L \) is perpendicular length of the tumor nodule in mm. Tumor growth ratio (TGR) was calculated as:

\[
TGR = \frac{T_w \text{ on day 30}}{T_w \text{ on day 0}}
\]

Hematological examinations were also performed. Statistical evaluations were carried out by Student's \( t \) test.

RESULTS

Early Histological Changes after Drug Administration. In group 1, marked fatty degeneration of tumor cells was observed 2 days after treatment with SMANCS (Fig. 2C); this change was not evident in group 2 (Fig. 2A). In group 2, although no remarkable changes were observed by light microscopy, pyknosis, karyorrhexis, and chromatolysis of nuclei in tumor cells were observed in electron micrographs (Fig. 2A). These degenerative changes were infrequently observed in fibroblasts of connective tissue, but they were not seen in other cells. In the blood vessels of the tumor, deposition of platelets and fibrin and thrombi were frequently observed, together with leakage of blood cells out of the vascular bed (Fig. 2B). In group 3, no changes in tumor cells were observed (not shown).

Antitumor Effects Based on Changes in Tumor Weight. In group 4 (treated with 0.9 mg/kg of SMANCS), tumor size decreased in 27 of 28 rats; in the remaining rat, the tumor weight did not change. In group 5, tumor size decreased in 8 of 9 rats; in the remaining rat, tumor size enlarged. In group 6 (NCS group), the tumors shrank in 8 of 14 rats, were larger in 5, and were unchanged in 1. In group 7 (control), all tumors were enlarged. In groups 4 and 5 (SMANCS groups), tumor weights on day 30 were significantly smaller than those on day 0 \( (P < 0.001 \text{ and } P < 0.05, \text{ respectively}) \). Tumor weights of groups 4 and 5 was significantly smaller than that of group 7, which showed no sign of decrease (Table 1). The tumor growth ratios of groups 4 and 5 were significantly smaller than those of groups 6 and 7 (Table 2).

Histological Evaluation of Antitumor Effects on Day 30. In groups 4 and 5, atrophy of cytoplasm, pyknosis of nuclei, and cystic degeneration of tumor cell cytoplasm were observed, and bleeding was often seen in the tumors (Fig. 1B). In groups 6 and 7, these degenerative findings were not present, and viable cancer cells occupied tumor tissue. Bleeding was infrequent in group 7.

New Tumor Development at Day 30. There was no newly developed tumor at day 30, other than the tumors noted on day 0 in groups 4 and 5. In group 6, new tumors were seen in 3 of 14 rats near or remote from the site of the original tumor during the observation period (day 0–day 30). In group 7, new tumors developed in 4 of 10 rats (Table 2).

Side Effects. Leukopenia and thrombocytopenia were not observed in all tested SMANCS groups (Table 3). RBC and hemoglobin values in the NCS group (group 6) were significantly lower than those in the control group, but this was not
Table 1 Antitumor effect of SMANCS and NCS given i.v. on solid DMBA-induced mammary carcinoma in rats
Values were compared with control. See text for details.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Total dose (mg/kg)</th>
<th>Before administration of drug</th>
<th>30 days after initial administration of drug</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>SMANCS</td>
<td>0.9</td>
<td>536 ± 265*</td>
<td>160 ± 120</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>SMANCS</td>
<td>0.3</td>
<td>587 ± 128</td>
<td>NS</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>NCS</td>
<td>0.9</td>
<td>556 ± 430</td>
<td>NS</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>None</td>
<td>612 ± 386</td>
<td>1501 ± 1349</td>
<td>10</td>
</tr>
</tbody>
</table>

* Mean ± SD.
† NS, not significant.
‡ P < 0.05.
§ P < 0.02.

Table 2 Tumor growth ratios of DMBA-induced tumors in drug-treated rats and subsequent tumor development
See text for details.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Total dose (mg/kg)</th>
<th>Tumor growth ratio (TGR)</th>
<th>No. of rats with new tumor after day 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>28 SMANCS</td>
<td>0.9</td>
<td>0.33 ± 0.23*</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>9 SMANCS</td>
<td>0.3</td>
<td>0.56 ± 0.34</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>14 NCS</td>
<td>0.9</td>
<td>1.90 ± 3.68</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>None</td>
<td>3.01 ± 2.66</td>
<td>4</td>
</tr>
</tbody>
</table>

* TGR = tumor weight on day 30/tumor weight on day 0.
* Mean ± SD.
‡ P < 0.05.
§ P < 0.02.
† NS, not significant.
‡ P < 0.001.

the case for the SMANCS groups (groups 4 and 5). Histological examination of the liver and the kidney on day 30 revealed no pathological damage in all tested groups including control.

DISCUSSION

Both SMANCS and NCS has a common molecular mechanism of action, i.e., inhibition of DNA synthesis (12), but their pharmacokinetic properties and immunopotentiating effect of SMANCS described later are completely different. We previously found that SMANCS and other macromolecules, with an apparent molecular size larger than 50 kDa, accumulated in tumor tissue more than did NCS, which is only 12 kDa (6, 8). These high-molecular-weight proteins and SMANCS can easily permeate the vasculature of tumor tissue, but they can barely do so in normal blood vessels. The architectural defect of tumor vessels is well known (15–17): hypervasculature of the neovasculature is usually observed in solid tumors with a diameter larger than 1–2 mm. The defect of tumor vessels will account for a part of the EPR effect of macromolecules and lipids in solid tumors. This effect is now known to be mediated by a
number of factors: bradykinin (18–20); permeability-enhancing factors, which are generated in situ (21); and tumor necrosis factor and lymphokines (22). This retention of SMANCS and lipids in tumor tissue continued for a long time. One reason for this retention is a lack in tumor tissue of the reticuloendothelial system, which removes lipids and high-molecular-weight substances from normal tissue (1–6, 8–10). This greater accumulation of SMANCS in tumor tissue results in a strong therapeutic effect and less side effects. It was also proved previously that SMANCS is more stable than NCS in blood (13). Namely, NCS portion was stabilized by styrene-maleic acid/anhydride conjugation. Thus, it is reasonable that SMANCS has more potent antitumor activity and less adverse effects because of the EPR effect, compared with its mother compound NCS. Furthermore, the increased hydrophobic character of SMANCS resulted in quicker cellular uptake and a higher cell-binding constant (23, 24). Thus, SMANCS requires only a short contact time with tumor cells to be effective (23, 24). Indeed, it became clear in the present experiments that antitumor activity of SMANCS was superior to that of NCS (Fig. 1; Table 1). Namely, the administration of SMANCS (0.3 mg/kg 3 times) caused remarkable tumor regression in 27 of 28 rats, and in the remaining rat the tumor size was unchanged. On the other hand, the administration of NCS (0.3 mg/kg 3 times) resulted in tumor regression in 8 of 14 rats, tumor growth in 5, and no change in 1. The superiority of SMANCS to NCS was also proved by the histological findings with sequential slices of tumors on day 30. Thus, the difference in antitumor activity between SMANCS and NCS is thought to be due primarily to the higher stability and more selective accumulation of SMANCS in tumor tissue. The long-lasting effect and large accumulation in tumor tissue are attributed to the EPR effect.

The prevention of subsequent tumor development was observed after the administration of SMANCS (Table 2). This effect appears to be another advantage of this drug. It may be explained by the fact that a newly developed tumor mass with a diameter larger than 1–2 mm, which had neoangiogenesis because of tumor angiogenesis (25), was reduced by the high drug concentration caused by the EPR effect. In contrast, nonvascularized tumor less than 1–2 mm in diameter was destroyed by freely accessible and stable activity of SMANCS which permeated from the circulating blood. Immunological activation by SMANCS involving macrophages and T-lymphocytes, as well as induction of γ-interferon, may contribute to this effect (26–29), or they have not reached a stage of palpable size.

Ebihara et al. (30) reported that the volume of rat mammary tumor induced by DMBA was enlarged despite the administration of Adriamycin, pepleomycin, and bleomycin (growth ratio, 1.15–3.49), and shrinkage of the tumor by a single drug administration of anticancer agent is thought to be very difficult to achieve. In contrast, regression of the majority of these DMBA-induced mammary tumors treated with SMANCS was an indication of definite antitumor activity of this drug. Few adverse side effects of SMANCS (Table 3), especially on WBC and platelet counts and on liver and kidney functions, may be explained by the fact that this drug leaks out much less from blood vessels in normal tissue than from those in tumor tissue. Further, the relative resistance of hepatocytes, macrophages, spleen, and other normal cells to SMANCS reported in vitro (12) made the drug less toxic in the organs concerned in vivo. All these results warrant further evaluation of SMANCS in the clinical setting.

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