A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs

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ABSTRACT

We previously found that a polymer conjugated to the anticancer protein neocarzinostatin, named smancs, accumulated more in tumor tissues than did neocarzinostatin. To determine the general mechanism of this tumoritropic accumulation of smancs and other proteins, we used radioactive (65Cr-labeled) proteins of various molecular sizes (M, 12,000 to 160,000) and other properties. In addition, we used dye-complexed serum albumin to visualize the accumulation in tumors of tumor-bearing mice. Many proteins progressively accumulated in the tumor tissues of these mice, and a ratio of the protein concentration in the tumor to that in the blood of 5 was obtained within 19 to 72 h. A large protein like immunoglobulin G required a longer time to reach this value of 5. The protein concentration ratio in the tumor to that in the blood of neither 1 nor 5 was achieved with neocarzinostatin, a representative of a small protein (M, 12,000) in all time. We speculate that the tumoritropic accumulation of these proteins resulted because of the hypervasculature, an enhanced permeability to even macromolecules, and little recovery through either blood vessels or lymphatic vessels. This accumulation of macromolecules in the tumor was also found after i.v. injection of an albumin-dye complex (M, 69,000), as well as after injection into normal and tumor tissues. The complex was retained only by tumor tissue for prolonged periods. There was little lymphatic recovery of macromolecules from tumor tissue. The present finding is of potential value in macromolecular tumor therapeutics and diagnosis.

INTRODUCTION

We have reported that a copolymer of styrene and maleic acid conjugated to the anticancer protein NCS, which we designated as smancs, accumulated more effectively in the tumor than normal tissues as measured by biological activity (3). Furthermore, its pronounced lymphotropic character and hence antilymphatic metastasis have been noted (4, 5).

Of a prime importance in cancer chemotherapy is the highly tumoritropic nature of the anticancer agent. Few compounds have been shown to exhibit this characteristic. No general rule to accomplish this goal has been ever described for the purpose of drug targeting in cancer chemotherapy. Most of the anticancer agents used or studied widely have a low molecular weight (less than 1500). We have successfully synthesized the polymer-conjugated protein, smancs, to improve its in vivo half-life and to enhance its lymphotropism (1, 3). We subsequently found that it was more tumoritropic than the parental compound, NCS, and thus exhibited greatly improved antitumor properties in animals (2, 3, 5) and humans (6, 7). During our pharmacological investigations, we realized that the predominant tumor accumulation was due to the unique vascular characteristics of the tumor tissue and the lack of a lymphatic recovery system in the solid tumor. The purpose of the present report is to clarify this tumoritropism of macromolecules in the tumor tissue.

MATERIALS AND METHODS

Animals and Tumor. Ten-wk-old ddY mice weighing about 35 g each were used. Sarcoma 180 tumor cells grown for 7 days in the ascites fluid of the mice were injected into an intracutaneous site on the back or the femoral skin of about 11-wk-old mice, at a dose of 1 x 10⁶ cells per site. The tumor-bearing mice received regular food, and the tumors were allowed to grow for about 8 to 10 days until the tumor diameter reached 8 to 10 mm.

Materials. NCS was obtained from Kayaku Antibiotic Laboratories Co., Ltd., Tokyo, Japan; smancs was prepared in our laboratories as described (2). Mouse serum albumin and BSA were from Sigma Chemical Co., St. Louis, MO, or were prepared and purified in our laboratory. Mouse IgG was purified with a Protein A column (Pharmacia A.B., Stockholm, Sweden) after partial purification in our laboratory. Chicken ovomucoid was from Dr. R. E. Feeney, University of California, Davis, CA. Both DTPA anhydride and EDPC were from Dojindo Laboratories, Kumamoto, Japan. Evans blue was from E. Merck, Darmstadt, West Germany. Radioactive 51CrCl3 was from New England Nuclear Inc., Boston, MA. Lysine and all other chemicals were from commercial sources.

Labeling of Radioactive Metal to Proteins. Proteins labeled with radioactive metal through the use of the chelating agent DTPA (8). The anhydride portion in DTPA was covalently linked by reaction through an amide bond. This bonding is more stable than iodination with tyrosine residues, which frequently yields unreliable values (9). To label proteins with 51Cr, their free amino groups were first reacted with equimolar amounts of DTPA anhydride within 5 min at pH 7.0 in 0.1 M 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid buffer (6). Smancs, which has no free amino group, was first conjugated with lysine by using a water-soluble carbodiimide (EDPC) at pH 6.0 (10). The conjugate was purified on a Sephadex G-50 column, and the lysine content was found to be about 3.6 residues per mol of smancs. This conjugate was referred to as Lys-smancs. DTPA anhydride was linked to Lys-smancs as described above. For all proteins, DTPA treatment resulted in about 1 mol of amino group reacting as determined by the decrease in free amino groups (11). For albumin and IgG the decrease in the amino group was not apparent, because there are about 60 and 85 free amino groups per mol of protein, respectively.

Radiolabeling with 51Cr was carried out as follows. To 1 ml of solution containing approximately 20 mg of DTPA-conjugated protein in 0.1 M 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid buffer at pH 7.0 except IgG (2.0 mg/ml) were added 50 μl of solution of 51CrCl3 (1 mCi/ml); chelation with 51Cr at room temperature proceeded overnight. The solution was separated on a column of Sephadex G-50 (1.5 x 40 cm), which had been equilibrated with 0.1 M acetate buffer at pH 6.0. The radioactivity and protein content of each fraction were determined. Radioactivity was measured by using a well-type gamma counter (Packard Model 5130). The protein peak with radioactivity was obtained and lyophilized after dialysis against deionized water, or it was kept frozen until used. 51Cr was incorporated to a significant degree in the proteins. Each DTPA-conjugated protein had the following specific radioactivity (cpm/mg) after labeling with 51Cr as described below: NCS, 4.7 x 10⁵; ovomucoid, 3.9 x 10⁵; smancs, 2.3
x 10^5; BSA, 9.5 × 10^5; mouse serum albumin, 4.7 × 10^5; and mouse IgG, 0.9 × 10^2.

Tissue Distribution and Accumulation of ^51Cr-labeled Proteins in Tumor-bearing Mice. ^51Cr-labeled proteins with about 1 × 10^5 cpm in 0.2 ml were injected into the tail vein of mice with the S-180 tumor (approximately 8 to 10 mm). The mice were then killed at 1, 3, 5, 10, 30, and 60 min; 3, 6, and 12 h; and 1, 2, and 3 days for removal of tumors and other tissues to determine the radioactivity. First blood specimens were removed by cardiac puncture; specimens of various organs or tissues were then removed and weighed, and radioactivity was counted. An average of 2 mice was used for each time point.

Plasma Clearance and Accumulation of Albumin-Evans Blue Complex in the Tumor Tissue. This experiment was conducted primarily to visualize and also to quantitate the behavior of macromolecules at the tissue level. Evans blue (0.18 ml) dissolved at 0.2% in 0.8% saline solution was injected into the tail vein of the tumor-bearing mice at a dose of 10 mg/kg. At this dose level there was no free dye in the plasma; it was found mostly bound to albumin as revealed by Bio Gel P-10 gel filtration. The blood samples from the mice under ethyl ether anesthesia were obtained 0.2 ml at a time by cardiac puncture with a syringe fitted with a 27/32 gauge needle. The blood samples were immediately mixed with 2.8 ml of Isoton II (Coulter, Inc.) followed by centrifugation at 150 × g for 5 min. Then the concentration of Evans blue was determined spectrophotometrically at 620 nm. Solid tumors were removed, weighed, and immersed in 3 ml of formamide followed by incubation at 60°C for 48 h to extract the dye. The concentration of the dye was similarly determined spectrophotometrically. In the same experiment, the tumor specimen on the mouse skin was removed and photographed before the extraction procedure.

For the photography with monochromatic film an R2 filter was used to remove the red component of the blood and vessels in the background; the blue component thus selectively appeared as black in the picture.

To reveal the efficient retention and lack of recovery from the tumor, 0.05 ml of Evans blue complexed with BSA were injected as a single dose into the center of the tumor with a 27/32 gauge needle. To prepare the complex, 1 mg of Evans blue was mixed with 8 mg of BSA in 1 ml of saline in a test tube; all of the dye was found to complex with albumin. The tumor was then removed at different times for quantification of the remaining content of the dye in the tumor tissue as described above. Injections into normal skin and quantification were carried out similarly.

RESULTS

Plasma Clearance and Accumulation of ^51Cr-labeled Proteins. When NCS was injected i.v. it was cleared very rapidly; a plasma half-life of about 2 min was obtained. In contrast, the half-life of smancs was 18 min (Fig. 1A). Nonautologous glycoprotein, ovomucoid, had a half-life of 6 min. The t½ of smancs was 10 times longer than NCS. These values are given in detail in Table 1. We found that albumin from different animals exhibited somewhat different clearance rates, although t½ values were more similar.

We then compared the radioactivity of the proteins as the T/B ratio. The times required to reach a value of 1 and 5, which would reflect the efficiency of accumulation and retention in the tumor, respectively, were calculated (Table 2). NCS never reached a T/B of 1 or 5. Ovomucoid reached efficiently to a T/B of 1 within 1 h but not to 5. Smancs reached a T/B of 1 at 3.2 h and 5 at 19 h, whereas albumin and IgG took a longer time (Table 2).

A study of the distribution of these proteins was also carried out. Smancs showed a predominant accumulation in the liver, spleen, and tumor. The apparent increase in the T/B ratio after 24 h was largely a result of the decrease in the plasma concentration, while the intratumor concentration as well as that of the liver and spleen remained unchanged (Table 3). A similar tendency was found for albumin and IgG. However, the highest values for IgG were in the tumor compared with the other tissues and organs as the time progressed (Table 3; Fig. 1C).

Accumulation, Retention, and Recovery as Revealed with Evans Blue-Albumin Complex. Fig. 2, A to D, illustrates tumor distribution and accumulation of various proteins in tumor-bearing mice. Plasma clearance of various proteins with molecular weights ranging from 12,000 to 160,000 during short and long time periods is shown in A and B, respectively. Their intratumor concentration is shown in C: O, NCS (M, 12,000); ●, smancs (M, 16,000); ▲, ovomucoid (M, 29,000); □, BSA (M, 69,000); ■, mouse serum albumin; △, mouse IgG (M, 160,000). Radioactive proteins were injected i.v. at time zero. Values are based on radioactivity. See text for detail.
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Table 1. Plasma clearance time of various \(^{111}\text{Cr}-\text{labeled proteins in tumor-bearing mice quantified by radioactivity (cpm) by gamma counter (see text). Values are the average of two experiments.}

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size (M, (10^{-3}))</th>
<th>(t_h) (min)(^a)</th>
<th>(t_{1/2}) (h)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neocarzinostatin</td>
<td>12</td>
<td>2</td>
<td>0.28</td>
</tr>
<tr>
<td>Smans</td>
<td>17</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>Ovomucoid, chicken</td>
<td>29</td>
<td>6</td>
<td>0.47</td>
</tr>
<tr>
<td>Albumin, bovine</td>
<td>68</td>
<td>60</td>
<td>32.5</td>
</tr>
<tr>
<td>Albumin, mouse</td>
<td>68</td>
<td>180</td>
<td>35</td>
</tr>
<tr>
<td>IgG, mouse</td>
<td>150</td>
<td>72</td>
<td>33</td>
</tr>
</tbody>
</table>

\(^a\) Time required to reach to half-concentration of time zero by interpolation. 
\(^b\) Time required to reach to 10% of the concentration of time zero.

Table 2. Intratumor accumulation of various \(^{111}\text{Cr}-\text{labeled proteins as revealed by T/B ratio quantified by radioactivity.}

<table>
<thead>
<tr>
<th>Protein administered</th>
<th>Molecular wt ((10^{-3}))</th>
<th>Time (h) to reach T/B ratio of 1</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neocarzinostatin</td>
<td>12</td>
<td>Not attained(^*)</td>
<td></td>
</tr>
<tr>
<td>Smans</td>
<td>16</td>
<td>3.2</td>
<td>19</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>29</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Albumin, bovine</td>
<td>68</td>
<td>9.3</td>
<td>56.7</td>
</tr>
<tr>
<td>Albumin, mouse</td>
<td>68</td>
<td>15.7</td>
<td>683</td>
</tr>
<tr>
<td>IgG, mouse</td>
<td>150</td>
<td>15.3</td>
<td>72</td>
</tr>
</tbody>
</table>

\(^*\) Maximum T/B ratio was 0.8 at 10 min after i.v. injection.
\(^b\) Those values of more than 1% of injected dose in the blood concentration were calculated, but others less than 1% were omitted from calculation as marked.

tissue after i.v. injection of Evans blue; the tumor is blue compared with the normal tissue in the background. Quantification of Evans blue at different times in plasma, tumor tissue, and normal tissue (skin and muscle) is illustrated in Fig. 2E, which shows a gradual increase in the intratumor concentration. This concentration became much higher than that of plasma 12 h after injection, while plasma concentration progressively decreased. The high intratumor concentration was retained over a prolonged period. This retention was confirmed by the following experiment.

Evans blue-albumin complex was injected into the solid tumor, and disappearance of the complex from the tumor tissue was evaluated visually and quantitatively. Fig. 3, A and B, illustrates that the albumin-dye complex was retained in the tumor for a long period once the macromolecules reached the tumor tissue. On the contrary, rapid clearance was demonstrated in the normal tissue (Fig. 3, C and D). Quantification confirmed a very low clearance rate from the tumor but a high rate for normal skin (Fig. 3E).

DISCUSSION

It is now well known that tumor cells secrete angiogenesis factors (angiogenin) (12–15) and that tumor angiogenesis becomes visible when solid tumor foci are larger than 2 to 3 mm (12, 13), and when extensive neovascularization (hypervascularization) develops. This massively developed tumor vasculature can be routinely seen by angiography (16) or other X-ray systems in animal and human solid tumors. Furthermore, many solid tumors with a diameter of several millimeters or more can be located by radioscintigraphy using radioemitting gallium or other metals (17). We believe that these data are interrelated in the present context, as discussed below. However, there is no general rule or mechanism known to explain these facts, or applications to cancer chemotherapy or diagnosis based on these facts.

In this paper we have reported that many proteins tend to accumulate in the cancer tissues much more than in circulating blood (Figs. 1C and 2; Tables 2 and 3). The Evans blue-albumin complex was injected into the solid tumor, and disappearance of the complex from the tumor tissue was evaluated visually and quantitatively. Fig. 3, A and B, illustrates that the albumin-dye complex was retained in the tumor for a long period once the macromolecules reached the tumor tissue. On the contrary, rapid clearance was demonstrated in the normal tissue (Fig. 3, C and D). Quantification confirmed a very low clearance rate from the tumor but a high rate for normal skin (Fig. 3E).
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Fig. 2. Clearance of Evans blue-albumin complex from blood plasma and its accumulation in tumor tissue and normal skin in tumor-bearing mice. Tumor S-180 was injected into the skin. A to D provide a macroscopic picture of the tumor in the skin taken at 0, 6, 24, and 72 h, respectively, after i.v. injection of Evans blue. T (indicated by an arrow) is the site of tumor inoculation; the dark area indicates the tumor, which became progressively blue after injection. E shows quantification of the concentration of the Evans blue-albumin complex at different times for plasma (C), normal skin (D), and tumor (E). See text for details. Values in the muscle were very similar to the skin.

complex, although its dye is not covalently bound, clearly showed that progressive accumulation occurs in the tumor (Fig. 2). Furthermore, recovery via the blood capillary or the lymphatic system is far slower than that from the normal tissue (Fig. 3). Some organs, such as the liver and spleen, which have a well-developed reticuloendothelial system, may have taken up many of these proteins, particularly those of heterologous origin including DTPA-Lys-smanos, which shows less biocompatibil-
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Fig. 3. Clearance of Evans blue-albumin complex from tumor (A, B) and from normal skin (C, D). Dye-albumin complex was injected into the tumor (A, B) or into the normal skin (C, D), which were removed for macroscopic observation at 1 h (A, C) and 72 h (B, D) after in situ injection. The arrow in D indicates the site of injection into the normal skin. The retention of the dye-albumin complex and its clearance were quantified (E). □, ■, and □, the recovered concentration of dye complex in the tumor, the skin at the site of injection, and the normal skin (no injection), respectively, at the indicated times. Quantification was done after extraction by a spectrophotometer. See text for details.

ity (Fig. 1; Tables 1 and 3). In addition, ovomucoid (M, 29,000), with a high content of sialic acid and other carbohydrates (35%, wt/wt), may be rapidly recovered by a different mechanism, such as a galactose or mannose receptor, during circulation in the liver.

The semisynthetic protein drug smancs was shown previously to have an in vivo half-life of biological activity in the blood that was 10 times longer than that of the parental NCS. This tailor-made protein (M, 16,000) is shown here to have a similarly prolonged plasma half-life in vivo (Table 1). It is more interesting that it accumulated in the tumor much more than did the parent compound: a T/B ratio of 1 or 5 was never
observed for NCS, whereas smancs attained these values in 3.2 or 19 h, respectively (Table 2). When one compares the time to reach a T/B value of 5 and the respective molecular weights, smancs accumulated more rapidly in the tumor than did albumin or IgG. The latter two, with higher molecular weights of about 69,000 and 160,000, required a longer time, which indicated less efficient vascular leakage to the tumor tissue because of their large size. On the other hand, a relatively well-maintained plasma concentration for a long period would eventually result in a high T/B value at 72 h (see Table 3, albumin). These results may indicate that macromolecules of a certain molecular range (M, 15,000 to 70,000) and with certain properties can effectively accumulate in a solid tumor.

Four vascular characteristics support this: (a) hypervascularization; (b) enhanced vascular permeability as elicited by a factor (18–20); (c) little recovery of macromolecules via the blood vessels; and (d) little recovery from the lymphatic system. The last is attributed to poor development of the lymphatics in the tumor tissue, which was previously demonstrated by using the lipid lymphographic agent Lipiodol (21). If one injects small molecules, which can traverse barriers freely, into the tumor or the normal tissue, they will immediately disappear from the tumor or the normal tissue by diffusion, primarily into blood capillaries. Macromolecules contrast sharply with the small molecules, as shown in Figs. 2 and 3. Normal tissue (represented by the skin), however, carried the Evans blue-albumin complex rapidly compared with the tumor tissue (Fig. 3). Thus, both the anatomical difference and the molecular size and property of the drug appear to be critical for selective tumor targeting.

Macromolecules and lipids in the interstitial space are known to be recovered via lymphatics in normal tissues (22). The present findings demonstrated that a clear contrast exists in the tumor tissue which has a highly enhanced leakiness and no lymphatic clearance.

We previously used smancs dissolved in lipid contrast medium and showed a marked retention of lipid (a T/B of 1200) in the tumor when we administered smancs/Lipiodol via a tumor-feeding artery (21). This, as a consequence, resulted in unprecedented clinical benefit with few side effects for patients with hepatoma and lung cancer (6, 7, 23). Furthermore, the method has diagnostic value: use of various X-ray systems permits a highly sensitive diagnosis, determination of subsequent dose regimen, and long-term follow-up (24). The basic mechanism operating here with lipid is again attributed to hypervascularization, enhanced permeability, little recovery from lymphatics, and perhaps an architectural uniqueness at the neovascularature level where more lipid adhered on the vascular endothelium than in normal counterpart.

All these data can be used to explain the general mechanism for the tumoritropism of smancs and γ-emitting metals used in radioscintigraphy for the diagnosis of solid tumors. Radioactive gallium or other γ-emitting metal citrates injected into the general circulation are bound to serum transferrin (M₈ 90,000) (25), the radioactive transferrin tends to accumulate more in the tumor. The highly tumoritropic properties of macromolecular anticancer agents as seen with smancs suggest a direction for the future development of new anticancer agents based on this prototype drug.

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