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Disposition Characteristics of Macromolecules in the Perfused Tissue-isolated Tumor Preparation

Hirofumi Imoto, Yumi Sakamura, Kazuhiro Ohkouchi, Ryo Atsumi, Yoshinobu Takakura, Hitoshi Sezaki, and Mitsuru Hashida

ABSTRACT

Disposition characteristics of model macromolecules with different physicochemical characteristics and macromolecular prodrugs of mitomycin C, namely mitomycin C-dextran conjugates, were studied in tissue-isolated tumor preparations of Walker 256 carcinoma with the use of a single-pass vascular perfusion technique. In constant infusion experiments, all radiolabeled macromolecules accumulated in the tumor tissue, but the degree and pattern of distribution greatly varied, depending on their electric charges. Positively charged macromolecules were markedly accumulated compared with those that were neutral or negatively charged. In addition, their concentrations were significantly higher in viable than in necrotic regions, while neutral and negative compounds were distributed in necrotic rather than in viable regions. Pharmacokinetic analysis of tissue concentration-time courses of positively charged diethylaminoethyl and neutral dextrans showed that their movement occurred by convective fluid flow, and that high tissue accumulation of positively charged macromolecules could be explained by strong binding due to electrostatic interaction. For neutral and anionic macromolecules with negligible affinity to the tissue, it was suggested that the final concentration gradient between the viable and necrotic regions was decided by their tissue fluid content. Thus, the present study revealed the basic disposition characteristics of macromolecules in tumor tissue relative to their physicochemical properties.

INTRODUCTION

In cancer chemotherapy, it is important to deliver antitumor drugs selectively to the tumor with minimum exposure of other normal tissues, and various drug carrier systems have been developed for achieving this. Conjugation of antitumor drugs to macromolecules seems to be one of the most promising approaches, since diverse physicochemical properties and functions of macromolecules can be utilized for control of drug disposition (1-3).

In a series of investigations, we developed macromolecular prodrugs of MMC, which are MMC-dextran conjugates having cationic and anionic charges as well as a variety of molecular weights, and examined their physicochemical, pharmacokinetic, and pharmacological characteristics (4-11). The disposition and subsequent pharmacological effects can be controlled by selecting specific physicochemical properties. Tumor targeting in vivo was achieved by systemic injection of MMCDan in mice (10). These results were explained by the general relationships between the physicochemical characteristics and in vivo behavior, including tumor localization, which we established for various model macromolecules (12). We further developed a monoclonal antibody-MMC conjugate by using MMCDan as an intermediate. Immunotargeting was achieved, based upon these findings (13).

On the other hand, we established an experimental system for the evaluation of the drug disposition in the tumor at the organ level by using a tissue-isolated tumor preparation and moment analysis (14). This system was applied to the pharmacokinetic analysis of the disposition of antitumor drugs, including MMC and its lipophilic prodrugs. The present study aimed to clarify the disposition characteristics of model macromolecules and MMCD in tumor tissue with the use of this system and to construct a strategy of tumor targeting with a macromolecular drug carrier system.

MATERIALS AND METHODS

Chemicals. MMC was kindly supplied by Kyowa Hakko Kogyo Co., Tokyo, Japan. Dextrans were purchased from Pharmacia Fine Chemicals Co., Uppsala, Sweden, and had average molecular weights of about 10,000 (T-10) and 70,000 (T-70). BSA (Fraction V) was obtained from Armour Pharmaceutical Co., United Kingdom. The sources of radiochemicals were as follows: γ-amino[U-14C]butyric acid (74 MBq/mg); [U-14C]sucrose (0.11 MBq/mmol); and [methoxy-14C]ulin (0.35 MBq/g), New England Nuclear, Boston, MA; potassium [14C]cyanide (29.5 MBq), Amersham Japan, Tokyo; indium chloride (111 In Cl3), Nihon Medipysics, Takarazuka, Japan. All other chemicals were of reagent grade, obtained commercially.

Preparation of Model Macromolecules. DEAED(T-70) and CMD(T-70) were synthesized by reacting (diethylamino)ethyl chloride hydrochloride and monochloroacetic acid with dextran(T-70) in alkaline solutions, respectively (12). Radiolabeled DEAED(T-70) and CMD(T-70) were synthesized by using [carboxy-14C]dextran(T-70). Both [carboxy-14C]dextran(T-10) and [carboxy-14C]dextran(T-70) were prepared by reacting potassium [14C]cyanide with dextran according to the method of Isbell et al. (15), as reported previously (12).

cBSA was synthesized by coupling hexamethylenediamine to BSA with a carbodiimide-catalyzed reaction according to the published method (12). BSA and cBSA were labeled with 111 InCl3, using diethyleneetriaminepentacetic acid anhydride (Dojindo Labs, Kumamoto, Japan), according to the method of Hnatovich et al. (16).

Preparation of MMCD. MMCDan and MMCDcat were synthesized as reported previously (4, 9). MMC was radiolabeled by coupling γ-[14C]aminobutyric acid to dextran activated by cyanogen bromide, together with ε-aminocapric acid ([14C]MMCDan) or to spacer-introduced dextran together with MMC by a carbodiimide-catalyzed reaction ([14C]cMMCDan) (8). The radiolabel in MMCDs was stable and had behavior consistent with that of a dextran backbone.

Preparation of Tissue-isolated Tumors. According to the method of Gullino and Grantham (17, 18), rat ovarian tissue-isolated tumor was...
prepared as reported previously (14). In brief, three small blocks of Walker 256 carcinoma were inoculated into the adipose tissue around the ovary, enclosed in a Sealon film envelope (Fuji Film, Tokyo, Japan), then placed in the s.c. pouch of the abdomen. The tumor was grown in a "tissue-isolated" form and the film was changed after 1 week. About 2 weeks after tumor inoculation, tumor weighing 4-8 g was used for perfusion.

Perfusion Experiment. The tissue-isolated tumor was perfused according to the previous method (14) with slight modifications. Fig. 1 shows a diagram of the perfusion experiment. At first, all blood vessels supplying nontumorous tissues around the tumor (left renal artery and vein, left adrenal artery and vein, and aorta) were ligated. Immediately after ligation of the aorta, vinyl tubing (inside diameter, 0.5 mm; outside diameter, 0.9 mm; Dural Plastics & Engineering, Dural, Australia) was inserted into the aorta and the perfusate was infused at a rate of 0.8 ml/min. Vinyl tubing (inside diameter, 0.8 mm; outside diameter, 1.2 mm) was inserted into the tumor vein for outflow sampling. Finally, the tumor was transferred onto a plate in the experimental system. The tumor mass was perfused with Tyrode's solution (a mixture of 137 mM NaCl, 2.68 mM KCl, 1.80 mM CaCl₂, 11.9 mM NaHCO₃, 0.362 mM NaH₂PO₄, 0.492 mM MgCl₂, and 5.55 mM d-glucose) containing BSA at a concentration of 4.7% (w/v). The same medium was used for drug administration. The perfusate was gassed with 95% O₂-5% CO₂ and maintained at 37°C. Viability of the perfused tumor was confirmed by measurement of lactic acid production. The rate of production during a 3-h perfusion experiment was within the range of 1.3-2.2 mmol/h/100 g, which was comparable to that reported by Gullino and Grantham (17). In addition, the viable region subjected to s.c. inoculation to normal rats after a 3-h perfusion showed almost the same growth rate as freshly prepared tumor, suggesting its viability.

Indicator Dilution Experiment. Drug solution (0.1 ml) was introduced from the arterial side of the tumor by using a six-position valve injector as a pulse function. The concentrations of the injection solutions were 1 mg/ml for MMC and 100 μg/ml for [14C]MMCDcat(T-70). EB/BSA was used as a VRS and was applied prior to the drug injection to obtain basic physiological information for each tumor preparation. The drug solution was injected into the same tumor in the second run. The outflow was collected into previously tared tubes at appropriate time intervals (at first 5 s, subsequently 10 to 15 s). The sample volume was derived to describe the concentration change in the viable (v) and necrotic (n) components. The degree of the interaction is expressed in terms of the osmotic reflection coefficient for the macromolecule is assumed to be an independent convection flow with rates of Jv and Jn (ml/min/g). The macromolecule moves through the interstitial space, and finally oozes from the tumor space at a fixed concentration (Co), permeates across the vascular wall, and enters the intravascular space. The radioactivity of the 111In-BSA was directly determined by a NaI well-type scintillator (ARC-500, Aloka, Tokyo, Japan).

Pharmacokinetic Analysis of Indicator Dilution Experiment. Moment analysis was used to analyze dilution curves and the statistical moment parameters are calculated as follows (19);

\[ AUC = \int_0^\infty C \, dt \]  
\[ MTT = \int_0^\infty t \cdot C \, dt / AUC \]

where \( t \) is time and C is the concentration of a test substance normalized by injection dose with a dimension of percentage of dose/ml. AUC and MTT are the area under the concentration-time curve and the mean transit time, respectively. The disposition parameters representing the recovery ratio (F) and intrinsic clearance (CLint) are calculated from the moment parameters based on well-stirred conditions as follows (19);

\[ F = \frac{AUC}{AUC_{inj}} \]

\[ CL_{int} = \frac{Q(1 - F)}{F} \]

where \( Q \) and \( AUC_{inj} \) are mean inflow rate and AUC of VRS.

Constant Infusion Experiment. The drug-containing perfusate (10 μg/ml) was infused at a rate of 0.8 ml/min into the tumor preparation for up to 180 min. At the end of infusion, drug-free perfusate was infused for 5 min to remove drugs remaining in the intravascular space. During these procedures, the venous outflow was collected into tared counting vials at appropriate time intervals. The sum of the outflow perfusate and exudate from the tumor was almost the same as the inflow rate and no significant weight gain of the tumor during the perfusion was detected. After washing the intravascular space, the tissue was excised and carefully divided into viable and necrotic regions based upon macroscopic observation. The viable region is the whitish peripheral part of the tumor while the reddish and flabby necrotic region was usually found in the center. Histological observation confirmed that both samples essentially represented viable and necrotic tissues. These samples were minced into small pieces. To measure the level of 14C-labeled macromolecules, tissue sample (approximately 0.1 g) was placed into a counting vial and 0.7 ml of Soluene-350 (Packard, Downers Grove, IL) was added. The mixture was heated overnight at 50°C, cooled to room temperature, and 0.18 ml of 2 n hydrogen chloride was added. The radioactivity in the tissue sample and outflow were determined in a liquid scintillation counter after adding 5 ml of scintillation fluid. The radioactivity of the 111In-BSA was directly determined by a NaI well-type scintillator (ARC-500, Aloka, Tokyo, Japan).

Pharmacokinetic Analysis of Constant Infusion Experiment. Time courses of accumulation of [14C]dextran(T-70) and [14C]DEAE-D(T-70) in the tumor tissue were analyzed based upon a parallel flow model (Fig. 2). Here, a test compound, which is initially found in the vascular space at a fixed concentration (Co), permeates across the vascular wall, moves through the interstitial space, and finally oozes from the tumor tissue along with the convective fluid flow. In this model, the macromolecule is directly distributed to the viable and necrotic regions by an independent convection flow with rates of Jv and Jn (ml/min/g). The osmotic reflection coefficient for the macromolecule is assumed to be zero throughout all processes. The macromolecule passes through the interstitial water space with a volume of V and interacts with tissue components. The degree of the interaction is expressed in terms of the distribution volume (K). Based upon the model, the following equations were derived to describe the concentration change in the viable (v) and necrotic (n) regions.

\[ \frac{dC_v}{dt} = (Vv + Kn) \frac{dC_v'}{dt} = Jv(Co - C_v') \]  
\[ \frac{dC_n}{dt} = (Vn + Kn) \frac{dC_n'}{dt} = Jn(Co - C_n') \]

where \( C_v \) and \( C_n \) are concentrations in the viable and necrotic regions.
DISPOSITION OF MACROMOLECULES IN ISOLATED TUMOR

Constant Infusion

Co

Kv Cv' Vv

Viable Region

Kn Cn'

Necrotic Region

V

Jv

Jv

[Cv + Kn][1 - \exp(-\frac{Jv}{Vv + Kv}t)]

[Cn + Kn][1 - \exp(-\frac{Jn}{Vn + Kn}t)]

The concentration profiles were then fitted to Equations G and H by using the nonlinear regression program MULTI (20), to estimate the pharmacokinetic parameters.

Vascular and interstitial water space volumes of the tissue-isolated tumor preparation were determined by using [14C]dextran(T-70) and [14C]sucrose as intravascular and extracellular markers, respectively. The tumor preparation was infused with perfusate containing [14C]dextran for 5 min or [14C]sucrose for 60 min, and the radioactivity in each region of the tumor and in the outflow samples was measured without washing with drug-free perfusate. Vascular and interstitial water space volumes were calculated by using the following equations

Vascular space (ml/g) = \frac{[14C]Dextran in tissue (dpm/g)}{[14C]Dextran in outflow (dpm/ml)}

Extracellular space (ml/g) = \frac{[14C]Sucrose in tissue (dpm/g)}{[14C]Sucrose in outflow (dpm/ml)}

Interstitial space (ml/g) = Extracellular space − vascular space.

RESULTS

Indicator Dilution Experiment. Fig. 3 shows a typical dilution curve of [14C]MMC(T-70) together with VRS (EB/BSA). EB/BSA; O; [14C]MMCD(T-70). Each compound was injected separately into the same tumor.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>AUC (%)</th>
<th>MTT (s)</th>
<th>F</th>
<th>CL_int (ml/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB/BSA</td>
<td>7774.2</td>
<td>44.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>MMC</td>
<td>6850.3</td>
<td>70.1</td>
<td>0.885</td>
<td>0.0114</td>
</tr>
<tr>
<td>[14C]MMCD(T-70)</td>
<td>7528.7</td>
<td>35.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>[14C]MMCD(T-70)</td>
<td>7538.4</td>
<td>32.4</td>
<td>1.00</td>
<td>0</td>
</tr>
</tbody>
</table>

Each entry is the mean of at least three experiments.
tissue with time, and cationic [14C]DEAED(T-70) was accumulated at higher levels.

Prior to the analysis, the vascular volumes and the interstitial water space volumes of viable and necrotic regions were determined. The vascular volume in the viable region [0.0372 ± 0.0200 (SD) (N = 3)] was slightly larger than that in the necrotic region [0.0282 ± 0.0178 (N = 3)] (no significant difference), whereas the extracellular space volume determined as equilibrated sucrose space at 60 min in the necrotic region [0.281 ± 0.048 (N = 3)], was significantly larger (P < 0.05) than that in the viable region [0.194 ± 0.019 (N = 3)]. Intersitial water space volumes calculated as the difference between these volumes are shown in Table 2 and are used for the pharmacokinetic analysis.

Table 2 summarizes the parameters estimated by data fitting the tumor concentration profiles based on the pharmacokinetic model. The total distribution volumes of [14C]dextran(T-70) for viable and necrotic regions were 71.7 and 57.7% of the interstitial water space volumes, respectively. On the other hand, those of [14C]DEAED(T-70) were 9.8- and 7.0-fold larger than the interstitial water space volumes, respectively. The convection flow rates were estimated to be 0.011 and 0.005 ml/min/g in the viable and necrotic regions, respectively.

In Fig. 6, simulation curves generated by using the parameters in Table 2 are also shown and relatively good agreement with experimental data was obtained.

DISCUSSION

Generally, tumor tissues are characterized by enhanced vascular permeability, high interstitial diffusion coefficients of macromolecules, and lack of a functional lymphatic system (21, 22). These characteristics seem to act as favorable factors for passive transport of blood-oriented large molecules to the tumor, and several investigators have reported the accumulation of macromolecules in tumors after i.v. injection (12, 23–25). Thus, tumor targeting using macromolecular drug carriers is rationalized by the physiological and anatomical characteristics of tumor tissues. On the other hand, it has also been suggested that elevated interstitial pressure in tumor tissues restricts the extravasation and interstitial transport of macromolecules and leads to their heterogeneous distribution (26–28). For monoclonal antibodies, furthermore, antigen-antibody interaction was shown to result in retarded penetration and nonuniform distribution in the tumor (29–32). However, detailed information on the actual disposition of macromolecules in the tumor tissue remains to be elucidated. We performed perfusion experiments by using tissue-isolated tumor preparations to assess the effect of physicochemical properties of macromolecules on their disposition in the tumor.

Table 2 Interstitial space volumes and pharmacokinetic parameters for [14C]dextran(T-70) and [14C]DEAED(T-70) in the tissue-isolated tumor preparation after constant infusion. Numbers in parentheses are percentages to the respective interstitial space volume. The abbreviations are: J, convection flow rate; V + X, total distribution volume.

<table>
<thead>
<tr>
<th></th>
<th>Viable region</th>
<th>Necrotic region</th>
</tr>
</thead>
<tbody>
<tr>
<td>V (ml/g)</td>
<td>0.157</td>
<td>0.253</td>
</tr>
<tr>
<td>J (ml/min/g)</td>
<td>0.0112</td>
<td>0.0050</td>
</tr>
<tr>
<td>V + X (ml/g)</td>
<td>1.530 (975)</td>
<td>1.73 (701)</td>
</tr>
<tr>
<td>[14C]dextran(T-70)</td>
<td>0.113 (71.7)</td>
<td>0.146 (57.7)</td>
</tr>
<tr>
<td>[14C]DEAED(T-70)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
We at first performed indicator dilution experiments, with which we previously evaluated the disposition of low molecular weight antitumor drugs (14), whose extraction by the tumor occurred depending on their lipophilicity as shown for MMC (Table 1). However, the macromolecules tested in this study had similar dilution curves to that of VRS (Fig. 2), indicating similar behavior to VRS at the extraction step. In addition, only a slight decrease in the perfusate concentration of cationic macromolecules was observed during single passage under the steady-state condition in the constant infusion (Fig. 4). These results are contrasted to our previous findings in the liver, where cationic macromolecules were extensively taken up through discontinuous endothelium, which allows free contact of macromolecules with the surface of the parenchymal cells (33). The present facts indicated that architecture of vascular wall is essentially tight in the tumor, unlike the liver, although tumors should have permeable vasculature. The binding of macromolecules to capillary endothelium was also suggested to be negligible.

In the tumor tissue, convection is considered to be the dominant mechanism of macromolecular transport (34), although it is a relatively slow process. Therefore, we measured the accumulation of macromolecules in the constant infusion system to elucidate the effects of their physicochemical properties (Figs. 5 and 6). Positively charged macromolecules were markedly accumulated in the tumor, in particular in the viable region, which was a well-perfused part. In contrast, those negatively charged or neutral macromolecules were accumulated at lower levels, and were characterized by being at a relatively higher level in the necrotic region. The molecular weight had little effect in the tested range (M, 5,000–70,000).

From the data in Fig. 5, the apparent tissue uptake clearance can be calculated by dividing the tissue concentration by the AUC of the compound in the perfusate (11). The values for cationic [14C]DEAED(T-70), which was markedly accumulated in the tumor, were 6.2 and 3.8 μl/min/g for the viable and necrotic regions, respectively. Then, recovery ratio (F) of a macromolecule in the outflowed perfusate during the constant infusion is calculated as follows

\[ F = \frac{\text{Inflow rate} - \text{tissue uptake clearance}}{\text{Inflow rate}}. \]

Assuming the tumor weight to be 6 g and weight ratio of viable and necrotic regions to be 5:1, F is calculated to be 0.957 for [14C]DEAED(T-70). At first glance, this value appears to be inconsistent with the results described in Fig. 4, where the outflow concentration was almost equal to inflow concentration. Furthermore, indicator dilution experiments showed no significant difference in uptake of [14C]MMCDcar(T-70) and VRS. These discrepancies can be explained by the hypothesis that the macromolecule is conveyed into the interstitial space by convective flow. The concentration in the perfusate would not change before and after passage through the tumor and the macromolecule would have the same dilution curves as that of VRS.

The tissue uptake clearance is an apparent index offering only phenomenological information equivalent to that in Fig. 5 and no information about the accumulation mechanism. Therefore, we analyzed the accumulation process of macromolecules with a pharmacokinetic model (Fig. 2), assuming convective transport and tissue binding.

We determined the vascular and interstitial water volumes of viable and necrotic regions for comparison with the apparent distribution volumes of macromolecules. The reported values of the vascular space in Walker 256 carcinoma varied to some extent, depending on the marker substance (35). Song and Levitt (23) reported that the average vascular volume of s.c. implanted Walker 256 carcinoma (0.01–4 g tumor) was 0.8%, using 51Cr-labeled erythrocytes. Guillino and Grantham (36) determined the vascular volume of tissue-isolated Walker 256 carcinoma (3.5–13 g tumor) with dextran 500 (M, 37,500) to be 10.8%. In a previous study, we reported the vascular volume of tissue-isolated Walker 256 carcinoma (1–20 g tumor; mean, 8.71 g) to be 7.0% using EB/BSA (17). The vascular volumes determined in the present study with dextran(T-70) (3.7 and 2.8% for viable and necrotic regions, respectively) were within these values. However, leakage of dextran, especially in the necrotic region, or contamination between these regions, might be included in these values.

On the other hand, the interstitial water spaces determined in the present study (15.7 and 25.3% for viable and necrotic regions, respectively) were smaller than that reported by Guillino et al. (37) (38.7%) obtained by direct measurement of 24Na in tissue fluid of Walker 256 carcinoma (4.7–10.4 g). Although some differences were observed between these studies, we used our values since those for viable and necrotic regions were separately available.

Analysis of the accumulation pattern of cationic [14C]-DEAED(T-70) revealed that the apparent distribution volumes in the viable and necrotic regions were 7 to 10 times higher than those of the interstitial water volumes (Table 2), indicating strong binding to the tissue. On the other hand, the apparent distribution volumes for neutral [14C]dextran(T-70) were smaller than the interstitial volumes.

Our previous studies in vitro demonstrated that MMCDcat and other cationic macromolecules bound with leukemia cells (8) and solid tumor cell layers grown on microporous membranes (38) due to electrostatic interactions. The same mechanism can account for the interaction of positively charged macromolecules with the tumor cell surface and components in the interstitium. In contrast, negatively charged or neutral macromolecules would only be distributed in the interstitial fluid, without any tissue interaction.

The sum of the convective flow (0.061 ml/min) calculated in the viable and necrotic regions with a weight ratio of 5:1 approximately corresponds to 7.7% of perfusion flow rate for 6 g of tumor. In another study, we determined the rate of fluid loss in isolated tumors with a mean weight of 6.36 g to be 16.6% from the outflow recovery. Butler et al. (39) reported that extravascular fluid loss was 10.2% of the perfused plasma volume in Walker 256 carcinoma (mean, 3.8 g tumors). These values essentially correspond to our estimated convective flows, suggesting the reliability of considering convective flow as a driving force of macromolecular movement.

The heterogeneous distribution in the tumor shown for cationic macromolecules in this study may be an obstacle to tumor targeting with macromolecular carrier systems including monoclonal antibodies (26). A possible approach to overcome this problem may be the use of the prodrug concept; i.e., regeneration of the parent drug in the interstitial space. The regenerated free drug can readily distribute over the whole tumor mass by diffusion, even if the movement of the prodrug (drug-macromolecule conjugate) itself is prevented by strong binding or a
high interstitial pressure. MMCD was shown to liberate free MMC by simple chemical hydrolysis (5), and it is implied that local constant infusion of MMCD_{cat} might be effective for intensive exposure of the tumor tissue to MMC irrespective of its limited movement. However, further studies are required to confirm whether regenerated MMC actually reaches into the tumor cell itself.

Thus, the present study demonstrates the basic disposition characteristics of macromolecules in a tissue-isolated tumor preparation and the importance of their electric charge. These findings will be useful for the development of macromolecular prodrugs aiming at targeted cancer chemotherapy.

REFERENCES