Lymphatic Transport of Recombinant Human Tumor Necrosis Factor in Rats

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The lymphatic transport of recombinant human tumor necrosis factor (rHu-TNF) was investigated after its administration via various routes in anesthetized rats. After intravenous (i.v.) administration, the level of rHu-TNF in the lymph reached a peak at 2 h, and thereafter decreased exponentially in a pattern similar to that of the elimination from plasma. Intramuscular (i.m.) and subcutaneous (s.c.) administrations resulted in low levels of rHu-TNF both in the plasma and in the lymph. On the other hand, intraperitoneal (i.p.), intra-stomach wall (s.w.) and intra-gut wall (g.w.) administrations gave high lymph levels of rHu-TNF. After the s.w., g.w. and i.p. administrations, the ratios of rHu-TNF recovered from the thoracic duct to the cumulative amount estimated from the rHu-TNF concentration in plasma were about 25, 32 and 8 times higher than in the case of i.v. administration, respectively. These results suggest that the routes of local administration of rHu-TNF such as s.w., g.w. and i.p. may be highly effective for the treatment of lymphatic metastasis of cancer.

Keywords — recombinant human tumor necrosis factor; plasma concentration; lymph concentration; anticancer agent; lymphatic metastasis

Introduction

Tumor necrosis factor (TNF) was discovered originally in sera of mice injected with bacillus Calmette-Guerin (BCG) and subsequently challenged with a bacterial lipopolysaccharide (LPS). Recently, human TNF complementary deoxyribonucleic acid (cDNA) was cloned in our laboratories, and mature human TNF polypeptide was produced in Escherichia coli. The recombinant human TNF (rHu-TNF) has also been observed to exhibit the antitumor effect on various murine tumors transplanted in syngeneic mice and on human tumors heterotransplanted in nude mice, and also to prevent the metastasis of some kinds of transplanted murine tumors. When we consider the metastasis of tumor cells, on the studies pharmacokinetic behavior of rHu-TNF in lymph are of great significance because the lymphatic route is a major route of cancer metastasis. The pharmacokinetics of rHu-TNF in plasma after administration via various routes has been widely studied in animals and cancer patients but little information is available concerning its pharmacokinetic behavior in lymph. In the present study, therefore, we examined using a thoracic duct-cannulated rat model the lymphatic transport of rHu-TNF following its administration via various routes, i.e., intravenous (i.v.), intramuscular (i.m.), intraperitoneal (i.p.), subcutaneous (s.c.) intra-stomach wall (s.w.) and intra-gut wall (g.w.).

Materials and Methods

rHu-TNF — rHu-TNF was produced through expression of human TNF cDNA in Escherichia coli in our laboratories. rHu-TNF consisted of 155 amino acid residues and had a relative molecular weight of 45000 ± 5000 daltons (Da) by gel filtration and 17000 ± 1000 Da by Sodium dodecyl sulfate (SDS)-PAGE. The isoelectric point was 5.9 ± 0.3. The specific activity was 2.9 × 10⁶ units/mg by cytotoxicity assay using L-M cells in vitro.

Enzyme Immunoassay of rHu-TNF (EIA Assay) — The plasma and lymph concentrations of rHu-TNF were determined by EIA assay as reported by Sunahara et al. The procedure in brief is as follows. The plasma or lymph samples (0.1 ml) were added to tubes containing 0.1 ml of anti-rHu-TNF antibodies (1st antibody). The tubes were stirred on a mixer and incubated at 37 °C for 1 h. After addition of 0.2 ml of enzyme-labelled rHu-TNF solu-
tion, the tubes were incubated at 37 °C for 30 min and mixed with 0.2 ml of insolubilized 2nd antibody suspension. Then the mixture were kept at ambient temperature for 15 min, mixed with 4 ml of 0.9% NaCl solution and centrifuged at 1500 g for 10 min. The supernatant was aspirated and the precipitate was stirred with 0.5 ml of phosphate buffer solution (pH 7) containing 0.9% NaCl, 0.1% bovine serum albumin (BSA) and 0.1% NaN₃. After preincubation at 37 °C for 3 to 5 min, 0.1 ml of substrate solution was added and the reaction was allowed to proceed at 37 °C for 30 min. Then the reaction was stopped with 1.5 ml of the reaction terminator and the mixture was centrifuged at 1500 g for 10 min. The absorbance of the supernatant fluid was measured at 410 nm in a spectrophotometer to estimate the concentration of rHu-TNF in the samples by reference to the standard curve. All assays were made in duplicate.

Biological Assay of rHu-TNF (L-M Assay) — The cytotoxic activity of rHu-TNF in lymph was determined by means of a procedure reported by Nakano et al. The procedure in brief is as follows. The lymph samples were diluted with minimum essential medium (MEM; Flow Laboratories) containing 1% fetal bovine serum (Flow Laboratories). One-tenth milliliter of diluted solution was mixed with an equal volume of cell suspension (1 x 10⁵ cells/ml) of L-M cells (CCL 1.2, American Type Culture Collection). After 48 h of culture of L-M cells at 37 °C, the remaining viable cells were fixed with glutaraldehyde and stained with 0.05% methylene blue. The dye was extracted with 0.33N HCl and the absorbance at 665 nm was measured. The concentration of rHu-TNF at which 50% of L-M cells were killed after culture was defined as 1 U/ml.

Administration of rHu-TNF and Collection of Plasma and Lymph — Male Wistar rats weighing 280—300 g were anesthetized intraperitoneally with sodium pentobarbital and used for each experimental group. The rats were allowed free access to food and water before anesthesia with sodium pentobarbital. The preparation of rHu-TNF solution used in this study was diluted with phosphate-buffered saline containing 0.1% gelatin to desired concentrations just before administration. The i.v., i.m., s.c. and i.p. administrations of rHu-TNF were carried out by injecting it into a femoral vein, the center of the thigh muscles of the right leg, the back and the peritoneal cavity of rats, respectively. On the other hand, the s.w. and g.w. administrations were done by injecting the rHu-TNF into the wall of the stomach and large intestine, respectively. The dose of rHu-TNF was 3.3 x 10⁶ U/kg. Blood and lymph samples were taken from the jugular vein and the thoracic duct at designated times. The thoracic duct was cannulated using a heparin-filled flexible polyethylene catheter (i.d. 0.97 mm, o.d. 1.27 mm, Dural Plastics) as described by Bollman et al. The total volume of lymph collected and concentration of rHu-TNF in the lymph were determined at the end of each time interval. The concentration of rHu-TNF in plasma was also determined at the end of each time interval. The cumulative amount of rHu-TNF transferred into plasma after the administration was estimated by means of the following equation:

\[ A_T = V (C_T + K \int_0^T C \, dt) \]

where \( A_T \) is the cumulative amount of rHu-TNF transferred from the time of administration to time \( T \), \( V \) is the apparent volume of distribution,

![Fig. 1. Concentrations of rHu-TNF in Plasma (●) and Lymph (Continuous Line) after i.v. Administration to Anesthetized Rats](image)  
Each point is the mean ± S.E. of 5 rats.
Fig. 2. Concentrations of rHu-TNF in Plasma (●) and Lymph (Continuous Line) after s.w., g.w. and i.p. Administrations to Anesthetized Rats
Each point is the mean ± S.E. of 5 rats.

Fig. 3. Concentrations of rHu-TNF in Plasma (●) and Lymph (Continuous Line) after i.m. and s.c. Administrations to Anesthetized Rats
Each point is the mean ± S.E. of 4 rats.
$C_T$ is the plasma concentration at time $T$, $K$ is the elimination rate constant and the integral is the area under the plasma concentration-time curve between time zero and time $T$.

**Results**

In order to identify the administration route giving the most selective uptake of rHu-TNF into lymph, we examined the plasma and lymph levels of rHu-TNF after systemic and various local administrations.

When rHu-TNF was administered i.v., rHu-TNF disappeared rapidly from the plasma with half-life of 0.9 h just after administration. On the contrary, the lymph level of rHu-TNF increased gradually, reached a peak of 2110 U/ml at 2 h, and thereafter decreased exponentially in a pattern similar to that of the elimination from plasma (Fig. 1).

On the other hand, the lymph levels of rHu-TNF after the s.w., g.w. and i.p. administrations were significantly higher than that in plasma (Fig. 2). For example, the concentrations of rHu-TNF in lymph at 2 h after the g.w., s.w. and i.p. administrations were approximately 31, 25 and 8 times higher than that in plasma, respectively. The plasma and lymph levels of rHu-TNF after the i.m. and s.c. administrations are shown in Fig. 3. The plasma levels of rHu-TNF after the i.m. and s.c. administrations were rather low when compared with the levels after the i.v. administration. The levels of rHu-TNF in lymph after the i.m. administration were higher than that in plasma. In contrast to the i.m. administration, rHu-TNF in lymph after the s.c. administration was low relative to that in the plasma. Figure 4 shows the cumulative amount of rHu-TNF in lymph collected from the thoracic duct during the first 4 h after the administration. As shown in Fig. 4, the cumulative amounts of rHu-TNF in lymph after the s.w. and g.w. administrations were significantly higher than that in lymph after the i.v. administration. The ratio of rHu-TNF recovered from the thoracic duct to the cumulative amount estimated from the rHu-

**TABLE I.** Effect of Administration Route on the Lymphatic Delivery of rHu-TNF in Anesthetized Rats

<table>
<thead>
<tr>
<th>Administration route</th>
<th>Number of experiments</th>
<th>Cumulative amount estimated in plasma up to 4 h, $a)$ (U)</th>
<th>Lymph flow (ml/h)</th>
<th>Cumulative amount in lymph up to 4 h, (U)</th>
<th>$\frac{B}{A} \times 100$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>5</td>
<td>106167 ± 1253</td>
<td>0.55 ± 0.02</td>
<td>3355 ± 409</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>i.p.</td>
<td>5</td>
<td>24511 ± 10392</td>
<td>0.50 ± 0.05</td>
<td>4662 ± 1040</td>
<td>26.0 ± 6.7</td>
</tr>
<tr>
<td>s.w.</td>
<td>5</td>
<td>23894 ± 19335</td>
<td>0.58 ± 0.01</td>
<td>19335 ± 4085</td>
<td>79.5 ± 8.0</td>
</tr>
<tr>
<td>g.w.</td>
<td>5</td>
<td>11534 ± 3548</td>
<td>0.51 ± 0.05</td>
<td>9926 ± 2022</td>
<td>101.1 ± 18.7</td>
</tr>
<tr>
<td>s.c.</td>
<td>4</td>
<td>1202 ± 402</td>
<td>0.47 ± 0.02</td>
<td>25 ± 9</td>
<td>4.7 ± 3.4</td>
</tr>
<tr>
<td>i.m.</td>
<td>4</td>
<td>1462 ± 505</td>
<td>0.59 ± 0.03</td>
<td>1241 ± 393</td>
<td>116.4 ± 40.4</td>
</tr>
</tbody>
</table>

Dose of rHu-TNF was $3.3 \times 10^4$ U/kg for all experiments. Each value represents the mean ± S.E. $a)$ Cumulative amount in the plasma was estimated by the Wagner–Nelson method.
TNF concentration in plasma is summarized in Table I. The values of lymphatic uptake ratio of rHu-TNF after local administration, except the s.c. route, were $8 - 36$ times higher than that after the i.v. administration. These findings suggest that the rHu-TNF after the s.w., g.w., i.p. and i.m. administrations was transferred selectively into the lymph capillaries. On the other hand, the lymph flow was almost the same among the administration routes.

The correlation between immunoreactive activity determined by EIA assay and cytotoxic activity measured by L-M assay of rHu-TNF in lymph after the systemic and local administrations is shown in Fig. 5. The results showed good agreement between immunoreactive and cytotoxic activities.

**Discussion**

The prevention of lymphatic metastasis is very important for the treatment of cancer. Therefore, the selective lymphatic transport of anticancer agents might be of great significance for cancer chemotherapy. In general, watersoluble substances with relatively low molecular weight would behave similarly in both blood and lymph capillaries. However, lipophilic substances$^{17,18}$ and macromolecular substances$^{19,20}$ after intestinal or i.m. administration are absorbed mainly via the lymph capillaries. On the other hand, it is also well known that the permeation of macromolecular substances into lymph after i.v. administration falls by a factor of $10^4$ as the molecular weight increases from insulin to serum albumin.$^{21}$ Bocci et al.$^7$ reported that levels of rHu-TNF in lymph after i.v. administration to rabbits were always lower than that in plasma. However, this behavior was not found in the rats used in the present study (Fig. 1). No marked difference between rats and rabbits in elimination rate of rHu-TNF from plasma was found. Therefore, the above findings may indicate that after the i.v. administration, transport into lymph from plasma of rHu-TNF is species-specific. Talmadge et al.$^{22}$ reported that the i.p. administration of rHu-TNF has no therapeutic activity for the treatment of metastatic disease. However, this metastasis model was an experimental system via blood. As illustrated in Fig. 2, rHu-TNF after the i.p. administration showed high lymph levels compared to the plasma level. Therefore, the treatment of metastatic disease by i.p. administration might be most effective in models of metastasis via lymph. The administration of an anticancer drug via visceral organ wall is used to treat lymphatic metastasis in cancer chemotherapy.$^{23-25}$ In the present study, it is clear from the ratio of rHu-TNF recovered from the thoracic duct to the cumulative amount estimated from rHu-TNF concentration in plasma (Table I) that rHu-TNF given by local administration, except s.c., was transferred selectively into the lymph capillaries. Further, we showed that the s.w. and g.w. administrations provided a sustained supply of rHu-TNF to the lymph, and the levels were relatively high when compared with the levels after the i.v. administration (Fig. 2). These findings suggest that the s.w. and g.w. routes should be effective for the treatment of lymphatic metastasis of gastric and colorectal cancer patients, respectively. This is also supported by the data on the cumulative transport of rHu-TNF (Fig. 4). On the other hand, the i.p. administration could be useful as a route of administration for the treatment of lymphatic metastasis, although the
cumulative transport of rHu-TNF in lymph after the i.p. administration was approximately similar to that after the i.v. administration. The levels and cumulative amounts of rHu-TNF in lymph after the i.m. and s.c. administrations were rather low compared with those after the s.w., g.w. and i.p. administrations (Figs. 3 and 4). These results might be due to the paucity of lymph vessels in the subcutaneous and intramuscular tissues against the rich supply in the stomach wall, gut wall and intraperitoneal lymph vessels.26

In the present study, the concentrations of rHu-TNF in plasma and lymph after administration via various routes were measured by EIA assay. Sunahara et al.12) reported that immunoactive activity measured by EIA assay of rHu-TNF in plasma was in good agreement with cytotoxic activity determined by L-M assay. The rHu-TNF in lymph also showed good agreement between immunoactive and cytotoxic activities (Fig. 5). This finding indicates that the pharmacokinetic parameters of rHu-TNF in lymph can be employed as a measure of the cytotoxic activity.

Consequently, we expect that the local routes of administration of rHu-TNF such as s.w., g.w. and i.p. might be most effective for the treatment of lymphatic metastasis of cancer patients.

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References