Antitumor Effect of a Splenic Injection of 5-Fluorouracil on Metastatic Liver Cancer in Mice

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ABSTRACT

The regional administration of 5-fluorouracil (5-FU) has been the fundamental therapy against liver metastases for the improvement of patient prognosis; however, there have been few reports about the immunological effects of this agent. It is also unknown whether it affects the spleen, one of the major lymphoid organs. The objective of the present study was to determine the immunological effect of an intrasplenic injection of 5-FU against liver metastases. We investigated the effect of an intrasplenic injection of 5-FU on the formation of experimental liver metastasis resulting from an intraportal vein injection of colon 26 carcinoma cells in BALB/c mice and elucidated some of the underlying mechanisms involving the effects of this on cellular immunity. Liver metastases were significantly diminished by the splenic injection of 5-FU, particularly in comparison with the portal injection or systemic administration. This was followed by augmentation of the interleukin-12 (IL-12) level in the spleen and activation of hepatic mononuclear cells. In those cells, NK1.1+ (NKT) cells played a central role against metastases. A splenic injection of 5-FU is more effective on the involution of liver metastases than portal or systemic injection. This effect may be attributed to the augmentation of the IL-12 level in the spleen and of NKT cells in the liver rather than to the original effect of 5-FU, which is the so-called inhibition of DNA synthesis.

Liver metastasis, the most common recurrence of colon carcinomas, is assumed to be the best indicator of a patient's prognosis. Currently, although regional administration (hepatic arterial infusion) of anticancer agents is used to prevent postoperative recurrence (also in cases that have unresectable tumors), the results are not always satisfactory. In general, 5-fluorouracil (5-FU)-based chemotherapy is widely considered to be associated with the suppression of the immune function (Mitchell and DeConti, 1970; Berenbaum, 1979); however, other reports indicate that the natural killer (NK) cytotoxicity in the spleen is reinforced by 5-FU (Blomgren et al., 1965; Goto et al., 1981, Okamoto et al., 1998), which is one of the most frequently used anticancer drugs. Heretofore, there have only been a few reports about the immunological effect of 5-FU on cancer.

5-FU is a potent inducer of several cytokines, such as interferon-γ, tumor necrosis factor-α and -β, and interleukin-12 (IL-12) (Okamoto et al., 1998). Foremost, IL-12 has been described as a growth factor for activated NK cells and NK1.1+ (NKT) cells (Kobayashi et al., 1989; Perussia et al., 1992; Brunda et al., 1993; Trinchieri, 1994; Hashimoto et al., 1995; Smyth et al., 2000), and these cells have been known to be cytotoxic effector cells and to comprise the main antitumor lymphocyte population in the liver (Watanabe et al., 1995; Kobayashi et al., 2002). Meanwhile, systemic administration of the IL-12 protein was reported to augment the number of hepatic mononuclear cells (hMNCs) in mice, suppress the growth of a variety of established tumors in mice, and prolong the survival of tumor-bearing mice (Brunda et al., 1993; Hashimoto et al., 1995; Zou et al., 1995).

The objective of this study is to determine the immunological effect and mechanisms of the intrasplenic effect of 5-FU against liver metastases in mice and to establish whether the administration routes of 5-FU have some relationship with its effects. We investigated the cytotoxicity of splenic cells or hMNCs and the level of IL-12 in the spleen or liver. A subset of hMNCs having an anti-tumor effect was also identified using monoclonal antibodies.

Materials and Methods

This study was approved by the Animal Research Ethics Board of Gunma University, Graduate School of Medicine.

ABBREVIATIONS: 5-FU, 5-fluorouracil; NK, natural killer; IL-12, interleukin-12; hMNC, hepatic mononuclear cell; CTL, cytotoxic T lymphocyte; PBS, phosphate-buffered saline; Ab, antibody; T:E ratio, target-to-effector ratio; ASGM1, asialoGM1; TCN, total cell number; rmlIL-12, recombinant murine interleukin-12.
Mice. Normal inbred BALB/c mice aged 7 weeks were routinely used (Charles River Japan, Tokyo, Japan). All mice were housed in the Institute of Experimental Animal Research, Gunma University, Graduate School of Medicine, under conditions with a laminar airflow. They were maintained on standard laboratory feed and in 12-h light/dark cycles.

Preparation and Administration of Colon Tumor Cells. All mice were anesthetized using diethyl ether and laparotomized using a median incision on day 0. One hundred thousand colon 26 cells, a mouse colon carcinoma cell line capable of liver metastasis of BALB/c mice origin, were obtained by the in vivo selection method (Fidler, 1973) and injected into the portal vein. The wound was closed with continuous sutures of 3-0 silk in layers. Liver metastasis of colon tumor cells was demonstrated on the surface of the liver macroscopically 2 weeks after inoculation.

Administration Routes of 5-FU. The subjects were divided into four groups. On day 14, all mice were anesthetized, and laparotomy was performed again. In the first group, 25 mg/kg 5-FU (a kind gift from Kyowa Hakko Co., Tokyo, Japan) was injected directly into the spleen. The second group took the agent injected via the portal vein, and the third group, via the common iliac vein. Normal saline was injected into the spleen in the last group as a control. Each group consisted of 40 mice.

Survival Rates. Survival rates were calculated using 10 mice from each group from the day administered 5-FU or normal saline.

Collection of Samples and Measurement of Hepatic Metastases. The mice were sacrificed by decapitation under adequate anesthesia with pentobarbital according to the guidelines of Gunma University, Graduate School of Medicine, on day 18. The removed spleen and liver of 10 mice from each group were weighed. Two days after fixation in 10% formalin, the number of metastatic foci on the surface of each fixed liver was counted. The very small nodes spleen and the liver of 10 mice from each group were weighed. Two

To determine the significance of the differences between the experimental groups concerning total cell number and metastatic focus number, the Kruskal-Wallis test was used. Survival rates were calculated by the method of Kaplan-Meier. p values of <0.05 were judged to be statistically significant.

Results

Effect of 5-FU Injection into the Spleen Influences Spleen and Liver Weight after Inoculation of Tumor Cells. Spleen and liver weight were examined after the inoculation of colon 26 to investigate the effect of 5-FU injection into the spleen on liver metastases. As shown in Table 1, in three groups that were injected with 5-FU, the average weight of the spleen in the group with an intrasplenic injec-
Intrasplenic (saline) 2.1
Intravenous (saline) 2.6
Intravenous (5-FU) 0.7 7.4
Intraperoral (5-FU) 2.6 7.4
Intrasplenic (5-FU) 8.8 7.5
Intrasplenic (saline) 2.1 0.4

$^a$ Not significant versus the other groups.
$^b$ $p < 0.001$ versus intravenous (5-FU) and intrasplenic (saline).
$^c$ $p < 0.05$ versus the other groups.

Intrasplenic (saline) 217.6 ± 37.7
Intravenous (5-FU) 139.1 ± 36.5
Intraperoral (5-FU) 123.9 ± 29.8
Intrasplenic (5-FU) 183.7 ± 42.1

$^a$ $p < 0.001$ versus intraportal (5-FU) and intravenous (5-FU).
$^b$ Not significant versus intraportal (5-FU) and intrasplenic (saline).
$^c$ $p < 0.05$ versus the other groups.

intraportal injection of 5-FU or with an intrasplenic injection of normal saline. With respect to the total number of hMNCs, there was no significant difference among all groups. To expresses the relative increase or the capacity of hMNCs that attack metastatic foci, the number of hMNCs per liver weight was calculated. The number in the group injected with 5-FU into the spleen was significantly higher than that in the other groups ($p < 0.01$) (Table 3). These results suggest that the intrasplenic injection of 5-FU with a single 25-mg/kg dose plays a critical role in the involution of liver metastases and prolongs the survival period through the augmentation of hMNCs in comparison with the other groups. They also suggest that the injection to the spleen restrains the reduction of splenic cells as the systemic administration of 5-FU.

Cytotoxic Activities of hMNCs and Splenic Cells. We examined the cytotoxicity of splenic cells and hMNCs isolated from mice in all groups using Yac-1 lymphoma cells and colon 26 as targets. There was no significant difference in the CTL and NK cytotoxicity of splenic cells among the groups (data not shown). The same result was obtained with regard to the CTL cytotoxicity of hMNCs in each group (data not shown). On the other hand, the NK cytotoxicity of hMNCs in the group injected with 5-FU into the spleen was significantly increased in comparison with that in the other groups in any point of the T.E ratio ($p < 0.001$) (Fig. 2). In particular, the difference was nearly twice as high as that in the group with an intrasplenic injection of normal saline. These results suggest that the injection of 5-FU into the spleen augments the NK cytotoxicity of hMNCs but not that of splenic cells. Furthermore, they demonstrate that the intrasplenic injection of 5-FU does not augment the CTL cytotoxicity of both spleen and liver.

Up-Regulation of IL-12 by Treatment with 5-FU into the Spleen. Because IL-12 reportedly acts as a growth factor for activated NK cells, we examined the IL-12 level in spleen and liver homogenates of mice after 5-FU administration. As shown in Fig. 3, the IL-12 level in spleen or liver in the group

### TABLE 1

Organ weight and number of metastatic foci on the surface of the liver

<table>
<thead>
<tr>
<th></th>
<th>Spleen Weight</th>
<th>Liver Weight</th>
<th>No. of Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrasplenic (5-FU)</td>
<td>183.7 ± 13.7$^a$</td>
<td>2527.9 ± 359.2$^b$</td>
<td>22.9 ± 7.3$^c$</td>
</tr>
<tr>
<td>Intraperoral (5-FU)</td>
<td>144.8 ± 24.6</td>
<td>2880.0 ± 281.7</td>
<td>32.7 ± 6.4</td>
</tr>
<tr>
<td>Intrasplenic (5-FU)</td>
<td>119.1 ± 14.7</td>
<td>3616.0 ± 654.8</td>
<td>36.4 ± 6.5</td>
</tr>
<tr>
<td>Intrasplenic (saline)</td>
<td>217.6 ± 21.7</td>
<td>3503.5 ± 675.2</td>
<td>41.2 ± 7.4</td>
</tr>
</tbody>
</table>

$^a$ $p < 0.001$ versus the other groups.
$^b$ $p < 0.01$ versus intravenous (5-FU) and intrasplenic (saline), $p < 0.05$ versus intraportal (5-FU).
$^c$ $p < 0.05$ versus the other groups.

### TABLE 2

TCN of splenic cells and ratio of spleen weight

<table>
<thead>
<tr>
<th></th>
<th>TCN of Splenic Cells ($\times 10^5$)</th>
<th>TCN/Spleen Weight ($\times 10^7$/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrasplenic (5-FU)</td>
<td>82.3 ± 6.5$^a$</td>
<td>4.5 ± 0.2$^a$</td>
</tr>
<tr>
<td>Intraperoral (5-FU)</td>
<td>61.7 ± 7.1</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Intrasplenic (5-FU)</td>
<td>43.4 ± 8.0$^b$</td>
<td>3.6 ± 0.2$^b$</td>
</tr>
<tr>
<td>Intrasplenic (saline)</td>
<td>92.3 ± 15.3</td>
<td>4.3 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ $p < 0.001$ versus intraportal (5-FU) and intravenous (5-FU).
$^b$ $p < 0.01$ versus intraportal (5-FU) and intravenous (5-FU).
$^c$ $p < 0.05$ versus the other groups.
injected 5-FU into the spleen was significantly higher than the levels in the other groups (p < 0.001). Particularly, the IL-12 level in the liver was nearly twice as high in the group with intrasplenic injection with 5-FU as it was in the other groups. These facts support the findings that the splenic injection of 5-FU stimulates the splenic immune system to produce IL-12 and up-regulate the IL-12 level in the liver.

Involvement of NK Cells and NKT Cells. To investigate the principal cells that have high NK cytotoxicity, we used two antibodies, which depleted NK cells or both NK and NKT cells. Anti-ASGM1 Ab depletes only NK cells, and anti-NK1.1 Ab depletes NK and NKT cells. The liver weight and number of metastatic foci of the liver in the group pretreated with anti-ASGM1 Ab and injected with 5-FU into the spleen were significantly less than those in the other groups (p < 0.01) (Table 4). The NK cytotoxicity of hMNCs in the group injected with 5-FU into the spleen with ASGM1 Ab was significantly higher than that in other groups with Ab in any point of the T:E ratio (p < 0.001). The results described above suggest that rmIL-12 injected into the spleen activates the hMNCs and, secondarily, reduces the volume of metastases.

On the other hand, the IL-12 level in the liver (Fig. 4B) and the NK cytotoxicity of hMNCs (Fig. 5) in the group injected with rmIL-12 via the portal vein were higher than those in the groups that received an intravenous injection of rmIL-12 and an intrasplenic injection of normal saline (p < 0.05). These facts show that, in spite of the augmentation of the NK cytotoxicity by injection of IL-12 through the portal vein, the liver weight and the number of metastases did not diminish as they did when IL-12 was administered into the spleen.

Discussion

The liver is one of the most susceptible organs to metastasis of a digestive cancer, especially colon cancer. Currently, various treatments, such as resection, microwave coagulation, and local administration of anticancer agents, are used. Although it is obvious that resection is the most effective method to prevent metastasis and recurrence in the liver, the local administration of anticancer agents, such as 5-FU, is still potentially important for long-term prognosis.

The spleen is a large lymphoid organ that produces various kinds of cytokines (Hood et al., 1978), which are known to stream into the liver via the splenic and portal veins and to enhance NK cytotoxicity in the liver (Shiratori et al., 1995). NK cells have been known to mediate spontaneous cytotoxicity against tumor cells and their metastases (Gorelik et al., 1982). It has also been reported that hepatic NK cells act as the first line of defense in the hepatic metastasis of colon cancer (Shiratori et al., 1992; Bouwens et al., 1998).

The splenic cells from mice given 5-FU intraperitoneally...
exhibited higher NK activities (Okamoto et al., 1998). The present study was carried out to determine whether the immune system in the spleen is up-regulated and whether it has an anticancer effect when 5-FU is administered into the spleen. As a result, in the mice that received 5-FU into the spleen, the weight of the liver, and the number of metastases were diminished. Furthermore, hMNCs increased, and their NK cytotoxicity was significantly augmented. These results demonstrate that the splenic injection of 5-FU plays a critical role in the involution of liver metastases. Therefore, the survival in mice with an intrasplenic injection of 5-FU was significantly longer than that in mice injected with normal saline, and at least, no less than that in mice with an intraportal or intravenous injection of 5-FU. These facts reveal the possibility that an intrasplenic injection of 5-FU can prolong the survival period in combination with other treatments if it reduces the number and the volume of metastases.

Although 5-FU-based chemotherapy is widely considered to be associated with the suppression of immune function (at least, transiently) (Mitchell and DeConti, 1970; Berenbaum, 1979), other reports have described the increase in the immune function to result from the inhibition of suppressor T cell cytotoxicity associated with this drug, inversely (Blomgren et al., 1965; Goto et al., 1981, Okamoto et al., 1998). Thus, the effect of 5-FU on the immune system is controversial. In this study, the total number of cells per spleen weight was significantly diminished only in mice that

### TABLE 4
Number of metastatic foci on the surface of liver

<table>
<thead>
<tr>
<th>Liver Weight (mg)</th>
<th>No. of Metastases</th>
<th>NK Cytotoxicity of hMNCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T:E=1:2.5</td>
</tr>
<tr>
<td>ASGM1 + intrasplenic (5-FU)</td>
<td>2751.3 ± 339.1*</td>
<td>23.7 ± 7.6</td>
</tr>
<tr>
<td>ASGM1 + intraportal (5-FU)</td>
<td>3557.4 ± 423.5</td>
<td>36.0 ± 8.2</td>
</tr>
<tr>
<td>ASGM1 + intravenous (5-FU)</td>
<td>3311.7 ± 361.7</td>
<td>37.3 ± 8.5</td>
</tr>
<tr>
<td>NK1.1 + intrasplenic (5-FU)</td>
<td>3622.8 ± 541.8</td>
<td>36.4 ± 7.3</td>
</tr>
<tr>
<td>NK1.1 + intraportal (5-FU)</td>
<td>3730.4 ± 688.5</td>
<td>39.2 ± 6.7</td>
</tr>
<tr>
<td>NK1.1 + intravenous (5-FU)</td>
<td>3426.3 ± 484.0</td>
<td>37.9 ± 9.5</td>
</tr>
</tbody>
</table>

* p < 0.001 versus ASGM1 + intraportal (5-FU), NK1.1 + intrasplenic (5-FU), and NK1.1 + intraportal (5-FU), p < 0.01 versus ASGM1 + intravenous (5-FU), NK1.1 + intravenous (5-FU).

b p < 0.001 versus the other groups.

### TABLE 5
Effects of rmIL-12 on liver metastases

<table>
<thead>
<tr>
<th>Liver Weight (mg)</th>
<th>No. of Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrasplenic (rmIL-12)</td>
<td>2541.5 ± 289.3*</td>
</tr>
<tr>
<td>Intraportal (rmIL-12)</td>
<td>3235.8 ± 351.6</td>
</tr>
<tr>
<td>Intravenous (rmIL-12)</td>
<td>3485.5 ± 348.1</td>
</tr>
<tr>
<td>Intrasplenic (saline)</td>
<td>3505.3 ± 675.2</td>
</tr>
</tbody>
</table>

* p < 0.001 versus the other groups.

b p < 0.01 versus the other groups.

**Fig. 4.** Distribution of IL-12 after injection of rmIL-12 was examined using enzyme-linked immunosorbent assay. A, IL-12 level obtained from the splenic homogenate. B, IL-12 level obtained from the hepatic homogenate. The spleen and liver levels of IL-12 in the group injected with rmIL-12 into the spleen were significantly higher (+++, p < 0.001) than those in the other groups. Moreover, the liver level in the group injected with rmIL-12 into the portal vein was significantly higher (+++, p < 0.001) than that in the group with an intravenous injection of rmIL-12 and intrasplenic injection of normal saline. Error bars represent S.D.

**Fig. 5.** Effects of rmIL-12 for liver metastases. Groups shown are defined as follows: ●, intrasplenic injection of rmIL-12; ■, intraportal injection of rmIL-12; ▲, intravenous injection of rmIL-12; and ×, intrasplenic injection of normal saline. The NK cytotoxicity of hMNCs in the group injected with rmIL-12 into the spleen was significantly higher than that in the other groups (+++, p < 0.001; ***, p < 0.01). Moreover, that in the group injected with rmIL-12 into the portal vein was significantly higher than that in the group with an intrasplenic injection of rmIL-12 and intrasplenic injection of normal saline (+++, p < 0.001; +, p < 0.05). Error bars represent S.D.
were injected with 5-FU via the common iliac vein. And that in mice injected with 5-FU into the spleen is not diminished in comparison with the control. These results suggest that splenic cells are involved in the effect on the involution of liver metastases.

Until a certain size, a metastatic tumor in the liver grows by securing nutrients through the hepatic artery (Izumi et al., 1986). Archer and Gray (1990) reported that branches of the portal vein at the tumor periphery become invaded and compressed during the growth of metastases, which further diminishes drug delivery to the tumor site. In addition, 5-FU is an agent with a short half-life in serum of several tens of minutes. Therefore, it is conceivable that there is a new effect other than the original anticancer effect with this method. These findings suggest that the splenic administration of 5-FU reduces the metastatic lesion by augmenting the NK cytotoxicity of hMNCs rather than by the original effect of 5-FU, which is the so-called inhibition of DNA synthesis.

5-FU is a potent inducer of several T helper 1-type cytokines, such as interferon-γ, tumor necrosis factor-α and -β, and IL-12, and of effector cells carrying anticaner cytokoty mediated by NK cells as well as T cells; in addition, these abilities are closely-associated with the in vivo anticancer effect of this agent (Okamoto et al., 1998). Especially, IL-12 was originally described as an NK stimulatory factor (Kobayashi et al., 1989) or a cytokotic lymphocyte maturation factor (Trinchieri, 1994) produced principally by antigen-presenting cells. In this study, a 5-FU injection into the spleen was found to augment the level of IL-12 in the spleen or liver more than in the other groups. Furthermore, the fact that the increase of IL-12 is not followed by the augmentation of splenic cytokoty substancestes the view that most of the IL-12 produced in the spleen flows into the liver via the splenic vein and induces an increase in the number of hMNCs and augmentation of the cytokotic of hMNCs. Eberl and MacDonald (2000) reported that the proportion of NK and NKT cells made up about 8% of the total splenic cells and about 35% of the hMNCs. Their findings tend to substantiate this view. Actually, the cytokotic of hMNCs and the IL-12 level in the liver of mice that received injections of 5-FU into the spleen were significantly higher than those in mice that received 5-FU via the other administration routes.

Recent studies on IL-12 show that it acts as a growth factor for activated NK cells and NKT cells (Perussia et al., 1992; Smyth et al., 2000). Watanabe et al. (1995) reported that NKT cells, which proliferate in the liver after the administration of IL-12, are cytotoxic effector cells and comprise the main metastatic lymphocyte population in the liver. The systemic administration of IL-12 markedly activates hepatic NKT cells and induces the cytokotic activities of hMNC against targets (Brunda et al., 1993; Hashimoto et al., 1995; Smyth et al., 2000). Wiltrout et al. (1984) reported that metastatic lesions became enlarged in mice that were treated by anti-ASGM1 Ab. Seki and colleagues reported that in vivo anti-ASGM1 Ab treatment only depletes NK cells in mice, whereas anti-NK1.1 Ab treatment depletes NK and NKT cells (Seki et al., 1997; Kawamura et al., 1999). This study revealed that anticaner effects persist and NKT cells play a major role in preventing metastases, even when NK cells are depleted and only NKT cells exist.

Systemic administration of IL-12 protein significantly suppressed the growth of a variety of established mouse tumors and prolonged the survival of tumor-implanted mice (Brunda et al., 1993; Zou et al., 1995). The number of hMNCs in mice that received rmIL-12 increased more significantly than that in mice that did not receive it (Hashimoto et al., 1995). In this study, to determine whether IL-12 influences the activation of hepatic NKT cells, rmIL-12 was injected into the spleen in tumor-implanted mice. The results revealed that the rmIL-12 injected into the spleen activated the hepatic NKT cells and reduced the volume of metastases. However, in spite of the augmentation of the NK cytotoxicity by rmIL-12 flowing through the portal vein, the effect did not directly aid in tumor involution. These data imply that some other factor, which is produced in the spleen by IL-12, plays a supplementary role.

We believe that the use of the spleen as a potential reservoir for the activation of cell-mediated immunity in vivo should be taken into account when considering the development of clinical anticancer immunotherapeutic strategies.

References


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