Prevention of Growth of Metastases in Rat Liver by Perioperative Immunocaution

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INTRODUCTION

If a patient has undergone a successful operation for gastrointestinal cancer deemed curable at the time but later presents evidence of hepatic metastases, we are unable to tell at this time whether these lesions were preexisting microscopic metastatic foci or were metastases resulting from surgical intervention or both. This offers an important problem in the management of malignant tumor.

Major intraabdominal operations with anesthesia have been shown to cause decreased levels of immune function in normal patients, in patients with cancer, and in animals (1–3), and manipulation of malignant tumors may release tumor cells into the systemic and portal circulation. The additive effects of immunodepression and tumor cell release during surgical treatment for gastrointestinal cancer may increase the metastases of tumors to the liver. In this respect, the perioperative period may be critical for dissemination and implantation of metastatic disease, and transient immunodepression may favorably affect the development of metastatic disease and survival. This model may have relevance to the adjuvant treatment of human gastrointestinal cancer.

MATERIALS AND METHODS

Materials

Forty-four male HOS-Donryu rats weighing 200–250 g were used in this study and maintained on a standard rat pellet diet and tap water ad libitum.

Preparation of Tumor

Rat ascites hepatoma AH130 used for preparing hepatic metastases models was supplied from the Sasaki Institute, Tokyo, Japan. AH130 was maintained by serial i.p. implants in male Donryu rats every 7 days, and the ascites of the seventh day of passage was used in the experiments. More than 90% of ascites hepatoma AH130 was in the single cell state, and this facilitated the preparation of cell suspension to a great extent.

Preparation of ImmunoActivator

OK-432 (Picibanil) is an inactivated and lyophilized preparation of low virulence strain Su of Streptococcus pyogenes Group A (4). OK-432 supplied by Chugai Pharmaceutical Co. Ltd., Tokyo, Japan, was suspended in 0.9% NaCl solution at a concentration of 0.5 mg dried cell/0.2 ml. Rats were given an i.p. injection of 0.5 mg/0.2 ml OK-432.

Methods

Preparation of Experimental Groups

Rats were assigned to either of the following two groups: the group treated with OK-432 (Group A); and the controls, given no OK-432 (Group B).

Group A. OK-432 (0.5 mg per rat) (approximately 2.5 mg/kg) was dissolved in 0.2 ml of 0.9% NaCl solution and was administered to rats i.p. for 7 consecutive days before tumor implantation.

Group B. NaCl solution (0.9%) was administered to rats i.p. at a dose of 0.2 ml for 7 consecutive days before tumor implantation.

Induction of Hepatic Metastases

Under ether anesthesia, the rat abdomens were opened through a midline incision. With the duodenum deflected, a puncture was performed under direct vision into the main portal vein using a 26-gauge needle to inject AH130 suspended in Hanks' balanced salt solution at a concentration of $5 \times 10^6$ cells/ml. It was injected slowly at a dose of $10^6$ cells/0.2 ml so that no fluid would leak from the puncture site and, after the injection, light pressure was applied to the puncture site for approximately 5 s to achieve hemostasis.

Evaluation of the Inhibitory Effect of OK-432 on Hepatic Metastases

Comparison of the Number of Metastatic Lesions Between Groups A and B. On day 14 following the injection of AH130 into the portal vein, as described above, the rats were sacrificed for autopsy. The whole livers were removed, fixed in 10% formaldehyde, and dissected into 3-mm slices. The number of hepatic metastatic lesions on each dissected surface was counted and compared between Group A ($n = 8$) and Group B ($n = 8$). Pulmonary metastatic lesions were also examined.

Comparison of Survival Days. A part of the two groups of rats between which the number of metastatic lesions was compared, Group A ($n =$
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9) and Group B (n = 6), was organized. Ascites hepatoma AH130 was injected into them via the portal vein, and the survival days following the injection between the two groups were compared. Autopsy was performed on all rats of the two groups.

Changes in Peripheral Blood T-Cell Subsets due to the i.p. Administration of OK-432

Another pair of groups [Groups A (n = 7) and B (n = 6)] was organized, and the abdomens of those rats were opened under light anesthesia to collect 5-ml blood samples from the inferior vena cava. Blood samples were examined for T-cell subsets according to the flow cytometry technique by using monoclonal antibody in the Ortho-mune OK series (5).

Histological Findings in the Perifocal Tissues of Hepatic Metastases

Hepatic tissues obtained from Groups A and B were studied for histological changes in the perifocal area of the metastases. Hepatic tissue specimens were stained with hematoxylin-eosin and examined by optical microscopy.

RESULTS

Number of Lesions of Hepatic Metastases. Hepatic metastases were confirmed in all rats in Groups A (n = 8) and B (n = 8) which were killed for autopsy 14 days after the injection of ascites hepatoma AH130 at a dose of 10^6 cells/0.2 ml into the portal vein (Fig. 1).

The entire liver was removed and dissected into 3-mm slices, and the number of hepatic metastatic lesions appearing on each dissected surface amounted to 71.5 ± 45.9 in Group A and 149.3 ± 61.9 in Group B. Group A disclosed a significantly smaller number of metastatic lesions compared to Group B (P < 0.01) (Fig. 2). Pulmonary metastatic lesions were not detected in all experiments of two groups.

Survival Days. The mean numbers of survival days of rats in Groups A (n = 9) and B (n = 6) were 33.4 ± 8.1 and 21.8 ± 6.9, respectively (Fig. 3).

A significant prolongation in the number of survival days was achieved in Group A compared to Group B (P < 0.01). Autopsies at the time of death revealed marked hepatic metastases in both groups and the presence of ascites in many rats. Pulmonary metastases were detected in 1 of 9 rats in Group A and in 2 of 6 rats in Group B. These findings were not significantly different between the two groups. All rats died of cancer.

Changes in Peripheral Blood T-Cell Subsets. Peripheral blood T-cell subsets in rats in groups A (n = 7) and B (n = 6) were determined by using the Ortho-mune OK series. The values of OKT4 in Groups A and B were 51.9 ± 7.0 and 41.8 ± 7.2%, respectively, demonstrating a significantly higher value in Group A than that in Group B (P < 0.025) (Table 1). There was no significant difference between either group in the OKT3 and OKT8 values.

Histological Findings in the Perifocal Metastatic Tissues. Examination of the perifocal tissues of hepatic metastases in

![Fig. 1. Autopsy specimen of a nontreated rat. Marked metastatic lesions were revealed 14 days after the injection of AH130.](image1)

![Fig. 2. Number of metastatic lesions 14 days after AH130 injection (mean). Bars, SD.](image2)

![Fig. 3. Survival days (mean ± SD).](image3)

<table>
<thead>
<tr>
<th>T-cell subset</th>
<th>Group A: treated with OK-432 (%) (n = 7)</th>
<th>Group B: control (%) (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT 3^*</td>
<td>65.3 ± 7.3^*</td>
<td>58.8 ± 6.0</td>
<td>NS^a</td>
</tr>
<tr>
<td>OKT 4^*</td>
<td>51.9 ± 7.0</td>
<td>41.8 ± 7.2</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>OKT 8^*</td>
<td>16.2 ± 1.9</td>
<td>15.9 ± 4.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

^a Mean ± SD.
^a NS, not significant.
rats on the 14th day after the injection of ascites hepatoma AH130 into the portal vein disclosed a marked infiltration by small round cells around many of the metastatic lesions in Group A. Rats in Group B did not exhibit infiltration by as many small round cells as was seen in group A (Fig. 4).

**DISCUSSION**

Since the establishment of the metastasis forming method in experimental animals (6–9), a variety of practical studies on the inhibition of metastases has been conducted using anticancer agents, anticoagulant agents (10, 11), and, more recently, antiplatelet agents (12). However, long-term administration of anticancer drugs embraces many problems because of their strong cytotoxicity, and there are some reports to the effect that the administration of anticancer agents, paradoxically, accelerates metastases by their cytotoxic effect on the vascular endothelial cells and eventually depresses the resistance (13).

The primary action mechanism of anticoagulant drugs is inhibition of implantation of tumor cells, and, therefore, it is considered necessary to use this type of drug in combination with other anticancer drugs. However, anticoagulant drugs, when used at a large dose, produce hemorrhagic diathesis, implying great difficulty in their clinical application in the perioperative period.

Operation with anesthesia induces significant transient immunological depression. In addition, manipulation of malignant tumors may release tumor cells into systemic and portal circulations. The additive effects of immunodepression and tumor cell release may enhance the metastatic potential of tumors. We postulate that the perioperative period is critical for the implantation and development of metastatic disease. Immunity activators, although not dramatically powerful in antitumor activity, may be expected to show an adequate effect when used, especially in the perioperative period, which provides an immunocompromised host. In this respect, immune activators can be considered to be suitable drugs to prevent the implantation and growth of metastases, especially in the perioperative period.

OK-432, a widely accepted immunity activator, was studied for its inhibitory effect on hepatic metastases. OK-432 is a dried cell preparation, derived from Group A hemolytic Streptococci, Su strain (4), featuring two antitumor action mechanisms: i.e., a direct cytotoxic action on tumor cells; and an inhibitory action on tumor cells through activation of the immunological functions in hosts (14). The tumor response to the direct antitumor action of OK-432 varies according to tumor types, and others report that OK-432 has almost no direct action on AH130 (15).

In the present study, OK-432 was administered i.p. for 7 consecutive days prior to the injection of tumor cells into the portal vein. Some investigators report that most of the OK-432 administered i.p. remains in the vicinity of the organs in the abdomen, with only 6.4% distributed in the liver 30 min after administration. OK-432 may be administered by one of the following schemes: administration prior to the tumor cell injection; concomitant administration; or administration after the injection. Tanaka et al. (16) reported that the antitumor effect of OK-432 on ascites hepatoma AH7974 was confirmed only in cases of administration prior to the intraabdominal injection of the tumor cell, but the contradictory reports by other investigators describing the effectiveness only in cases of administration after the injection (17) resulted from a different experimental system. In our experiments, the antitumor effect of OK-432 was confirmed by administering prior to the tumor injection.

Numerous studies have been reported on the antitumor effect of OK-432 manifested in hosts. OK-432 has been reported to activate macrophages non-specifically in hosts (16, 18), and, thereby, activation of macrophages is considered to be one of the important action mechanisms for immunity activators (19). It is also reported that carbon clearance tests have shown activation of not only macrophages but also of the overall function of the reticuloendothelial system by OK-432 (20). OK-432 is reported to induce activation of non-specific killer cells (19, 21) and to activate the function of T-lymphocytes (21, 22). We determined the T-cell subsets to examine the accelerated differentiation of T-lymphocytes by OK-432. The group given i.p. administration of OK-432 for 7 consecutive days demonstrated an increased differentiation of T-lymphocytes and increased OKT4+ cells. In histological findings, the marked infiltration of the tumor cell, but the contradictory reports by other investigators describing the effectiveness only in cases of administration after the injection (17) resulted from a different experimental system. In our experiments, the antitumor effect of OK-432 was confirmed by administering prior to the tumor injection.

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**Fig. 4.** Perifocal lesion of hepatic metastases. Hematoxylin-eosin stain; × 400. A, OK-432 treated group. B, OK-432 non-treated group.
of small round cells into the surrounding tissues of metastatic lesions seen in the group treated with OK-432 is considered to be related to the OK-432-induced activation of cellular immunity. Regarding the mechanism of the inhibitory effect of OK-432 on liver metastases, therefore, activation of the host immune system by OK-432, especially the function of lymphocytes, may favorably affect growth of metastases. We suppose that almost all tumor cells injected into the portal vein are trapped in the liver, and thereafter growth of metastases is modified by various degrees of the host defense system.

In this study, metastases to the liver were induced artificially by portal vein injection of tumor cell suspension. Therefore the results of this study may be different from those due to conditions of spontaneous metastasis, and anticancer treatment for preexisting microscopic metastatic foci remains unsolved in this study. However, this model is relevant to the clinical setting in which metastases to the liver result from the operation and may have relevance to the adjuvant treatment of human gastrointestinal cancer.

Perioperative immune activation with OK-432 pretreatment reduced the incidence of liver metastases developed in animals injected with tumor cells, and survival was significantly affected in these animals. We believe that the perioperative period in therapy protocols of patients with gastrointestinal cancer is important in the development of adjuvant treatment, and correction of perioperative immune depression may favorably affect the development of metastatic disease and survival.

REFERENCES

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