Effect of Splenectomy on Antitumor Immune System in Mice

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Abstract. Background: The influence on the antitumor immune system after splenectomy in vivo are controversial. CD4+CD25+Foxp3+T-cells (regulatory T-cell: reg T) and natural killer (NK) cells play important roles in immunological tolerance and antitumor immunity. The influence of splenectomy on the antitumor immune system was evaluated in a metastasis induced mouse model. Materials and Methods: Experiment 1, splenectomy in a cancer-free model. The mice were divided into two groups, one control, and the other splenectomy group. At days 4, 7 and 10 after splenectomy, the mesenteric lymph node, the liver and the lung were harvested. The lymph nodes were analyzed by flow cytometric analysis and the number of reg T-cells and NK cells were calculated. Foxp3 mRNA in the liver and the lung was evaluated by reverse transcriptional polymerase chain reaction (RT-PCR). Experiment 2, splenectomy in a liver metastasis model. Colon 26 cells were injected into the spleen of mice and the mice were divided into two groups, a spleen preserved group, and a splenectomy group. Splenectomy was performed at day 4 after injection. At days 7 and 10 after injection, flow cytometric analysis, and at day 10 RT-PCR were performed. Ten days after injection, the number of liver metastases (>1 mm) was counted. Results: Experiment 1, in the splenectomy group the flow cytometric analysis showed a significant decrease in the number of reg T and NK cells in the mesenteric lymph nodes compared with the control group. In the splenectomy group, the Foxp3 mRNA increased significantly in the liver at day 10, and in the lung at days 4 and 7. Experiment 2, liver metastasis was observed in the splenectomy group. Flow cytometric analysis showed that splenectomy did not affect the number of reg T at day 7 and day 10. The number of NK cells increased in the splenectomy group at day 7, but at day 10, there was no significant difference between the groups. RT-PCR showed that at day 10, the Foxp3 mRNA in liver increased in the splenectomy group. Conclusion: The spleen plays a very important role in the antitumor immune system and splenectomy enhances liver metastasis through the increase of Foxp3 mRNA in the liver.

Colorectal cancer is one of the most common malignancies in the world and hematogenous metastasis to the liver and the lung, as well as lymph node metastases, are the most important factors that influence the prognosis of patients. Hematogenous liver metastases are found in 20% of patients at the time of surgery (1) and in 60-70% after operations (2, 3). Surgical resection of liver metastases is the best treatment for patients with solitary tumors, and the 5-year disease-free survival rate is 25-30% (4-10); on the other hand, for patients with multiple liver metastases, systemic or intraarterial infusion chemotherapy may be performed, but the prognosis is not good. To ascertain the mechanism of metastasis and its relationship with the immune system is the current challenge for improving clinical treatment. There are many factors that affect cancer metastasis and the antitumor immune system is one such important factors. The spleen is a large lymphoid organ that produces various kinds of cytokines (11), which are known to flow into the liver via the splenic and portal veins and to enhance natural killer (NK) cytotoxicity in the liver (12). NK cells have been known to mediate spontaneous cytotoxicity against tumor cells and their metastases (13). It has also been reported that hepatic NK cells act as the first line of defense in the hepatic metastasis of colon cancer (14, 15). The spleen also plays an important role in the immune system, and a relationship between splenectomy and tumor metastasis has been reported (12, 16, 17). However, the effect of splenectomy on the antitumor immune system in vivo remains controversial. It is only known that splenocytes cotraffic with the tumor cells to the liver and facilitate metastatic colony formation (18) but reports of the mechanism of the antitumor immune system in the spleen are limited.
CD4+CD25+Foxp3+T-cells (regulatory T-cells: reg T-cells) exist in malignant tumors (19-24), and play a role in immunological tolerance to self-antigens and in suppression of antitumor immunity. Foxp3 is a transcriptional factor that regulates the development of reg T-cells (25). We previously reported that the number of reg T-cells in the peripheral blood significantly increased in pancreatic cancer patients compared with healthy donors (26). Moreover, the simultaneous administration of CD4+CD25+ T-cells with hepatic parenchyma prolonged islet graft survival and induced donor-specific hyporesponsiveness (27). It is known that reg T-cells suppress proliferation of T-cells and autoimmunity in animals (29-31). They are also linked to cancer development because depletion of reg T-cells facilitates tumor rejection (32-35). Reg T-cells preferentially accumulate in tumor-draining lymph nodes and tumor masses and the presence of such cells is correlated with a poor prognosis in patients (36-38).

The present study was carried out to determine whether the host immune system is down-regulated and to investigate the influence on anticancer immunity when splenectomy was performed using splenic injection of tumor cells as a useful method for obtaining liver metastasis (28).

Materials and Methods

Animal preparation. Female Balb/c mice (6 to 8-weeks old) were purchased from Charles River Co. Ltd (Kanagawa, Japan). The animals were provided with water and standard laboratory diet for at least 7 days before use. Throughout the experiment, the animals were maintained behind barriers under controlled conditions and had free access to tap water and diet before and after surgery. The present study was conducted in compliance with the Division for Animal Research Resources, Institute of Health Biosciences, the University of Tokushima. The experiments and procedures were approved by the Animal Care and Use Committee of the University of Tokushima.

Cell line. The standard experimental mouse tumor cell line colon 26 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B. All the cells were kept at 37°C, in a humidified 5% CO2 atmosphere.

Experiment 1: Splenectomy in the cancer-free model. The mice were divided into two groups, a spleen preserved (control) group and a splenectomy group. The splenectomy procedure was as described elsewhere. The mice were sacrificed on days 4, 7 or 10 after splenectomy. The mesenteric lymph nodes, the livers and the lungs were harvested. Reg T-cells and NK cells in the mesenteric lymph nodes were analyzed by flow cytometric analysis. Pieces of the liver and the lung were put in RNA later (RNA Later RNA Stabilization Reagent, QIAGEN) and saved at –80˚C until RNA extraction for the reverse transcription-polymerase chain reaction (RT-PCR).

Experiment 2: Splenectomy in liver metastasis model. The outline of the experiment is shown in Figure 1. Under ether anesthesia, a small upper-quadrant incision was made to expose the spleen of each Balb/c mouse and 0.1 ml of the viable cell suspension (1x10⁶ cells/mouse) of colon 26 cells was injected into the lower splenic pole with a 27-gauge needle. Four days after the injection of tumor cells, the mice were divided into two groups, a spleen preserved group, and a splenectomy group in which splenectomy was performed with the same procedure as above.

Flow cytometric analysis. The following antibodies and reagents to detect reg T-cells and NK cells were purchased from BD Biosciences Pharmigen (San Diego, CA, USA): CD4 (FITC anti-mouse CD4), CD25 (APC anti-mouse CD25 (IL-2 Receptor alpha,
p55), Foxp3 (PE anti-mouse/rat Foxp3; e-Bioscience, San Diego, CA, USA). The reagent for NK cells (PE CD49b/Pan-NK Cells; e-Bioscience, San Diego, CA, USA) was obtained from BD Biosciences Pharmigen. A PE anti-Foxp3 staining set (e-Bioscience) was used to stain intracellular Foxp3, and Via-Probe (BD Biosciences Pharmigen) was used to discriminate viable from non-viable cells according to the manufacturer’s instructions. Immunofluorescence was analyzed with CellQuest software (BD Biosciences Pharmigen) with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Figure 1. Experimental protocol of liver metastasis model. 1×10^6 colon 26 cells were injected into spleen of Balb/c mice. Splenectomy was performed 4 days after cancer cell implantation in the splenectomy group. Reg T-cells and NK cells were detected by flow cytometric analysis and Foxp3 mRNA by RT-PCR on Day 7 and Day 10. Macroscopic metastases on the surface of the liver (>1 mm) were counted on Day 10.
**RT-PCR**. The mRNA in liver was evaluated using Foxp3-specific primers. Total RNA was extracted from the mouse liver and lung tissue using RNeasy Mini Kit (Qiagen, Hilden, Germany). The amount of purified RNA was measured by UV spectra at 260 nm, and its purity was determined by calculating the ratio of Foxp3 to β-actin by UV activity at 260/280nm. Using the total RNA isolated from each sample, the synthesis of cDNA was carried out with M-MLV Reverse Transcriptase (Promega Co., Madison, WI, USA), and then PCR was conducted using Foxp3-specific primers (TaqMan Gene Expression Assays; Applied Biosystems).

**Statistical analysis.** All the results are presented as mean±SD. Comparisons between two or multiple groups were performed with Student’s *t*-test using StatView-J 5.0 software (SAS, CA, USA). A *p*-value less than 0.05 was considered statistically significant.

**Results**

**Experiment 1.** In the splenectomy group, the percentage of reg T-cells in the mesenteric lymph nodes decreased compared with the control (spleen preserved) group on day 4 and day 10 as shown by flow cytometric analysis (Figure 2A, control group: day 4: day 10=6.144±0.375: 4.328±0.439: 4.984±0.389, *p*<0.01). In the splenectomy group, the percentage of NK cells in the mesenteric lymph nodes also decreased on days 4, 7 and 10 compared with the control group (Figure 2B, control group: day 4: day 7: day 10=1.542±0.089: 0.898±0.117: 0.795±0.099: 1.296±0.083, *p*<0.01).

RT-PCR showed that splenectomy significantly up-regulated the Foxp3 mRNA in the liver on day 10 (Figure 3A, *p*<0.01) and in the lung on day 4 (Figure 3B, *p*<0.05) and day 7 (Figure 3B, *p*<0.01).

**Experiment 2.** Macroscopic metastasis on the surface of the liver was seen in all cases, in both groups, and the number of hepatic metastases significantly increased in the splenectomy group (spleen-preserved group: splenectomy group=1.5±1.3: 19.2±9.1) (Figure 4).

The flow cytometric analysis showed that splenectomy did not affect the percentage of reg T-cells on both days 7 and 10 compared with the untreated control group (day 7, control group: spleen-preserved group: spleenectomy group=6.144±0.375: 5.198±0.646: 3.890±1.075; day 10, control group: spleen-preserved group: spleenectomy group=6.144±0.375: 5.988±0.857: 5.415±1.250) (Figure 5). The percentage of NK cells decreased in both the spleen-preserved group and the splenectomy group on day 7 and the spleen-preserved group showed a significant decrease in the number of NK cells compared with the splenectomy group (control group: spleen-preserved group: spleenectomy group=1.542±0.089: 0.560±0.177: 0.8875±0.127, *p*<0.05). On the other hand, on day 10, both the spleen-preserved group and the splenectomy group showed a low percentage of NK cells, but there was no significant difference between the groups (Figure 6). RT-PCR on day 10 showed that Foxp3 mRNA significantly increased (*p*<0.01) in both the spleen-preserved group and the splenectomy group compared to the control group, with the splenectomy group showing a significantly higher ratio compared with the spleen-preserved group (Figure 7, *p*<0.05).

**Discussion**

Our first hypothesis was that the proportion of CD4+CD25+Foxp3+ reg T-cells would be reduced after splenectomy, which would induce the up-regulation of NK cells. However, the splenectomy group showed a significant decrease
in the percentage of both reg T- and NK cells in the mesenteric lymph nodes, and also revealed a significant increase in the Foxp3 to β-actin mRNA ratio both in the liver and the lung compared with the control group. The present results were in contrast to those of Smyth et al. (59). But several reports have indicated a parallel decrease of reg T-cells and NK cells after splenectomy, so further investigation is needed to explain the meaning of these phenomena. A possible explanation as to why both reg T-cells and NK cells were reduced after splenectomy is the specificity of origin of the CD4+CD25+Foxp3+ reg T-cells. Some of the CD4+CD25+ reg T-cells may be generated from the CD4+CD25+ population in the spleen. Recent studies have shown that CD4+CD25+Foxp3+ reg T-cells are generated from the CD4+CD25− population in the adoptive transfer system. Karim et al. demonstrated that alloantigen-specific CD4+CD25− reg T-cells could be generated in the spleen of thymectomized mice. Chosa et al. reported that splenectomy in conjunction with anti-CD25 antibody therapy on post-heart-transplanted C57BL/6 mice resulted in rejection, which indicated that the CD4+CD25+ population in the periphery (other than the spleen) could not maintain alloantigen-specific reg T-cells. The CD4+CD25+ population present in the spleen might decrease after splenectomy, so the reg T-cells might be reduced.

The spleen has several unique functions in the immune system concerning not only CD4+CD25+Foxp3+ reg T-cells, but also NK cells and expression of TGF-β (transforming growth factor-β). It is well known that TGF-β suppresses the activation of CD4+CD25+Foxp3+ reg T-cells and CD4+CD25+Foxp3+ reg T-cells suppress the activation of NK cells. Smyth MJ et al. reported that activated reg T-cells directly suppressed NK cell function via the NKG2D pathway in vivo (59). They have extended the implications further by illustrating that the relief of that reg T-cell suppression could greatly enhance the functional activity of NK cells responding to the activating interleukin (IL)-12 cytokine, which is known to enhance the NKG2D pathway of NK cell activation (60). The suppressive effect of reg T-cell-expressed TGF-β on NK cell cytotoxicity was brought about by the soluble TGF-β reducing NK cell cytotoxicity and perforin gene transcription (57) and the role of perforin downstream of the NKG2D receptor function (60).

Liver metastasis by the intrasplenic injection of colon cancer cells as used in this study would appear to involve locomotion to distant tissue, invasion from blood vessels (extravasation) into the hepatic tissue and metastatic colonization and subsequent growth. It would seem that host
cells could have a strong promoting effect on the formation of liver metastasis. The inhibition of liver metastasis by hydrodynamics-based gene expression of NK4 cells indicated that NK4 might inhibit these distinct processes leading to metastatic colony formation and growth in the liver. As NK4 inhibited in vitro invasion of MC-38 colon cancer cells, NK4 gene expression inhibited invasive behavior of cells in the liver, and NK4 gene expression suppressed tumor growth primarily due to angiogenesis inhibition; NK4 gene expression may inhibit both invasion and colonization of colon cancer cells and the subsequent growth of colonies in the liver (61).
Regarding the enhanced liver metastasis by splenectomy, Shiratori et al. (12) and Imai et al. (16) reported that the number of hepatic and lung metastases increased in splenectomized mice. They focused on the relationship between the spleen and the NK cells, and explained the mechanism of up-regulation of liver and pulmonary metastasis as being due to decreased activity of NK cells after splenectomy. These results were compatible with our present results. NK activity per hepatic asialo GM-1 (ganglioside)-positive cell in the splenectomized mice was less than that of the sham-operated mice. This result suggested that the spleen was one of the sources of NK cells that can migrate to the liver or that the spleen might be a source of factors that allow stimulation of the endothelium in the liver.

On the other hand, using a metastasis mice model Sonoda et al. reported that the lung metastatic nodules were significantly smaller in size and number in the splenectomy group than in the control group (17) and concluded that this might be associated with a decrease in serum levels of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced by splenectomy. The results were in contrast to the present study, but in the present study VEGF and bFGF were not evaluated. These results suggest organ specificity in the influence of splenectomy on pulmonary and liver metastasis.

The present study suggested that one of the mechanisms by which splenectomy enhanced liver metastasis was by an increase of reg T-cells in the liver. Several reports were incompatible with these results including that of Shiratori et al. who reported that the NK activity of hepatic mononuclear cells in the splenectomized mice was less than that of the sham-operated mice (12).

In the present study, the reg T-cells and NK cells in the mesenteric lymph nodes decreased in parallel after splenectomy. Tumor cells were not transplanted in experiment 1, and the number and activity of NK cells and reg T-cells in mesenteric lymph nodes decreased. In experiment 2, splenectomy did not significantly affect the number of NK cells and reg T-cells in the mesenteric lymph nodes. There might be some difference in the role of reg T-cells in each organ.

In conclusion, the spleen has a very important role in the antitumor immune system, and splenectomy enhances liver metastasis through the increase of Foxp3 mRNA in the liver.

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