Antitumor Activity of an Interleukin-2 Monoclonal Antibody in a Murine Osteosarcoma Transplantation Model

KEISHI KOHYAMA, HIDEHI SUGIURA, EIJI KOZAWA, JUNJI WASA, KENJI YAMADA, AKIKO NISHIOKA, YUZURU KAMEI and OSAMU TAGUCHI

Abstract. Background: Injection of monoclonal antibody to interleukin-2 (S4B6) into mice depletes regulatory T-cells (Tregs) and exhibits antitumor activities mediated through an autoimmune reaction. In this study, we demonstrate that administration of S4B6 suppresses the murine osteosarcoma cell line, LM8. Materials and Methods: LM8 osteosarcoma cells were transplanted subcutaneously into C3H mice (n=58). C3H mice were injected intraperitoneally with S4B6 starting at 7 days before LM8 transplantation (pre-S4B6 group), 2 days after transplantation, or 5 days after transplantation. Control group mice were injected with normal rat IgG. Mice were sacrificed and examined 4 weeks later. Results: The number of pulmonary metastatic colonies and the tumor size were significantly reduced in the pre-S4B6 group compared to the control group. In addition, pulmonary metastases were inhibited in mice injected with S4B6 2 days, but not 5 days, after tumor transplantation. Control group mice were injected with normal rat IgG. Mice were sacrificed and examined 4 weeks later. Results: The number of pulmonary metastatic colonies and the tumor size were significantly reduced in the pre-S4B6 group compared to the control group. In addition, pulmonary metastases were inhibited in mice injected with S4B6 2 days, but not 5 days, after tumor transplantation. Conclusion: S4B6 administration inhibited metastasis even when injected 2 days after LM8 transplantation. Our data suggest that treatment with S4B6 might be suitable in a postoperative clinical setting.

Osteosarcoma is the most common form of bone malignancy which affects adolescents and young adults. Advances in multimodal therapy consisting of aggressive adjuvant chemotherapy and wide local excision have improved survival and limb salvage. Five-year survival rates have increased from approximately 20% in 1970 to about 70% at present (1).

Despite these results, 30-40% of patients experience disease relapse and manifest pulmonary metastasis (2). Unfortunately, only 20% of patients survive three years after relapse (3). In an effort to improve patient prognosis, the development of new therapies aimed at preventing pulmonary metastasis is crucial.

Immune response regulation has been proposed to be involved in the growth of some tumors, and various immunotherapies that inhibit tumor growth and distant metastasis have been reported. One potential candidate is the monoclonal antibody (mAb) to CD25 interleukin-2 receptor α (IL2Rα) (PC61). Several reports have shown that administration of PC61 leads to a reduction in CD4+CD25+ cells in peripheral lymphoid tissues and causes an effective tumor rejection response (4-6). These findings suggest that CD4+CD25+ immunoregulatory cells are involved in tumor growth. However, organ-localized autoimmune disease has been shown to develop concurrently with PC61 treatment (7).

It was recently shown that intraperitoneal injection of an interleukin-2 mAb (S4B6) depletes CD4+CD25+ cells and enhances antitumor activities in a murine melanoma model (8). However, there are no reports which evaluate the efficacy of S4B6 administration and osteosarcoma suppression. Therefore, the purpose of this study was to test whether S4B6 inhibits osteosarcoma cell growth and prevents distant metastasis in mice.

Materials and Methods

Mice. Two months old male C3H mice were purchased from CLEA (Osaka, Japan), and kept under pathogen-free conditions in an animal facility located at Aichi Cancer Center Research Institute. All mice were maintained in plastic cages and exposed to a 14-hour light/10-hour dark cycle along with standard feed and water provision. Animal experiments were approved by the Animal Care Committee of Aichi Cancer Center Research Institute, and were conducted according to institutional guidelines for animal care.
Antibody treatments. Aliquots of 0.2 mg S4B6 was injected intraperitoneally into C3H mice at -7, -2, 2, 7, and 14 days after LM8 transplantation (pre-S4B6 group); at 2, 7, 10, 14, and 19 days after transplantation (post-2d S4B6 group); or at 5, 10, 14, and 19 days after transplantation (post-5d S4B6 group). Normal rat IgG was used as a control group and injected in the same manner as for the pre-S4B6 group.

Evaluation. Mice were sacrificed and S4B6 inhibitory effects were examined 4 weeks after LM8 transplantation. The wet weight of removed subcutaneous tumors was measured, and excised lungs, livers, and kidneys were fixed with formalin. Fixed organs were then embedded in paraffin, sectioned (6 μm thickness), and stained with hematoxylin and eosin for histological observation. The number of metastatic colonies in lungs, livers, and kidneys were counted under a light microscope (Nikon ECLIPSE 80i; Nikon, Tokyo, Japan) with the selected midline section.

Flow cytometry analysis. Approximately 1×10^6 splenic cells obtained from post-2d S4B6 and normal rat IgG-injected mice were resuspended in 100 μl of PBS, and incubated with mAbs for 30 min at 4˚C in PBS containing 1% bovine serum albumin. Antibodies used included phycoerythrin -conjugated anti-CD4 mAb (GK1.5) and fluorescein isothiocyanate-conjugated anti-CD25 mAb (7D4) (Biomeda, Foster City, CA, USA). Intracellular forkhead helix transcription factor 3 (Foxp3) was detected following the manufacturer’s recommendations using a PE-Cy5 conjugated, anti-Foxp3 staining kit (eBioscience, San Diego, CA, USA). Following incubation, cells were washed, resuspended in 300 μl of PBS, and assayed using a fluorescence-activated cell sorter (FACSscan; BD Bioscience, San Jose, CA, USA). For this experiment, at least three mice were used.

Statistical analysis. All analyses were performed using the SPSS 12.0 software package (SPSS Inc, Chicago, IL, USA). Unpaired Student’s t-tests were used to assess statistical differences between control and S4B6-injected groups. P-values corresponding to <0.05 were considered to be statistically significant.

Results

Effects of S4B6 on subcutaneous tumor growth. The effects of S4B6 treatment on tumor growth were investigated. Treatment with S4B6 showed a tendency towards reduced tumor weight compared to the control group. Tumor weights harvested 4 weeks after tumor transplantation were significantly lower in the pre-S4B6 group, but not the post-S4B6 group, compared to the control group (p=0.016) (Figure 1).

Effects of S4B6 on distant metastasis. We also investigated the effect of S4B6 treatment on metastasis. The number of metastatic nodules within the lung was reduced by S4B6 administration. Metastasis of LM8 tumor cells into the lungs was observed in 0/13 pre-S4B6 group mice (0.0%), 3/15 post-2d S4B6 group mice (20.0%), 10/15 post-5d S4B6 group mice (66.7%), and 12/15 control group mice (80.0%). Typical metastatic colonies identified in the lung are shown in Figure 2. Significant differences were also evident between the mice of the control, pre-S4B6 (p=0.029), and post-2d S4B6 groups (p=0.034) (Figure 3). Although the post-5d S4B6 treatment could suppress the number of metastatic colonies to more than half of control group, the difference was not statistically significant (Figure 3). The number of metastatic colonies in the liver and kidneys was also lower, but the difference was not statistically significant (Figure 4).

Effects of S4B6 on CD4+CD25+ T-cell populations. We investigated the effect of S4B6 administration on CD4+CD25+ T-cell populations in the spleen using flow cytometry. As shown in Figure 5, the percentage of CD4+CD25+ T-cells in the spleen of a control mouse was 1.19%, while that of a post-2d S4B6-injected mouse was 0.02%. This corresponded to 9.1% and 0.09% CD25+ cells within the CD4+ lymphocyte population in control versus post-2d S4B6-injected mice. The percentage of Foxp3+CD25+ T-cells in a control mouse was 14.7% and 0.10% in a post-2d S4B6-injected mouse. These results showed that the Foxp3+CD4+CD25+ T-cell population was reduced by in vivo S4B6 administration.

Discussion

In this study, we show that tumor weight in a murine osteosarcoma (LM8) transplantation model is significantly
lower in the mice of the pre-S4B6 group (Figure 1), and that the number of pulmonary metastatic nodules is significantly lower in mice of the pre-S4B6 and post-2d S4B6 groups (Figure 3) compared to those from the control group. Flow cytometric analysis further revealed that CD4+CD25+ regulatory T-cells (Tregs) were depleted in S4B6-injected mice compared to control group mice (Figure 5). Our findings indicate that administration of S4B6 leads to a reduction in the number of Tregs in peripheral lymphoid tissues, and causes suppressive effects on tumor growth and distant metastasis in a murine osteosarcoma transplantation model.

IL-2 is a cytokine normally produced by antigen-activated T-cells and by a subset of CD4+ T-cells and is a potent growth factor necessary for immune cell function (8). Although IL-2 is not required for thymic Treg development, it is essential for physiological expansion and survival of natural CD4+CD25+ Tregs in the periphery. Furthermore, IL-2 is required for immunosuppression, which implies that constitutive IL-2 signaling maintains CD4+CD25+ Tregs in a ‘primed’ state (9). Because Tregs are not capable of producing IL-2 themselves, CD4+CD25low T-cells sustain proliferation and survival of CD4+CD25+ natural Tregs by secreting IL-2. Administration of neutralizing anti-IL-2 mAb to normal naïve mice selectively impairs this feedback mechanism, which can selectively reduce the number of Tregs of the immune system, particularly those in the periphery (10). Naturally occurring Tregs are identified by their constitutive expression of the IL-2 receptor α-chain (CD25), however, not all CD4+CD25+ T-cells function as Tregs. In addition, these cells express Foxp3, a more specific and unambiguous marker, which is a key regulatory transcription factor and crucial master control of Treg development and function (10).

Setoguchi et al. reported that neutralization of circulating IL-2 by anti-IL-2 mAb (S4B6) elicits autoimmune gastritis with circulating autoantibody directed against parietal cells and reduces peripheral CD4+CD25+ T-cells in BALB/c mice (10). However, despite the marked reduction in CD4+CD25+ cells observed in our study following S4B6 treatment, no inflammatory reactions were observed in the organs examined, which included the stomach and thyroid gland. Moreover, no autoantibodies to individual organs were detected in the blood of S4B6-treated mice using the indirect immunofluorescence technique. One possible reason for this contrasting observation may be explained by the different mouse strains used.

No studies have been reported which evaluate the efficacy of S4B6 administration and osteosarcoma suppression. In this study, we demonstrate that the administration of S4B6 causes a reduction in the number of CD4+CD25+ cells in peripheral
lymphoid tissues and causes a suppressive effect on tumor growth and metastasis in a murine osteosarcoma model. Previously, we reported similar osteosarcoma-suppressive effects using a mAb against IL2Rα (CD25) (PC61) (7). The antitumor activities by the pretreatments of S4B6 were very effective, like the ones of PC61. However, PC61 treatment is more effective at suppressing tumor growth following tumor transplantation, but not pulmonary metastasis, compared to S4B6 treatment. This may reflect that PC61 treatment may reduce Tregs quickly compared to S4B6 treatment. The target of PC61 is surface Ag of Tregs, on the other hand, the target of S4B6 is the cytokine IL-2. Therefore, Tregs in mice injected with PC61 are inactivated immediately, while several days may elapse before a reduction in Tregs is observed in mice injected with S4B6. Tumor metastasis is a lengthy process, which may explain why S4B6 treatment is effective. Due to the slow decrease of Tregs by post-2d S4B6 treatment, the transplanted tumor may not be injured by effector cells until the tumor has colonized, and it seems that it can defend against the attack of effector cells once colonized; however this S4B6 treatment was effective against pulmonary tumor metastasis, which took time.

Immunotherapy is an attractive approach to treat cancer due to the superior specificity of the immune system which selectively eliminates tumor cells. In this report, prevention of pulmonary metastasis was effective in both pre-and post-2d S4B6-treated mice. Furthermore, the onset of autoimmune disease was not induced by S4B6 administration. Taken together, our results suggest that S4B6 may be a useful tool for treatment in osteosarcoma patients.

**Acknowledgements**

This study was supported by a Grant-in-Aid for Scientific Research (C) by the Japanese Society for the Promotion of Science (JSPS) (22591672), Tokyo, Japan.

**References**


**Received December 12, 2011**
**Revised January 19, 2012**
**Accepted January 20, 2012**