Relationship between the Tissue Distribution and Antitumor Activity of Highly Branched (1→3)- β -D-Glucan, SSG

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Distribution of ^3H -labeled (1→3)- β -D-glucan(^3H]SSG) obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395, in various tissues in tumor-bearing mice was examined. ^3H]SSG administered intra-peritoneally was mainly detected in liver, spleen, kidney and tumor masses. In contrast to i.p. administration, intra-lesionally administered ^3H]SSG was not released from the tumor. Similarly, in a double grafted tumor system, ^3H]SSG was located in the administered tumor and not distributed in the distant site tumor, in spite of the fact that significant antitumor effect was shown in both tumor sites in this system. Winn assay confirmed the activation of the systemic antitumor immunity. These results suggested that the distribution of glucans would be one important factor in determining their antitumor effects. However, this would not always be necessary if systemic immunity could be induced.

Keywords antitumor glucan; *Sclerotinia sclerotiorum*; ^3H -(1→3)- β -D-glucan; double grafted tumor system; antitumor activity; tissue distribution

Antitumor (1→3)- β -D-glucans have recently been applied clinically in cancer immunotherapy.^{1,2)} It is well known that these glucans have no direct cytotoxicity on tumor cells, but show significant antitumor activity by producing modifications of the host immune systems,^{3,4)} i.e. activate macrophages, killer T cells, NK cells, etc.,^{5–9)} enhanced hematopoietic response,¹⁰⁾ complement system,¹¹⁾ blood coagulation system,¹²⁾ etc., and release cytokines.¹³⁾

We have studied the antitumor activities and immunomodulating activities of SSG obtained from the liquid-culture broth of a fungus *Sclerotinia sclerotiorum* IFO 9395.^{8,9,14)} SSG is a highly branched (1→3)- β -D-glucan, and forms a gel in physiological conditions.^{15,16)} The antitumor activities of SSG are also suggested to be mediated by the host immune systems. These concepts are supported by a great deal of experimental evidence, however, it is still difficult to explain the entire activation mechanism of their antitumor activity. As one of the approaches to clarifying this, it seemed important to be able to understand the relationship between the distribution of glucan and the antitumor activity. The distribution of glucans and their derivatives in tumor masses has been studied^{7–19)} and suggested that their distributions into the tumor was important. However, the role of glucan distributed to the tumor was not yet been defined.

In a previous study, we examined the tissue and cellular distribution of SSG in normal mice, and showed that much of the SSG was distributed and remained in liver and spleen for a long time but only a small fraction was incorporated into cells.²⁰⁾ Since it is well known that various conditions and factors in normal mice are different in tumor-bearing mice, it is necessary to examine the distribution of SSG in tumor-bearing mice to understand the mechanism of antitumor activity. Therefore, to understand the relationship between the distribution and antitumor activity of SSG, we have examined the tissue distribution of SSG in tumor-bearing mice.

MATERIALS AND METHODS

Mice Male ICR and BALB/c mice were obtained from Japan SLC, Inc., Shizuoka. These mice were 5–8 weeks of age and maintained under specific pathogen free conditions.

Tumor Sarcoma 180 and Meth A fibrosarcoma were maintained in the peritoneal cavities of ICR and BALB/c mice, respectively.

Preparation of ^3H -Labeled SSG Metabolically ^3H -labeled SSG (15–20 kBq/mg) was prepared by the procedure described previously.²¹⁾

Assay for Distribution of ^3H]SSG in Various Tissues After the administration of ^3H]SSG, the mice were sacrificed by exposure to ether. The mouse blood was collected by cardiac puncture and several tissues were removed. Each tissue was dried in air overnight, and then oxidized by combustion using an automatic sample oxidizer (ASC-113, Aloka). The ^3H]H₂O formed after combustion was measured by liquid scintillation counting.

Preparation of Tumor-Bearing Mice Cells of Sarcoma 180 were inoculated subcutaneously (s.c.) into the right hind groin of ICR mice (day 0). Seven or 14 d later, ^3H]SSG was intra-peritoneally (i.p.) or intra-lesionally (i.l.) administered and the distribution examined. These administration schedules are optimum for showing antitumor activity by SSG.

Evaluation of Antitumor Activity in a Double Grafted Tumor System ICR mice received simultaneous s.c. inoculations of Sarcoma 180 tumor cells into both right (5×10^6 cells) and left (1×10^6 cells) flanks on day 0. BALB/c mice received simultaneous s.c. inoculations of Meth A fibrosarcoma cells into both right (5×10^5 cells) and left (1×10^5 cells) flanks on day 0. Two hundred and fifty μg of SSG was administered into the right flank tumor on days 7, 9, 11, 13, and 15. Control mice were given physiological saline instead of the sample solution. On day 35, the mice were sacrificed and each tumor was weighed. The significance (p value) of any difference between the

control and treated groups was evaluated using the Student's *t*-test.

Winn Assay Meth A fibrosarcoma cells were inoculated s.c. into both the right (5×10^5 cells) and the left (1×10^5 cells) flanks of BALB/c mice on day 0. SSG was administered into the right flank tumor on days 7, 9, 11, 13, and 15. These mice were sacrificed on day 35, and the spleen cells of cured mice were obtained. Meth A fibrosarcoma cells (1×10^5) were admixed with the spleen cells (5×10^6) obtained from the tumor-regressed mice or normal mice. The mixture was inoculated s.c. into BALB/c mice. The maximum and minimum diameter of tumor masses were measured every week, and 5 weeks later, the mice were sacrificed and the tumor masses measured.

RESULTS

Tissue Distribution of [^3H]SSG in Tumor-Bearing Mice In order to examine the distribution of SSG in tumor-bearing mice, 250 μg of [^3H]SSG was administered i.p. or i.l. into ICR mice (days 14 or 7, respectively) bearing the solid form of Sarcoma 180 cells. As shown in Fig. 1A, the majority of SSG was distributed in liver, kidney, and spleen after i.p. injection. Similarly to normal mice,²⁰⁾ [^3H]SSG remained at a high concentration (e.g. about 35% in liver) in these tissues at least for one month (data not shown). Although the distribution pattern was similar to that seen in normal mice, the disappearance of SSG from the peritoneal cavity was faster. Distribution of SSG to tumor masses was also observed (about 4%). To clarify the specificity of distribution, the concentration of SSG in each tissue was calculated relative to tissue weight. As shown in Fig. 1B, SSG seemed to be distributed to spleen with a high degree of specificity. SSG in tumor slightly increased with time, although the specific activity was low

compared with other tissues.

On the other hand, with i.l. administration, which also shows strong antitumor activity similar to i.p. administration,⁸⁾ a remarkable observation was obtained (Table I). In the lesion, which was the site where SSG was administered, most of administered SSG remained, about 93% of the recovered [^3H]SSG (corresponding to about 70% of the administered [^3H]SSG) was still there even at 48 h after administration. In contrast, a negligible amount was distributed to liver, spleen, and kidney. The difference in distributions between i.p. and i.l. administration suggested that the antitumor mechanisms of the two administration routes were different.

Antitumor Activity and Tissue Distribution in the Distant Site Tumor The distribution of (1 $\rightarrow$ 3)- β -D-glucans into tumors has been thought to be important for their antitumor activity. In order to examine whether this idea plays critical role in the antitumor activity, the antitumor activity and tissue distribution in double grafted tumor system²²⁻²⁴⁾ were examined. This system was developed by Ebina *et al.* to examine the antimetastatic effect of antitumor agents, especially biological response modifiers (BRMs). The system used in this paper, however, has been modified partially to examine the antitumor effect of SSG under optimal conditions.^{8,25)} ICR mice received simultaneous s.c. inoculations of Sarcoma 180 tumor cells in the right (5×10^6 cells) and the left (1×10^6 cells) flanks. Similarly BALB/c mice received simultaneous s.c. inoculations of Meth A tumor cells in the right (5×10^5 cells) and the left (1×10^5 cells) flanks. SSG (250 μg) was injected into the right tumor from day 7 for 5 times every other day. After 35 d, weights of the right and the left tumors were recorded. As shown in Table II, a significant antitumor effect against Sarcoma 180 was observed not only in the right flank tumor, which received

Fig. 1. Distribution of i.p. Administered [^3H]SSG in Tumor Bearing Mice

Sarcoma 180 (5×10^6 cells) was inoculated into the right groin of ICR mice. Two weeks later, 250 μg of [^3H]SSG [about 100000 dpm (total [^3H]SSG)] was administered i.p. to the mice. Four (▨), 8 (■), 24 (□) or 48 h (▤) later, each tissue was removed and oxidized. The data are shown as the average of three experiments, and expressed as follows: percent to total = $\frac{\text{dpm [tissue]}}{\text{dpm [total } ^3\text{H]SSG}} \times 100$. Sup is the supernatant fraction after centrifugation of peritoneal exudate cell suspension recovered by washing mouse peritoneal cavities with Hanks' solution. PEC is the peritoneal exudate cell pellet obtained after centrifugation. (A) Distribution

TABLE I. Distribution of i.l. Administered [^3H]SSG in Various Tissues in Tumor-Bearing Mice

	4 h	8 h	24 h	48 h
		(%)		
Blood	0.34	0.30	3.37	0.00
Liver	0.11	0.33	0.40	0.36
Kidney	0.02	0.37	0.05	0.06
Heart	0.01	0.10	0.00	0.03
Spleen	0.04	0.23	0.03	0.08
Stomach	0.03	0.58	0.00	0.02
Intestine	4.02	2.51	0.09	0.32
Lung	0.06	0.07	0.00	0.04
Tumor	94.55	94.03	94.12	92.71
Feces	0.14	0.12	0.56	4.99
Excrement	0.68	1.35	0.59	1.15

Sarcoma 180 tumor cells (5×10^6) were inoculated in the right groin of ICR mice. Seven days after inoculation, 250 μg of [^3H]SSG was administered to the tumor. Four, 8, 24 or 48 h later, each tissue was removed and oxidized. The data are shown as the average of the three experiments and expressed as follows; percent = {dpm [tissue]/dpm [total recovered [^3H]SSG]} $\times 100$.

TABLE II. Antitumor Effect of SSG against Sarcoma 180 in the Double Grafted Tumor System

	No. of mice	Right tumor		Left tumor	
		Tumor weight (g $\pm$ S.D.)	CR ^{a)}	Tumor weight (g $\pm$ S.D.)	CR ^{a)}
Control	17	4.57 $\pm$ 2.27	0/17	4.08 $\pm$ 1.75	0/17
SSG	21	0.11 $\pm$ 0.22 ^{b)}	15/21	0.44 $\pm$ 0.81 ^{b)}	14/21

Sarcoma 180 tumor cells were inoculated in right (5×10^6 cells) and left (1×10^6 cells) flanks of ICR mice simultaneously on day 0. SSG (250 μg) was injected into the right flank tumor on days 7, 9, 11, 13, and 15. ^{a)} Complete regression/total number of mice. Significant difference from the control group was evaluated according to Student's *t*-test; ^{b)} $p < 0.001$.

TABLE III. Antitumor Effect of SSG against Meth A Fibrosarcoma in the Double Grafted Tumor System

	No. of mice	Right tumor		Left tumor	
		Tumor weight (g $\pm$ S.D.)	CR ^{a)}	Tumor weight (g $\pm$ S.D.)	CR ^{a)}
Control	15	4.72 $\pm$ 1.76	0/15	3.64 $\pm$ 1.83	0/15
SSG	17	0.78 $\pm$ 1.35 ^{b)}	10/17	1.96 $\pm$ 1.89 ^{c)}	5/17

Meth A fibrosarcoma cells were inoculated in right (5×10^5 cells) and left (1×10^5 cells) flanks of BALB/c mice simultaneously on day 0. SSG (250 μg) was injected into the right flank tumor on days 7, 9, 11, 13, and 15. ^{a)} Complete regression/total number of mice. Significant difference from the control group was evaluated according to Student's *t*-test; ^{b)} $p < 0.001$, ^{c)} $p < 0.05$.

SSG, but also in the left. The inhibition ratios of right and left tumors calculated from tumor weights were 97.6% and 89.2%, respectively. Furthermore, complete regressions of both right and left tumors were observed in 14/21 of the SSG treated mice.

Similarly, the results in the syngenic tumor system (Meth A in BALB/c mice) are shown in Table III. The antitumor effect against the left tumor was slightly weaker than the results in the Sarcoma 180 tumor system. However, significant antitumor effects were observed in both the right tumor, which received SSG, and the left. The inhibition ratios of the right and left tumors were 83.5% and 46.2%, respectively, and complete regression of both

TABLE IV. Tissue Distribution of [^3H]SSG Administered i.l. in Double Grafted Tumor System

	4 h	8 h	24 h	48 h
		(%)		
Blood	1.64	0.09	0.05	0.03
Liver	0.40	0.30	0.25	4.94
Kidney	0.05	0.05	0.02	1.53
Heart	0.01	0.01	0.00	0.02
Spleen	0.03	0.07	0.03	1.17
Stomach	0.45	0.51	0.01	0.10
Intestine	4.95	1.56	0.12	0.61
Lung	0.02	0.04	0.04	0.07
Right tumor (treated tumor)	92.16	96.43	98.69	88.80
Left tumor (non-treated tumor)	0.12	0.48	0.08	0.19
Feces	0.10	0.21	0.52	1.59
Excrement	0.08	0.25	0.19	0.94

Sarcoma 180 tumor cells were inoculated in the right (5×10^6) and the left (1×10^6) flanks of ICR mice, simultaneously. Seven days after inoculation, 250 μg of [^3H]SSG was administered to the right flank tumor. Four, 8, 24 or 48 h later, each tissue was removed and oxidized. The data are shown as the average of the three experiments, and expressed as follows; percent = {dpm [tissue]/dpm [total recovered [^3H]SSG]} $\times 100$.

tumors was observed in 5/17 of the SSG treated mice. These results suggest that intratumoral administration of SSG inhibits the growth of tumors at the distant site.

In the first section, it was shown that [^3H]SSG administered i.l. is not released from the tumor. To clarify the relationship between the antitumor effect and the distribution of SSG in a double grafted tumor system, [^3H]SSG was administered. ICR mice were s.c. inoculated with Sarcoma 180 cells in both right (5×10^6 cells) and left (1×10^6 cells) flanks, simultaneously, and 7 d later, 250 μg of [^3H]SSG was administered into the right flank tumor. As shown in Table IV, most of the [^3H]SSG was recovered in the right flank tumor which received [^3H]SSG (corresponding to 80% of administered [^3H]SSG), and a negligible amount was distributed to the left flank tumor. In addition, similar to Table I, a negligible amount was distributed to the other tissues. These results strongly suggest that the antitumor effect of SSG could be exhibited without direct distribution to the tumor in this case.

Mechanism of the Antitumor Effect on the Distant Site Tumor As shown in Table I, SSG was not released from the injected site by i.l. administration, despite the fact that significant antitumor effects were found in both the SSG-administered and distant tumors. These facts suggest the possibility that i.l. administered SSG activates the systemic host immune system and results in antitumor effects on the distant site. In order to confirm this, Winn assay was performed. A spleen cell suspension was prepared from BALB/c mice, which were free from Meth A tumor, in a double grafted tumor system by SSG treatment. The spleen cell suspensions (5×10^6) were admixed with 1×10^5 of Meth A fibrosarcoma and the mixture was inoculated into the right groin of BALB/c mice s.c. The spleen cells of tumor-bearing and tumor-untreated normal mice are used as controls.

Figure 2 shows the changes in the average diameter of the tumor masses. Apparently, there was hardly any growth of Meth A tumor cells admixed with splenocytes

Fig. 2. Growth Inhibition of Meth A Fibrosarcoma in Winn Assay

Meth A fibrosarcoma cells were inoculated s.c. in the right (5×10^5) and the left (1×10^5) flanks of BALB/c mice on day 0, simultaneously. SSG (250 μ g) was administered to the right flank tumor on day 7, 9, 11, 13 and 15. These mice were sacrificed on the 35th day after tumor inoculation and spleen cells of tumor-regressed mice were obtained. Meth A fibrosarcoma cells (1×10^5) were admixed with spleen cells (5×10^6) obtained from the tumor-regressed mice, tumor-bearing control mice or normal mice. The mixture was inoculated s.c. into BALB/c mice. Number of mice used in this assay are shown in Table V. The diameter was expressed as the square root of the product of maximum diameter multiplied by minimum diameter. —●—, SSG-treated cured mice; —○—, non-treated tumor-bearing mice; —△—, normal mice.

TABLE V. Growth Inhibition of Meth A Fibrosarcoma in Winn Assay

Source of spleen	No. of mice	Weight of tumor (g $\pm$ S.D.)	Inhibition ratio (%) ^{a)}
Normal mice	9	2.63 $\pm$ 1.45	—
Tumor-bearing mice ^{b)}	7	1.62 $\pm$ 0.88	38.40
Tumor-regressed mice ^{c)}	11	0.02 $\pm$ 0.04 ^{d, e)}	99.24

Meth A fibrosarcoma cells were inoculated s.c. in both the right (5×10^5) and the left (1×10^5) flanks of BALB/c mice, simultaneously, on day 0. SSG was administered to the right flank tumor on days 7, 9, 11, 13 and 15. These mice were sacrificed 35 d after tumor inoculation and spleen cells of tumor-regressed and tumor-bearing mice were obtained. Meth A fibrosarcoma cells (1×10^5) were admixed with spleen cells (5×10^6) of these mice or normal mice. The mixture was inoculated s.c. into BALB/c mice. a) Inhibition ratio was determined on day 35 after tumor inoculation. b) The saline treated mice in Meth A-BALB/c double grafted tumor system. c) The SSG treated mice and regressed both the right and the left tumor in Meth A-BALB/c double grafted tumor system. Significant difference was evaluated according to Student's *t*-test; d) significant difference from control, $p < 0.001$, e) significant difference from tumor-bearing mice, $p < 0.01$.

from SSG-treated mice. In contrast, Meth A tumor cells admixed with splenocytes from normal mice or non-treated tumor-bearing mice were grow. Table V shows the weight of tumor at 5 weeks after inoculation. The growth of tumor admixed with splenocytes from SSG-treated mice was significantly inhibited compared with those of normal and non-treated tumor bearing mice. These results suggest that SSG raises systemic host immune responses even after i.l. administration and results in the inhibition of distant tumor growth.

DISCUSSION

In this paper, we examined the tissue distribution of [3 H]SSG in tumor bearing mice and compared the relationship between this distribution and the antitumor effect. We demonstrated that 1) SSG was mainly distributed to liver, spleen, kidney and tumor masses

following i.p. administration, 2) SSG was not released from the tumor by i.l. administration, 3) in spite of the negligible amount of SSG distribution into the distant tumor site, SSG exhibited an antitumor effect at both tumor sites in a double grafted tumor system, and 4) i.l. administered SSG enhanced the host immune system of the systemic site and resulted in inhibition of tumor growth at the distant site in a double grafted tumor system.

In a previous paper,²⁰⁾ we demonstrated that SSG administered i.p. to normal mice was mainly distributed in liver, spleen and kidney. Similar results were obtained in the tumor-bearing mice. Furthermore, SSG was also distributed to the tumor masses, to an extent equivalent to the kidney (about 3.5% to administered [3 H]SSG) as shown in Fig. 1A. However, SSG was not selectively distributed to the tumor, and the selectivity (μ g/g tissue) of distribution in tumor was much lower than in other tissues. In contrast to the tumor, it is interesting that SSG showed considerable selectivity to spleen. These results suggested the possibility that the antitumor immunity induced by the activation of the systemic immune response could be the main mechanism of the antitumor effect of i.p. administered SSG. However, since there is also a report that demonstrated the importance of the distribution of the glucan to the tumor in terms of the antitumor effect,¹⁷⁻¹⁹⁾ distribution of SSG to the tumor could not be ignored and may play an important role in the antitumor effect. It is interesting to speculate as to the causes of the difference in distribution among glucans.

In spite of the fact that SSG was not released from the tumor by i.l. administration, and not distributed to the distant site tumor in a double grafted tumor system, significant antitumor effect was observed in both tumors. Since SSG did not show direct cytotoxicity to the tumor, the antitumor effect on not only the distant, but also the SSG-treated tumor, might be mediated by the host immune system. This concept was supported by the result of the Winn assay and the host-mediated antitumor mechanism in the distant site tumor could be mainly dependent on tumor (or tumor-antigen) specific cytotoxicity.

In the tumor tissue, many immunologically important cell populations, such as tumor infiltrating leukocyte (TIL),²⁶⁾ tumor associated macrophage (TAM)^{27,28)} and polymorphonuclear leukocyte (PMN), exist. Since it has been reported that systemically administered (1 $\rightarrow$ 3)- β -D-glucan is distributed to the tumor and is located in macrophages around the tumor,^{17,18)} the relationship between (1 $\rightarrow$ 3)- β -D-glucans and these cell populations is very interesting. Considering the fact that i.l. administered SSG enhanced the systemic immune system without distribution to the whole body, the cell populations mentioned above could play important roles in the antitumor effect of SSG.

In this paper, we have shown the distribution of (1 $\rightarrow$ 3)- β -D-glucan, SSG, in tumor-bearing mice, and considered the relationship between the antitumor effect and distribution of SSG. The results of the i.l. administration of SSG suggest that systemic immune responses are induced without distribution to the whole body. Therefore, it is suggested that the distribution of glucans to the tumor is one important factor in their

antitumor effect. However, their distributions is not always necessary if systemic tumor-specific (tumor antigen specific) immune responses can be induced.

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