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Antitumor Effects of an Antiangiogenic Polysaccharide from an *Arthrobacter* Species with or without a Steroid

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ABSTRACT

A sulfated polysaccharide-peptidoglycan complex, DS-4152, isolated from the culture supernatant of an *Arthrobacter* species inhibited angiogenesis and tumor growth and enhanced the antiangiogenic activity of 11 steroid hormones by 2 to 100 times. In the presence of cortisone acetate or tetrahydro S, DS-4152 suppressed chick chorioallantoic membrane angiogenesis and murine tumor M5076 cell-induced s.c. angiogenesis. The antitumor effects of DS-4152 administered in combination with a steroid whose dose level did not affect tumor growth were examined. DS-4152 significantly inhibited the growth of s.c.-implanted B16 melanoma in combination with cortisone acetate. DS-4152 plus tetrahydro S inhibited the growth of s.c. solid tumors and prolonged the survival time of mice bearing highly metastasizing M5076. The body weight increase was not affected by any administration. On the other hand, the survival of mice with ascitic M5076 tumors was not affected by the combination of DS-4152 plus tetrahydro S.

The antiangiogenic activity of DS-4152 was more potent than that of heparin. Furthermore, DS-4152 is an angiogenesis inhibitor by itself, without steroid hormones. Successive s.c. treatment with heparin caused hemorrhagic death, but with DS-4152, suppressed tumor growth without reducing body weight.

INTRODUCTION

Solid tumors require capillary proliferation for their growth. In the avascular phase, tumor masses are no more than a few millimeters in diameter (1, 2). After capillarization, tumors rapidly grow into large masses. These findings suggested that inhibition of tumor angiogenesis might be a rational therapeutic approach to the prevention of tumor growth. Several angiogenesis inhibitors have been found to suppress both angiogenesis and tumor growth (3–5). Cartilage was first reported by Eisenstein *et al.* in 1973 (3) to be a potent inhibitor of neovascularization, and cartilage extracts were then shown to inhibit angiogenesis and tumor growth (4). Extracts of vitreous (6), a guanidine extract of bovine aorta (7, 8), and protamine (9, 10) have been shown to inhibit neovascularization and/or tumor growth. Of these, protamine was used in clinical trials for breast cancer patients (11, 12), but has proved to be toxic at high doses (12). Tumor necrosis factors (13) and transforming growth factor β (14) inhibited the growth of cultured endothelial cells.

In 1983, Folkman *et al.* (15) reported that cortisone or hydrocortisone administered with heparin or a heparin fragment inhibited the growth of new capillary blood vessels in the chick embryo, in the rabbit cornea, and in some murine tumors. This combination brought about complete regression of rapidly growing metastasizing murine tumors and of some human tumors. However, the effectiveness of heparin plus cortisone is still controversial, because other groups of investigators have failed to confirm the effects (16, 17).

In the previous study (18), we tested some sulfated polysaccharides including heparin and found that a SP-PG[2] complex from an *Arthrobacter* species potently inhibited angiogenesis of chick CAM in the presence of cortisone acetate. The SP-PG complex was composed of three fractions, SP-PG-H, SP-PG-M, and SP-PG-L, as separated by gel filtration (18). The major fraction SP-PG-L had the most potent antiangiogenic activities, which were essentially equal to those of SP-PG-LM (18). The SP-PG-LM (DS-4152) is principally a D-gluco-D-galactan sulfate derived from the bacterial cell wall, and it contains organic phosphates and peptidoglycan as minor components (19, 20). The purpose of this study is to confirm the antiangiogenic effect of DS-4152 in chick CAM as well as in murine dorsal air sac fascia, and to examine its antitumor effects on syngeneic metastasizing tumors.

MATERIALS AND METHODS

Agents. The preparation and fractionation of the SP-PG complex, DS-4152, have been described previously (18, 19). Three lots of DS-4152 were equal in the percentage of D-gluco-D-galactan sulfate, organic phosphates, and peptidoglycan contents. Heparins were obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan); Sigma Chemical Co. (St. Louis, MO); Carl Roth BmbH Co. (Karlsruhe, West Germany); and Dainippon Seiyaku Co., Ltd., (Osaka, Japan). All steroid hormones were purchased from Sigma Chemical Co.: cortisone acetate; hydrocortisone; prednisolone; 6α-methyl-prednisolone; betamethasone; progesterone; 6α-methyl-17β-medroxyprogresterone acetate; 17β-estradiol; 5α-androstane; tetrahydro S; and fluoxymesterone.

CAM Assay for Angiogenesis Inhibition. The details of the CAM assay method were mentioned elsewhere (21). To examine the direct activity of the agents, a mixture of 5 μl of saline solution containing them and 5 μl of 1% (w/v) saline solution of methylcellulose was added to the 5-day CAM of fertilized Norin Cross chicken eggs (Funabashi Farm, Funabashi, Japan). After 2 days, CAM angiogenesis of treated CAM was compared with that of the control. The doses required to inhibit 50% of CAM vascularization (ID_{50} values) were calculated by Probid analysis on the basis of T/C%.

Tumors and Animals. The origin and maintenance of B16 melanoma have been described previously (20). Murine ovarian ascites tumor M5076 was kindly provided by Dr. Tashiro, Cancer Chemotherapy Center, Tokyo, Japan; the original tumor was derived from the National Cancer Institute, Bethesda, MD. Male C57BL/6 mice were obtained from Shizuoka Animal Center, Shizuoka, Japan.

Dorsal Air Sac Method. As described previously (23), M5076 ascites cells were suspended in 0.1 M phosphate-buffered saline at a concentration of 1×10^8 cells/ml. A Millipore chamber containing 0.2 ml of the suspension was implanted into a dorsal air sac of a C57BL/6 mouse. Tetrahydro S (50 mg/kg/day) and/or DS-4152 (30 mg/kg/day) were s.c. administered from the day of implantation. Four mice per group were sacrificed on Day 5 to determine the antiangiogenic effects, because fibrin exudates surrounding the chamber disturb the judgment at a later point; capillarization of the fascia was determined using a Magiscan II image analyzer (Joyce-Loebl, Ltd., Vickers Co., Gateshead, England).

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[2] The abbreviations used are: SP-PG, sulfated polysaccharide-peptidoglycan; CAM, chorioallantoic membrane; ID_{50}, 50% inhibitory dose; T/C%, data of treatment group/those of control group × 100%.

Treatment for Tumor-bearing Mice. Groups of eight mice were each inoculated s.c. with 5×10^5 B16 melanoma cells into the right inguinal lesions. Groups of ten mice were given 1×10^6 M5076 cells s.c. or i.p. The mice with B16 were administered s.c. with 30 mg/kg/day of heparin (Daiichi Pure Chemicals Co., Ltd.) or DS-4152 from Day 4. At the same time, a tapering dose of cortisone acetate was administered p.o.: 250 mg/kg/day for 3 days followed by 100 mg/kg/day for 3 days; 50 mg/kg/day for 3 days; and then a constant dose of 1 mg/kg/day until autopsy. The administration started in the mice with M5076 on Day 5. Thirty mg/kg/day of DS-4152 were given s.c., and a tapering dose of tetrahydro S was administered s.c. or p.o. One course consisted of 250 mg/kg/day for 4 days, 100 mg/kg/day for 4 days, 50 mg/kg/day for 4 days, and 1 mg/kg/day for 4 days. B16-bearing mice were then sacrificed on Day 22, and the tumor weight and the number of pulmonary metastatic foci were determined. The body weights and estimated tumor weights of the solid M5076 tumor-bearing mice were examined twice a week during the treatment. The estimated tumor weights were calculated as the length $\times$ (width)^2 of each tumor mass $\times$ 0.5. Postmortem examinations were conducted on all the mice.

RESULTS

Antiangiogenic Activities with and without Steroid Hormones. Since heparin was reported to vary in antiangiogenic activities with manufacturers (15), the activities of heparin from 4 manufacturers and those of 3 lots of DS-4152 were determined. As shown in Table 1, 2 to 5 ng of DS-4152 were enough to inhibit 50% of CAM capillarization in the presence of 0.5 μg of cortisone acetate, while 8,660 to 60,800 ng of heparin were required for inhibiting it. Furthermore, DS-4152 potently decreased CAM angiogenesis in the absence of cortisone acetate, giving an ID_{50} value of 160 to 180 ng/egg, whereas a dose of 100,000 ng of heparin did not affect CAM angiogenesis at all. The ID_{50} values of several steroid hormones for inhibiting CAM vascularity were markedly decreased by the addition of 10 ng of DS-4152, the dose which did not affect angiogenesis when used alone (Table 2). A 2- to 100-fold increase of antiangiogenic activities of the steroid hormones was observed in the presence of 10 ng of DS-4152.

A Millipore chamber containing M5076 tumor cells was transplanted into the dorsal air sac of a mouse. Five days after transplantation, a dense capillary network developed on the dorsal air sac fascia of the mouse, though no tumor mass external to the chamber was observed (Fig. 1*a*). Used alone, tetrahydro S or DS-4152 slightly decreased capillarization, while the combination of these agents greatly diminished the vascular development induced by M5076 tumor cells (Fig. 1*b*; Table 3).

Synergistic Antitumor Effects of DS-4152 with a Steroid Hormone. Among steroid hormones shown in Table 2, cortisone acetate was chosen to clarify antitumor effects in combination with DS-4152, because the agent was frequently used in antiangiogenic studies. Cortisone acetate used alone did not affect the tumor growth of B16 at all (Table 4) but accelerated the development of pulmonary metastasis. Four of 8 mice with

Table 1 *CAM angiogenesis inhibition by DS-4152 or heparin with and without cortisone acetate*

Agent	Lot	Supplier	ID_{50} (ng/egg) Alone	ID_{50} (ng/egg) In combination with cortisone acetate[a]
DS-4152[b]	1		170	3
	2		160	2
	3		180	5
Heparin	0701	Dainippon Seiyaku	>100,000	8,660
	111FEH	Daiichi Pure Chemicals	>100,000	10,900
	25-F0683	Sigma Chemical Co.	>100,000	60,800
	4331203	Carl Roth	>100,000	30,600

[a] The activities were determined with 0.5 μg of cortisone acetate, a dose which did not affect CAM angiogenesis.
[b] The DS-4152 samples were prepared as described in "Materials and Methods."

Table 2 *Increase of antiangiogenic activities of steroids by DS-4152*

Steroid hormone	ID_{50} of steroid (μg/egg)[a] Alone	With 10 ng of DS-4152[b]
Cortisone acetate	1.20	0.17
Hydrocortisone	1.10	0.16
Prednisolone	1.30	0.08
6α-Methylprednisolone	1.15	0.03
Betamethasone	0.80	0.05
Tetrahydro S	1.00	0.01
Progesterone	1.02	0.49
Medroxyprogesterone acetate	1.12	0.42
17β-Estradiol	1.96	0.28
Fluoxymesterone	1.24	0.12
5α-Androstane	2.32	0.29

[a] Three doses of each steroid within the range of 10, 1, 0.1 to 0.01 μg/egg were added to CAM alone or with 10 ng of DS-4152 (Lot 2), a dose which did not affect CAM angiogenesis, by itself.
[b] The ID_{50} value of a steroid was determined in combination with 10 ng of DS-4152.

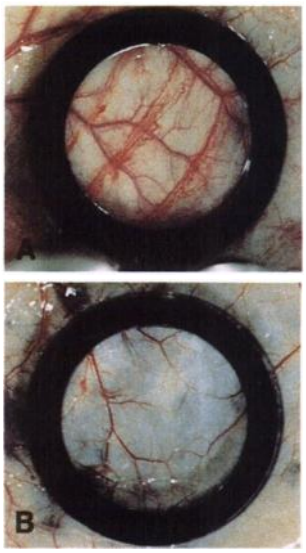

Fig. 1. Effect of DS-4152 plus tetrahydro S on M5076-induced angiogenesis. A Millipore chamber containing 2×10^7 M5076 cells was implanted into a dorsal air sac of a C57BL/6 mouse. Four mice of a group were given 30 mg/kg of DS-4152 and/or 50 mg/kg of tetrahydro S s.c. once a day for 5 days from the day of transplantation. The *area circled* with a black ring corresponds to the exact portion of the air sac fascia that was in contact with a Millipore chamber containing M5076 cells. The air sac fascia of a control mouse was densely capillarized (*a*), but the development of capillaries was very slight in the fascia of a mouse treated with DS-4152 plus tetrahydro S (*b*).

Table 3 *Effects of DS-4152 and tetrahydro S on M5076 tumor cell-induced s.c. angiogenesis of mice*

Agent (dosage/day)	Area of dense capillary network[a] Mean ± SE (mm^2)	T/C%
Saline	19.3 ± 1.2	100.0
DS-4152 (30 mg/kg)	16.4 ± 2.7	85.0
Tetrahydro S (50 mg/kg)	18.2 ± 0.8	94.3
DS-4152 (30 mg/kg) + tetrahydro S (50 mg/kg)	4.2 ± 1.1[b]	21.8

[a] A Millipore chamber containing 2×10^7 M5076 cells was implanted into four C57BL/6 mice per group, to which, then, agents were administered s.c. Four days after transplantation, capillarization of the air sac fascia was determined by an image analyzer.
[b] Statistical significance was determined by Student's *t* test ($P < 0.01$).

Table 4 *Combined effects of DS-4152 with cortisone acetate on tumor growth of B16 melanoma*

A group of 8 male C57BL/6 mice were inoculated s.c. with 5×10^5 B16 cells into right inguinal lesions on Day 0. From Day 4 each was administered with 30 mg/kg of DS-4152 once a day and/or cortisone acetate as a tapering dose (250 mg/kg/day for 3 days followed by 10 mg/kg/day for 3 days, 50 mg/kg/day for 3 days, and then a maintenance dose of 1 mg/kg/day).

Agent (mg/kg)	Cortisone acetate	Tumor wt. (g)	Body wt. (g)	No. of metastatic foci
Saline	–	4.83 ± 0.40[a] (100)[b]	25.1 ± 0.5	0
	+	4.48 ± 0.20 (93)	24.9 ± 0.2	0–11
DS-4152	–	3.58 ± 0.31[c] (74)	24.5 ± 0.3	0
	+	2.30 ± 0.14[c] (48)	25.0 ± 0.5	0–1

[a] Mean ± SE.
[b] Numbers in parentheses, T/C%.
[c] Statistical significance was evaluated by Student's *t* test ($P < 0.01$).

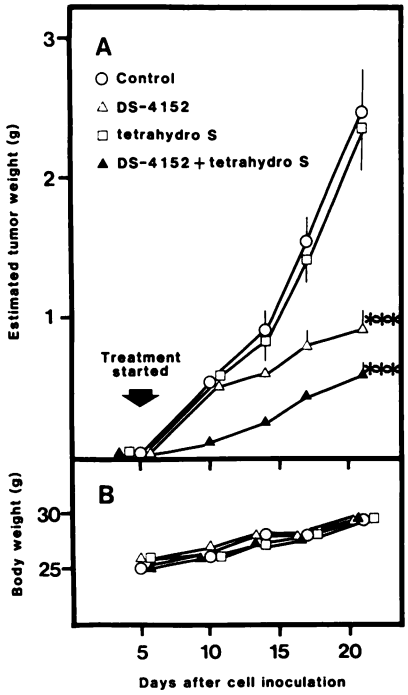

Fig. 2. The synergistic effect of DS-4152 and tetrahydro S on the growth of M5076. Groups of ten mice were implanted s.c. with 1×10^6 M5076 cells and from 5 days after inoculation given 30 mg/kg of DS-4152 and/or tapering doses of tetrahydro S. *Points*, mean of growth curves of s.c.-implanted M5076 tumors (*A*) or of growth curves of body weights (*B*); *bars*, S.E. Statistical significance was determined by Student's *t* test: ***, $P < 0.001$.

cortisone acetate had pulmonary metastasis, while none of the control mice did on Day 22. On the other hand, the treatment with 30 mg/kg of DS-4152 by itself inhibited the growth of B16 slightly, and the combination of DS-4152 with cortisone acetate significantly suppressed the growth of B16. The combination slightly decreased both the incidence of mice with metastasis (2 of 8 mice) and the number of metastatic foci compared with cortisone acetate treatment alone. The body weight of the mice was not changed by the administration. Injection of 30 mg/kg of heparin s.c. caused hemorrhagic death of all tumor-bearing mice. Subsequently tetrahydro S, a tetrahydrometabolite of cortisone acetate, was used in combination with DS-4152, because the steroid lacks glucocorticoid activity and does not enhance metastasis (25). The administration of DS-4152 and/or tetrahydro S against s.c.-implanted M5076 tumors yielded the following results (Fig. 2). The groups treated with tetrahydro S plus DS-4152 were prominent in decreases of solid tumor growth. Treatment s.c. with DS-4152 alone also suppressed the s.c. growth of M5076 significantly. Furthermore, the DS-4152 and tetrahydro S combination induced significant prolongation of survival times of mice bearing solid M5076 (Fig. 3). On Day 21, when 50% of the control mice with saline were dead and developed disseminated metastasis in the visceral organs, none of the mice treated with DS-4152 either with or without tetrahydro S had died. The s.c. combination of DS-4152 and tetrahydro S gave the best results of all the treatments. Tetrahydro S administration did not result in growth inhibition at all. On the other hand, neither treatment affected the survival time of mice with ascitic M5075. No treatment affected the body growth of the tumor-bearing mice (Fig. 2).

DISCUSSION

Heparin and heparin fragments were previously reported by Folkman *et al.* (15) to have antiangiogenic activity and antitumor effects in the presence of certain steroids. In the previous study (18), we found that a bacteria-derived SP-PG complex and its fractions had potent antiangiogenic and antitumor activities in the presence of cortisone acetate, although some other sulfated polysaccharides tested lacked those activities. In the present study, the antiangiogenic and antitumor activities of the major fraction of the SP-PG complex (DS-4152) were further investigated. DS-4152 possesses several outstanding characteristics that heparin and its fragments lack. (*a*) DS-4152 by itself inhibited angiogenesis in the absence of a steroid, while heparin showed antiangiogenic activity only in the presence of a steroid. (*b*) The anticoagulant activity of DS-4152 was markedly lower than that of heparin, even though its antiangiogenic activity was more potent. Successive s.c. treatment with heparin caused hemorrhagic death of B16-bearing mice. On the other hand, s.c. treatment with DS-4152 suppressed the tumor growth of B16 without affecting body weight increases.

In the previous study (24), the combination of heparin and cortisone acetate, a glucocorticoid, suppressed the growth of 3LL but did not result in a prolongation of survival time. This lack of effectiveness on survival time was due to the metastasis enhancement by cortisone acetate. The antiangiogenic activity of steroids is shown to be completely different from the glucocorticoid activity which tends to increase metastasis (25). Tetrahydro S lacks structural components essential for glucocorticoid activity but does have antiangiogenic activity (25, 26). It was therefore used in the present study to determine the effect of antiangiogenic agents on the survival time of M5076-bearing

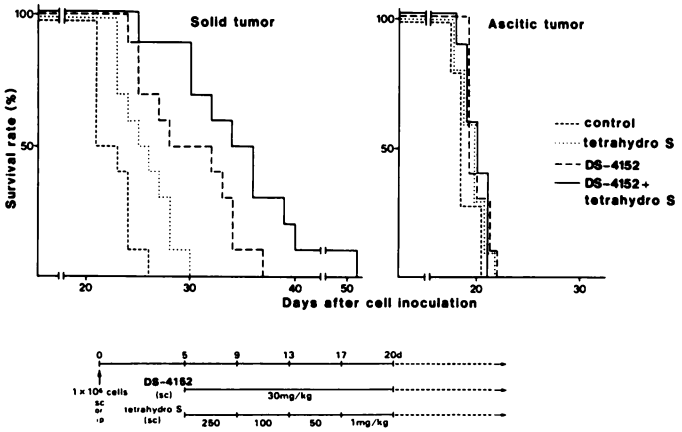

Fig. 3. The survival curves of s.c. or i.p. M5076-implanted mice with DS-4152 and/or tetrahydro S. The survival curves of solid M5076 were obtained from the mice of which growth curves were shown in Fig. 2. Treatment schedules were given under the *curves*.

mice. When M5076 tumor cells are implanted into mice, they metastasize to several organs including the liver, kidney, ovary, spleen, and bone marrow, the liver being the most frequent site of metastasis of M5076 (27). Considerable prolongation of the survival time of mice with solid M5076 was obtained by the combination of DS-4152 and tetrahydro S, which suggests that, in combination, these agents inhibit both s.c. tumor growth and metastasis formation. Pharmacokinetics remains to be studied in order to clarify the synergistic mechanisms of the two agents.

As stated previously, the synergistic antitumor effect of heparin and cortisone acetate was caused by an antiangiogenic effect mediated by the specific inhibition of endothelial cell DNA synthesis and not by the direct inhibition of tumor cell growth. The combination of DS-4152 and tetrahydro S also inhibited M5076 tumor cell-induced angiogenesis and further suppressed the growth of s.c.-implanted M5076 but not the growth of i.p.-implanted M5076. Angiogenesis is essential for the growth of solid tumors but not for that of ascitic tumors. These data indicate that the mechanisms of inhibition of tumor growth by the combination of DS-4152 and tetrahydro S are the same as those of the combination of heparin and cortisone acetate.

In conclusion, DS-4152 was shown to be a potent antiangiogenic agent by itself and to enhance the antiangiogenic activity of a steroid hormone. The combination of DS-4152 with certain potent angiostatic steroids may develop an antiangiogenic therapy of several diseases including cancers.

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