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Suppression of tumor growth by a new glycosaminoglycan isolated from the African giant snail *Achatina fulica*

Yeon Sil Lee[a], Hyun Ok Yang[b], Kuk Hyun Shin[a], Hyung Seok Choi[a], Sang Hoon Jung[a], Yong Man Kim[b], Deok Kun Oh[c], Robert J. Linhardt[d], Yeong Shik Kim[a,*]

[a] *Natural Products Research Institute, College of Pharmacy, Seoul National University, 28 Yeonkun-Dong, Jongno-Ku, Seoul 110-460, South Korea*
[b] *Department of Molecular Medicine, Asan Institute for Life Sciences and College of Medicine, University of Ulsan, Seoul 138-736, South Korea*
[c] *Department of Biotechnology and Bioscience, Sejong University, Seoul 143-741, South Korea*
[d] *Division of Medicinal and Natural Products Chemistry, Department of Chemistry and Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA 52242, USA*

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Abstract

Acharan sulfate is a new type of glycosaminoglycan from the giant African snail, *Achatina fulica*. Acharan sulfate, which has a primary repeating disaccharide structure of α-D-*N*-acetylglucosaminyl-2-*O*-sulfo-α-L-iduronic acid, was studied as a potential antitumor agent in both in vivo and in vitro assays. The antiangiogenic activity of acharan sulfate was evaluated in the chorioallantoic membrane assay and by measuring its effect on the proliferation of calf pulmonary artery endothelial cells. In vivo, a matrigel plug assay showed that acharan sulfate suppressed basic fibroblast growth factor (bFGF)-stimulated angiogenesis and lowered the hemoglobin (Hb) content inside the plug. Acharan sulfate was administered s.c. at two doses for 15 days to C57BL/6 mice implanted with murine Lewis lung carcinoma in the back. It was also administered i.p. to ICR mice bearing sarcoma 180 at a dose of 30 mg/kg. Subcutaneous injection of acharan sulfate at doses of 10 and 30 mg/kg decreased tumor weight and tumor volume by 40% without toxicity or resistance. Intraperitoneal injection of acharan sulfate also decreased tumor weight and volume by 40% in sarcoma 180-bearing mice. These results suggest that the antitumor activity of acharan sulfate may be related to the inhibition of angiogenesis.

Keywords: Glycosaminoglycan; Acharan sulfate; Tumor suppression; Angiogenesis; Matrigel assay

1. Introduction

Angiogenesis, or neovascularization, is the formation of new capillaries from pre-existing blood vessels and is a fundamental process involved in a number of physiological (Folkman, 1971, 1972; Folkman and Shing, 1992) and pathophysiological processes (Folkman, 1995; Carmeliet and Jain, 2000). In cancer, this process contributes to the progressive growth and metastasis of solid tumors (Liotta et al., 1991). Tumor angiogenesis is regulated by the production of angiogenic stimulators including members of the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF) families (Colville-Nash and Willoughby, 1997; Kim et al., 1993). Drugs that interfere with angiogenesis, by halting the action of angiogenic proteins, might reduce the size of tumors and keep them in a dormant state. Angiogenic inhibitors such as angiostatin and endostatin can modulate angiogenesis both at the primary site and at the downstream sites of metastasis (O'Reilly et al., 1994, 1997). The potential use of these and other natural and synthetic angiogenesis inhibitors is currently being studied intensively by many laboratories (Mohan et al., 2000; Suh et al., 1997; Minamiguchi et al., 2001; Kim et al., 2000). Such agents may have reduced toxicity and may be less likely to generate drug resistance than conventional cytotoxic drugs (Keshet and Ben-Sasson, 1999).

Heparin/heparan sulfate interacts with various angiogenic growth factors (Capila and Linhardt, 2002). Angiogenic growth factors induce a response in target endothelial cells by binding to cognate cell-surface tyrosine kinase receptors (Gale and Yancopoulos, 1999). The interaction of heparin-binding growth factors to tyrosine kinase receptors is

* Corresponding author. Tel.: +82-2-740-8929; fax: +82-2-765-4768.
E-mail address: kims@plaza.snu.ac.kr (Y.S. Kim).

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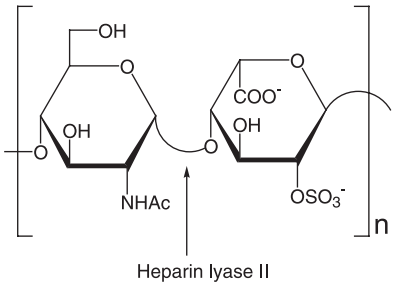

Fig. 1. Structure of acharan sulfate. The average number of disaccharide units (*n*) is approximately 300. Heparin lyase II can cleave the α1→4 linkage, giving a repeating disaccharide unit.

modulated by heparan sulfate proteoglycans. Acharan sulfate isolated from the giant African snail, *Achatina fulica*, is a novel member of the glycosaminoglycan family (Kim et al., 1996). This GAG has a major repeating disaccharide structure of →4)-α-D-GlcNpAc(1→4)-α-L-IdoAp2S(1→, where GlcNpAc is 2-acetamido 2-deoxyglucopyranose, IdoAp is idopyranosyluronic acid, Ac is acetyl and S is sulfo (Fig. 1). This polysaccharide has a molecular weight of 135,000, based on high-performance liquid chromatography–gel permeation chromatography (HPLC–GPC) analysis. Recently, we observed that acharan sulfate interfered with the bFGF mitogenicity of heparin in vitro, suggesting its possible utility as an angiogenesis inhibitor (Wang et al., 1997).

In the present study, we carried out experiments to evaluate the antiangiogenic activity of acharan sulfate. We demonstrated that acharan sulfate inhibited new blood vessel formation in the in vivo matrigel and chorioallantoic membrane assays. Moreover, we observed a substantial antitumor activity against sarcoma 180-induced solid and primary tumors in Lewis lung carcinoma-bearing C57BL/6 mice.

2. Materials and methods

2.1. Materials

Trypan blue solution (0.4%), *N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid), methylthiazol-2-yl-2,5-diphenyl-tetrazolum bromide (MTT), phosphate-buffered saline (PBS), DEAE-Sepharose Fast Flow, heparin, Drabkin's reagent kit 525, *p*-nitrophenyl phosphate, 5-fluorouracil (5-FU), and all-*trans*-retinoic acid were purchased from Sigma (St. Louis, MO, USA). Trypsin–EDTA, penicillin–streptomycin, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), HBSS, Roosevelt Park Memorial Institute Medium (RPMI) 1640 medium, and bFGF were from GIBCO (Gaithersburg, MD, USA). Alcalase was from Novo Korea (Seoul, Korea). Doxorubicin was kindly provided from Boryung Pharmaceuticals (Seoul, Korea).

2.2. Preparation of acharan sulfate

Acharan sulfate was isolated from the soft body tissue of the giant African snail by proteolysis of defatted tissue and purified by fractional precipitation and ion-exchange chromatography as previously described (Kim et al., 1996; Jeong et al., 2001). In brief, 500 mg of the crude sample was dissolved in 50 ml of 50 mM sodium phosphate buffer (pH 7.0) and applied to a column (2.5 × 50 cm) of DEAE-Sepharose equilibrated with the same buffer. The column was eluted with a stepwise gradient with 50 mM sodium phosphate buffer containing 0.0, 0.5 and 1.0 M NaCl. Elution was monitored at 210 nm and the flow rate was set at 30 ml/h. Each fraction was collected, dialyzed and freeze-dried to give a white powder. All samples were subjected to ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and agarose gel-electrophoresis. Simultaneously, the fractions were depolymerized by heparin lyase II and the resulting reaction product was analyzed by strong anion-exchange high-performance liquid chromatography (SAX-HPLC) as previously described (Jeong et al., 2001). The structure of the disaccharide repeating unit of acharan sulfate was determined by ^{1}H-NMR (Kim et al., 1996; Jeong et al., 2001).

2.3. Chicken chorioallantoic membrane assay

These assays were essentially carried out according to the previous reports (Tanaka et al., 1986; Oikawa et al., 1990). In brief, fertilized chicken eggs were kept in a humidified incubator at 37°C. On the third day of incubation, about 2 ml of egg albumin was aspirated by means of an 18-gauge hypodermic needle, to detach the developing chorioallantoic membrane from the shell. One and half days later, sample-loaded thermanox coverslips from Nunc (Naperville, IL, USA) were air-dried and applied to the chorioallontoic membrane surface for testing of angiogenesis inhibition by acharan sulfate. Two days later, 1 ml of 10% fat emulsion (Intralipose) was injected into the membrane and the avascular zone was observed under a dissecting microscope. Inhibition of angiogenesis was assessed when the avascular zone exceeded 3 mm. The heparinase II-depolymerized acharan sulfate was also tested in the antiangiogenic activity as negative control, to eliminate the possibility that a contaminant in acharan sulfate was responsible for the observed activity. The concentration of acharan sulfate used in this assay was selected based on the concentration of heparin that had previously been applied in the same assay (Collen et al., 2000).

2.4. Animals

Seven-week-old specific pathogen-free (SPF) male C57BL/6J mice were supplied from Japan SLC (Shizuoka, Japan) for the matrigel plug assay. Five-week-old SPF

male C57BL/6Ntac mice were purchased from Samtaco BioKorea (Osan, Korea). SPF male ICR mice were purchased from Daehan Biolink (Umsung, Korea). All animal studies were carried out in a pathogen-free barrier zone at Seoul National University Hospital in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals. Animals were fed on sterilized animal chow and water ad libitum and they were housed at 23 ± 0.5 °C, 10% humidity under a 12-h light–dark cycle.

2.5. In vivo matrigel plug assay

The matrigel plug assay was performed as previously described (Passaniti et al., 1992). Acharan sulfate, dissolved in water, bFGF and heparin, dissolved in 0.1% bovine serum albumin/PBS, were mixed with liquid matrigel from Collaborative Biomedical Products (Bedford, MA, USA) in proportions not exceeding 1% of the total volume of matrigel. A mixture of 0.5 ml matrigel with acharan sulfate or vehicle was injected s.c. into C57BL/6J mice. After injection, the matrigel rapidly formed a plug. Seven days later, the mouse skin was easily pulled away to expose the matrigel plug, which remained intact. The amount of hemoglobin (Hb) inside the matrigel was measured using the Drabkin method (Drabkin and Austin, 1932) and the Drabkin reagent 525 for the quantitation of blood vessel formation. The concentration of Hb was calculated based on Hb standard measured simultaneously.

2.6. Cell culture

Lewis lung carcinoma cells from American Type Cell Collection (Rockville, MD, USA) were maintained in DMEM supplemented with heat-inactivated 10% fetal bovine serum (Life Technologies, Grand Island, NY), 100 units/ml penicillin, and 100 μg/ml streptomycin. Calf pulmonary arterial endothelial cells and sarcoma 180 (Korea Cell Line Bank, Seoul, Korea) were cultured in RPMI 1640 media containing 10% FBS and 1% antibiotics in an incubator at 37°C and with a humidified atmosphere containing 5% CO_2. The viable cells alone were counted with a hemocytometer, using the Trypan blue dye exclusion test (Kaltenbach et al., 1958).

2.7. Proliferation of calf pulmonary artery endothelial cells

Calf pulmonary artery endothelial cells were seeded in a 24-well plate at a cell density of 10^5 cells/well in 90% RPMI and 10% FBS. After a 24-h incubation, cells were treated with various concentrations of acharan sulfate. Three days later, new media and MTT solution were added to each well. After incubation at 37 °C for 4 h, the absorbance at 540 nm of treated cells was compared to that of control cells.

2.8. Antitumor activity

Male C57BL/6 mice were inoculated s.c. in the back with Lewis lung carcinoma cells (1×10^6/animal) on day 0. When the tumor volume was at least 60–100 mm^3, acharan sulfate was administered into the subcutaneous region near the tumor mass at two doses of 10 and 30 mg/kg for 15 days. The size of tumors in all groups was measured using a digital-caliper and the volume of tumors was determined using the formula $width^2 \times length \times 0.52$ (Voest et al., 1995; Cao et al., 1995). The effects of acharan sulfate on tumor growth and host survival were also measured by evaluating tumor volume, tumor weight and percentage increase in the lifespan of tumor hosts (Oguchi et al., 1987; Kusumoto et al., 1991). For calculating the survival time, mice were inoculated i.p. with 10^6 sarcoma 180 cells/mouse on day 0 and the treatment with two doses of acharan sulfate (50 and 100 mg/kg, i.p.) was started 24 h after inoculation for 9 consecutive days. The control group was treated with saline. The median survival time (MST) for each group ($n = 7$) was observed and the antitumor activity of the test compounds was compared with that of the control group by measuring the increase of the lifespan. For solid tumor development, ICR mice were injected with 0.1 ml of sarcoma 180 suspension into the right hind limb. Six days after tumor transplantation, mice randomized into six groups were injected i.p. with acharan sulfate (50 and 100 mg/kg) and 5-FU (25 mg/kg) once a day for 9 days. Eight days after treatment, animals were killed by cervical dissociation, and solid tumors were removed and weighed.

2.9. Data analysis and statistics

Data are presented as means ± S.E. or as percentages of control. Statistical comparisons between groups were performed using the Student's *t*-test. The values at $P < 0.01$ and $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Characterization of acharan sulfate

Carbazole assay of the polysaccharide eluted at 1.0 M NaCl from a DEAE-Sepharose ion-exchange chromatography column showed that it contained uronic acid (Jeong et al., 2001). Azure A dye binding assay demonstrated the presence of sulfo groups in the structure, consistent with it being a glycosaminoglycan. ^{1}H-NMR analysis of the intact polysaccharide demonstrated the presence of two anomeric protons with chemical shifts corresponding to the H-1 of GlcNpAc at δ5.1 and H-1 of IdoAp2S at δ5.2, respectively. This upfield shift of the anomeric proton of GlcNpAc is attributable to the unusual →4)-α-D-GlcNpAc(1→4)-α-L-

IdoAp2S(1→ sequence of acharan sulfate (Kim et al., 1996). The insensitivity of this fraction to chondroitinase ABC strongly indicated that it was entirely composed of a new type of glycosaminoglycan. The heparinase II-depolymerized acharan sulfate afforded a single (>95%) repeating disaccharide product of the structure ΔUAp2S-GlcNpAcα, β, where ΔUAp is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid.

3.2. Effect of acharan sulfate on inhibition of angiogenesis

The chorioallantoic membrane assay was first carried out to determine whether acharan sulfate has antiangiogenic activity. On day 4.5, chorioallantoic membranes were treated with different doses of acharan sulfate for 2 days. The dose–response relationship for the appearance of an avascular zone was determined. The inhibitory effect of acharan sulfate on the treated chorioallantoic membrane is shown in Fig. 2 and Table 1. Compared to the effect of vehicle as control, which did not have antiangiogenic activity in the treated chorioallantoic membrane, acharan sulfate at doses of 5 and 10 μg/pellet showed antiangiogenic activity of 48.3% and 55.6%, respectively. The effect of acharan sulfate on chick embryonic angiogenesis decreased in a dose-dependent fashion. Retinoic acid strongly inhibited angiogenesis (80%) even at 1 μg/egg, but it may have a toxic effect

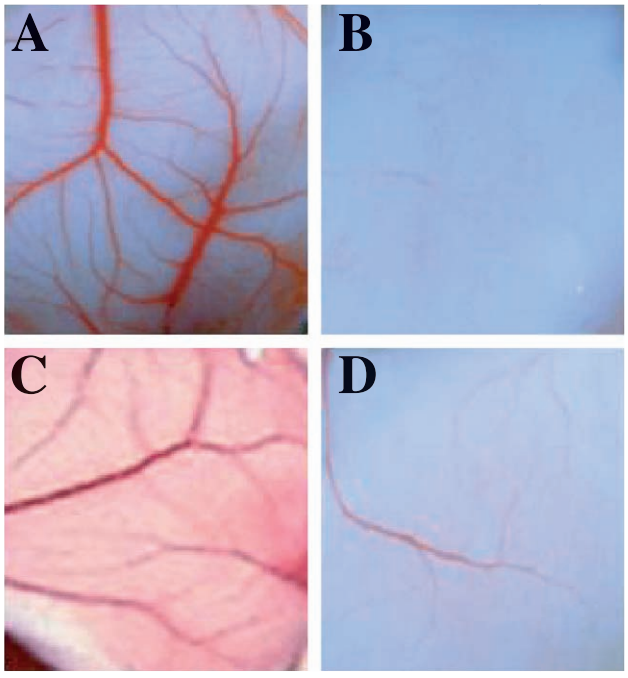

Fig. 2. Photographic image of control egg and one treated with acharan sulfate. Plastic coverslips immersed in samples were implanted on the top of growing chorioallantoic membranes at day 5 as described in Materials and methods. (A) Negative control (water); (B) retinoic acid (1 μg/ml); (C) heparinase II-depolymerized acharan sulfate (50 μg/ml); (D) intact acharan sulfate (10 μg/ml) (magnification 2 ×).

Table 1
Effect of acharan sulfate on chick embryonic angiogenesis

Compounds	Concentration (μg/egg)	Eggs showing antiangiogenesis[a]	Total eggs tested	% Inhibition
Control (H_2O)	–	3	54	5.6
Retinoic acid[b]	1	20	25	80.0
Acharan sulfate depolymerization mixture	50	1	30	3.0
Acharan sulfate I	5	14	29	48.3
Acharan sulfate II	10	15	27	55.6

[a] Antiangiogeneis was assessed when the avascular zone exceeded 3 mm.
[b] Retinoic acid was used as positive control.

on cells. The heparin lyase II-depolymerized acharan sulfate did not inhibit angiogenesis, indicating that a contaminant within the intact acharan sulfate was not acting as an angiogenic inhibitor. We then evaluated the effect of acharan sulfate on ongoing angiogenesis process in the mouse matrigel plug assay. Matrigel, heparin (10 units/500 μl), and bFGF (100 ng/500 μl), with or without acharan sulfate were injected s.c. into C57BL/6 mice. Seven days later, matrigel plug was excised to allow clear visualization of the intact vessels of the matrigel. The control samples in the matrigel assay had no vessels. A combination of 100 ng/ml bFGF and 10 units/ml (~ 65 μg/ml) of heparin pulled many vessels from the surrounding tissues into the matrigel. The new vessels were abundantly filled with intact red blood cells, indicating the formation of a functional vasculature inside the matrigel and blood circulation in newly formed vessels by angiogenesis induced by bFGF and heparin. Fifty micrograms of acharan sulfate in combination with bFGF and heparin slightly prevented vessel induction, indicating that acharan sulfate suppressed bFGF-stimulated angiogenesis. We next measured the Hb content inside the matrigel plugs to quantify angiogenesis. Whereas bFGF and heparin increased the Hb content to 11.8 g/dl and that of the control was 0.3 g/dl, acharan sulfate decreased the heparin and bFGF-elevated Hb content to about 8.6 g/dl (Fig. 3). Antiangiogenesis in this assay did not result from the effect of the bFGF vehicle and the injection sites showed no signs of inflammation or hemorrhage.

3.3. Effect of acharan sulfate on in vitro cell proliferation

We examined the effect of increasing concentrations of acharan sulfate on calf pulmonary artery endothelial cell viability. Acharan sulfate showed no cytotoxic effect on calf pulmonary artery endothelial cells (data not shown). We then examined the effect of acharan sulfate on the proliferation of calf pulmonary artery endothelial cells in the MTT assay. Acharan sulfate exhibited an inhibitory effect in a concentration-dependent fashion. After a 3-day treatment, the growth inhibitions of 12.5%, 15.2%, and 24.9% were

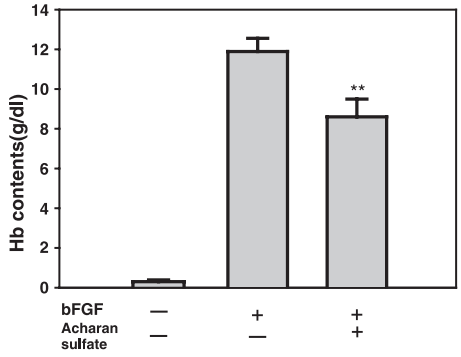

Fig. 3. Effect of acharan sulfate on bFGF-induced angiogenesis in mouse model. Matrigels (4 μl) mixed with 100 ng/ml bFGF and 10 units/ml of heparin in a vehicle of 0.1% PBS with or without 50 μg/ml of acharan sulfate were injected s.c. into C57BL/6J mice. Matrigel alone was used as negative control and 100 ng/ml bFGF and 10 units/ml heparin were used as positive control. After 7 days, mice were killed and the skin was pulled back to expose the matrigel plug. The content of Hb inside the matrigel was measured. Each value represents mean ± S.E.M. for at least five animals. The data are significantly different from the control; $^{**}P<0.01$.

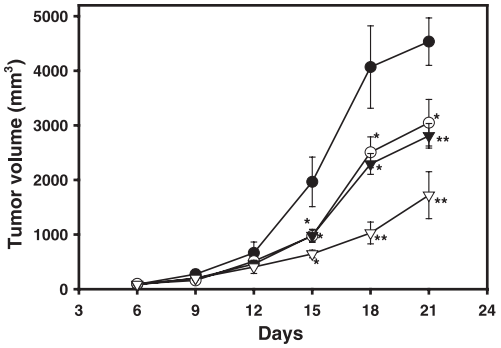

Fig. 5. Effect of acharan sulfate on tumor volume in Lewis lung carcinoma-bearing mice. C57BL/6 mice were implanted with Lewis lung carcinoma and the treatment with acharan sulfate was started when tumors were approximately 75–100 mg. Mice were treated at two doses of 10 and 30 mg/kg s.c. once daily. A 10-mg/kg dose of doxorubicin was injected i.v. every 5 days. Each point represents mean ± S.E.M. for 8 mice (saline, doxorubicin) or 12 mice (AS). The data are significantly different from those of the control group; $^{*}P<0.05$, $^{**}P<0.01$. ● saline; ○ acharan sulfate 10 mg/kg; ▼ acharan sulfate 30 mg/kg; ▽ doxorubicin 10 mg/kg.

observed at acharan sulfate concentrations as low as 0.1, 1 and 10 μg/ml, respectively (Fig. 4).

3.4. Inhibition of tumor growth in mice implanted with Lewis lung carcinoma cells

To evaluate the effect of acharan sulfate on tumor growth, it was used to treat C57BL/6 mice inoculated with Lewis lung carcinoma cells and the results are shown in Figs. 5 and 6. Daily subcutaneous injections of 10 and 30 mg/kg suppressed the growth of primary tumors during a 15-day treatment. At the end of treatment, tumor growth was inhibited by 32.8% (3049 mm^3) and 38.1% (2809 mm^3), at a dose of 10 and 30 mg/kg, respectively, as compared to that of control mice treated with saline alone (4534 mm^3). In contrast, the tumor grew rapidly to sizes >4000 mm^3 in saline-treated mice during the same 15-day treatment period. Doxorubicin, as positive control, was administered i.v. every 5 days at a dose of 10 mg/kg. It inhibited tumor growth by 62.0% (1721 mm^3). The acharan sulfate-treated mice did not lose weight during treatment, indicating that acharan sulfate showed little or no toxicity. On day 21, tumor tissues were removed and weighed. It was found that the tumor weight was reduced in a dose-dependent manner by acharan sulfate, as shown in Fig. 6. The mean tumor weight was reduced by 37.8% (2.8 ± 0.2 g) at 10 mg/kg and by 48.9% (2.3 ± 0.2 g) at 30 mg/kg, compared with the saline group (4.5 ± 0.7 g). Doxorubicin significantly reduced tumor weight by 68.0% (1.6 ± 0.2 g). However, the loss of tumor weight in the doxorubicin-treated mice was greater than that of the control mice and the injection area was significantly damaged by treatment.

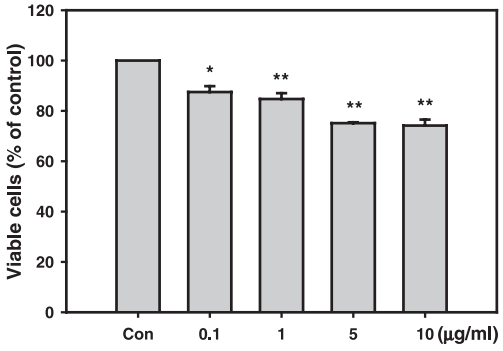

Fig. 4. Effect of acharan sulfate on calf pulmonary endothelial cell proliferation by MTT assay. The cells were seeded at a density of 2×10^5 cells/ml into each well. After 24 h, the indicated concentrations of acharan sulfate were given. After 3 days, the percentage of viable cells was measured by MTT assay. The data are significantly different from the control; $^{*}P<0.05$, $^{**}P<0.01$.

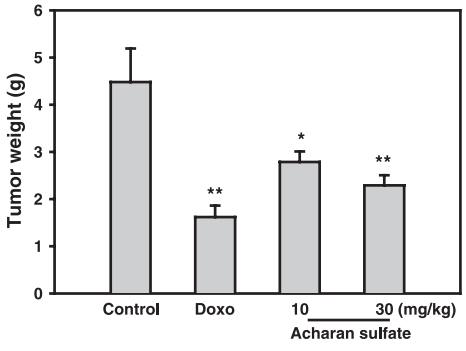

Fig. 6. Effect of acharan sulfate on tumor weight in Lewis lung carcinoma-bearing mice. C57BL/6 mice were implanted with Lewis lung carcinoma and treatment with acharan sulfate was started when tumors were approximately 75–100 mg. Mice were treated at two doses of 10 and 30 mg/kg s.c. once daily for 15 days. A 10-mg/kg dose of doxorubicin was injected i.v. every 5 days. At the end of experiment, tumors were dissected and weighed. The data are significantly different from those of the control; $^{*}P<0.05$, $^{**}P<0.01$.

3.5. Inhibition of tumor growth in mice bearing sarcoma 180 cells

The effect of acharan sulfate on solid tumor induced by sarcoma 180 tumor cells in ICR mice was tested and the results are shown in Fig. 7. As shown in Fig. 7A, the average tumor volume in the control was 8804 ± 465 mm^3. The tumor volume in groups treated with 5-FU decreased by 82.1% (1572 ± 202 mm^3), compared with the control level. Acharan sulfate at the dose of 50 mg/kg decreased the tumor volume by 45.0% (4799 ± 345 mm^3) and reduced tumor weight by 39.6% (4.3 ± 0.1 g), while 5-FU at the dose of 25 mg/kg decreased tumor weight by 75.1% (1.8 ± 0.3 g), compared with that of the control (7.1 ± 0.1 g) (Fig. 7B).

3.6. Enhancement of survival time in sarcoma 180 ascitic tumor

The effect of acharan sulfate on survival time in sarcoma 180-bearing mice was tested and the results are summarized in Fig. 8. The median survival time (MST) in the control mice was 22.4 ± 2.2 days, while it was increased in a dose-dependent manner by treatment with acharan sulfate at two doses of 30 and 50 mg/kg/day for 9 consecutive days. Acharan sulfate increased the lifespan by 1.5-fold (34.3 ± 2.6 days) compared with that of the control group at the dose of 30 mg/kg and it by 1.8-fold at the dose of 50 mg/kg (40.6 ± 3.1 days) (Fig. 8). The animal group treated with the 25 mg/kg dose of 5-FU, as positive control, showed a much stronger enhancement of MST (42.8 ± 4.2 days).

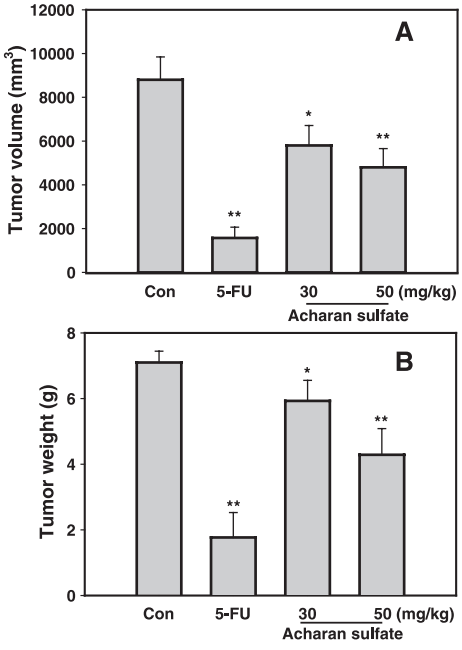

Fig. 7. Effects of acharan sulfate on tumor volume (A) and tumor weight (B) in sarcoma 180-bearing mice. Solid-type sarcoma 180 tumor was prepared by subcutaneous transplantation of 1×10^6 cells into the right groins of mice on day 0. After 6 days, the treatment with saline, acharan sulfate (30 and 50 mg/kg) and 5-FU (25 mg/kg) was started. The data are presented as means ± S.E.M. of nine mice. The data are significantly different from those of the control; $*P<0.05$, $**P<0.01$.

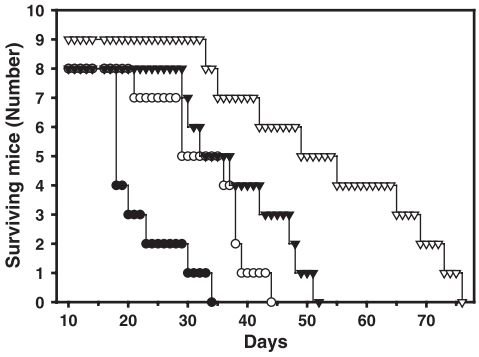

Fig. 8. Effect of acharan sulfate on survival time of mice sarcoma with 180 ascitic tumor. Sarcoma 180 cell suspensions of 1×10^7 cells/ml were prepared. Mice were inoculated i.p. with 0.1 ml of the above suspensions on day 0. 5-FU was used as positive control. Acharan sulfate (30 and 50 mg/kg) was i.p. administered for 8 consecutive days, starting 12 h after implantation of tumor cells. The symbols shown in this figure correspond to ● control, ○ acharan sulfate 30 mg/kg, ▼ acharan sulfate 50 mg/kg, ▽ 5-FU.

4. Discussion

We previously reported that, in the presence of heparin, acharan sulfate markedly decreased bFGF mitogenicity, without exhibiting anticoagulant activity (Wang et al., 1997), suggesting that acharan sulfate had antiangiogenic activity. Acharan sulfate did not reduce the number of living cells during a 40-h incubation with the same acharan sulfate concentrations that were used in the mitogenicity assay. Thus, the inhibition of mitogenicity was not due to toxicity. Based on the above findings, we decided to examine whether the inhibitory activity was specific for endothelial cells. We first assessed the proliferation of calf pulmonary artery endothelial cells by measuring the formation of a chromogen from MTT. Treatment of acharan sulfate at 10 μg inhibited cell growth by 25%. Such a result suggests that acharan sulfate does not directly influence the proliferation of endothelial cells. Next, we tested acharan sulfate in the chorioallantoic membrane assay to determine whether it could inhibit in vivo angiogenesis. We observed that acharan sulfate markedly inhibited the development of capillary networks in the chorioallantoic membrane at two concentrations (5 and 10 μg/membrane). The antiangiogenic activity of acharan sulfate was also confirmed by performing an in vivo mouse matrigel plug assay. Acharan sulfate inhibited the formation of neovessels induced by a combination of bFGF and heparin in the matrigel. These results are consistent with acharan sulfate having an effect on the mitogenicity of bFGF with heparin in F32 cells (Wang et al., 1997). The decrease in bFGF-enhanced angiogenesis in vivo implies that acharan sulfate may suppress abnormal,

excessive angiogenesis. To investigate the antitumor effect of acharan sulfate in mice bearing murine Lewis lung carcinoma tumors, it was given s.c. at a distant site from the primary tumor. We speculated that one of the mechanisms for the antiangiogenic action of acharan sulfate might be the inhibition of matrix metalloprotease activity. However, acharan sulfate shows no detectable antiprotease activity (data not shown). Acharan sulfate also showed substantial antitumor activity against sarcoma 180-induced solid tumor and primary tumor in Lewis lung carcinoma-bearing C57BL/6 mice. A remarkable increase of the life-span was observed in mice bearing sarcoma 180 ascitic tumors. Ascites fluids are direct nutritional sources for tumor cells. According to our recent results, acharan sulfate decreases VEGF-induced capillary tube formation and inhibits the activity of VEGF without affecting its production (Ghosh et al., 2002). Because the dimer of VEGF is stabilized by heparin (Fairbrother et al., 1998), it is possible that acharan sulfate binds the dimer of VEGF and inhibits its mitogenic activity. Heparan sulfate proteoglycans are involved in transforming a normal cell to a tumor cell (Blackhall et al., 1991). Thus, a specific sequence of acharan sulfate might interfere in the oncogenic transformation of the process. Acharan sulfate may also bind to specific proteins on the cell surface and the resulting complex may transfer signals into the cell (Sasisekharan et al., 2002). For example, cytokines, such as pleiotrophin and midkine (Zhang and Deuel, 1999), are known to bind to glycosaminoglycans and acharan sulfate may free these molecules from the cell surface or the extracellular matrix.

In conclusion, we demonstrate that acharan sulfate may act as an angiogenesis inhibitor and as an antitumor agent. Further studies are necessary to elucidate the mechanisms of antitumorigenic and antiangiogenic actions of acharan sulfate.

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References

Blackhall, F.H., Merry, C.L., Davies, E.J., Jayson, G.C., 1991. Heparan sulfate proteoglycans and cancer. Br. J. Cancer 85, 1094–1098.

Cao, Y., Chen, C., Weatherbee, J.A., Tsang, M., Folkman, J., 1995. Gro-β, a C-X-C chemokines, is an angiogenesis inhibitor that suppresses the growth of Lewis lung carcinoma in mice. J. Exp. Med. 182, 2069–2077.

Capila, I., Linhardt, R.J., 2002. Heparin–protein interactions. Angew. Chem. 41, 390–412.

Carmeliet, P., Jain, R.K., 2000. Angiogenesis in cancer and other diseases. Nature 407, 249–257.

Collen, A., Smorenburg, S.M., Peters, E., Lupu, F., Koolwijk, P., Van Noorden, C., Van Hinsbergh, V.W., 2000. Unfractionated and low molecular weight heparin affects fibrin structure and angiogenesis in vitro. Cancer Res. 60, 6196–6200.

Colville-Nash, P.R., Willoughby, D.A., 1997. Growth factors in angiogenesis: current interest and therapeutic potential. Mol. Med. Today 3, 14–23.

Drabkin, D.L., Austin, J.H., 1932. Spectrophotometric constants for common hemoglobin derivatives in human, dog, and rabbit blood. J. Biol. Chem. 98, 719–722.

Fairbrother, W.J., Champe, M.A., Christinger, H.W., Keyt, B.A., Starovasnik, M.A., 1998. Solution structure of the heparin-binding domain of vascular endothelial growth factor. Structure 6, 637–648.

Folkman, J., 1971. Tumor angiogenesis: therapeutic implications. N. Engl. J. Med. 285, 1182–1186.

Folkman, J., 1972. Anti-angiogenesis: new concept for therapy of solid tumors. Ann. Surg. 175, 409–416.

Folkman, J., 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med. 1, 27–31.

Folkman, J., Shing, Y., 1992. Angiogenesis. J. Biol. Chem. 267, 10931–10934.

Gale, N.W., Yancopoulos, G.D., 1999. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev. 13, 1055–1066.

Ghosh, A.K., Hirasawa, N., Lee, Y.S., Kim, Y.S., Shin, K.H., Ryu, N., Ohuchi, K., 2002. Inhibition by acharan sulfate of angiogenesis in experimental inflammation models. Br. J. Pharmacol. 137, 441–448.

Jeong, J.A., Toida, T., Muneta, Y., Kosiishi, I., Imanari, T., Linhardt, R.J., Choi, H.S., Wu, S.J., Kim, Y.S., 2001. Localization and characterization of acharan sulfate in the body of the giant African snail *Achatina fulica*. Comp. Biochem. Physiol. 130, 513–519.

Kaltenbach, J.P., Kaltenbach, M.H., Lyons, W.B., 1958. Dyes for differentiating live and dead ascites cells. Exp. Cell Res. 15, 112–117.

Keshet, E., Ben-Sasson, S.A., 1999. Anticancer drug targets: approaching angiogenesis. J. Clin. Invest. 104, 1497–1501.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., Ferrara, N., 1993. Inhibition of vascular endothelial growth factor induced angiogenesis suppresses tumor growth in vivo. Nature 362, 841–844.

Kim, Y.S., Chang, I.M., Toida, T., Park, Y., Linhardt, R.J., 1996. A new glycosaminoglycan from the giant African snail *Achatina fulica*. J. Biol. Chem. 271, 11750–11755.

Kim, M.S., Lee, Y.M., Moon, E.J., Kim, S.E., Lee, J.J., Kim, K.W., 2000. Anti-angiogetic activity of torilin, a sesquiterpene compound isolated from *Torilis japonica*. Int. J. Cancer 87, 269–275.

Kusumoto, T., Maehara, Y., Sugimachi, Y., Kohnos, S., Sugimachi, K., 1991. Hyperthermia potentiates the cytotoxic activity of 1-hexylcarbamoyl-5-fluorouracil in sarcoma-180 cells and human malignant tumor cells. Anticancer Res. 11, 1963–1966.

Liotta, L.A., Steeg, P.S., Stetler-Stevenson, W.G., 1991. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 64, 327–336.

Minamiguchi, K., Kumagai, H., Masuda, T., Kawada, M., Ishizuka, M., Takeuchitin, T., 2001. Thiolutin, an inhibitor of HUVEC adhesion to vitronectin, reduces paxillin in HUVEC and suppresses tumor cell-induced angiogenesis. Int. J. Cancer 93, 307–316.

Mohan, R., Sivak, J., Ashton, P., Russo, L.A., Pham, B.Q., Kasahara, N., Raizman, N.B., Fini, M.E., 2000. Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor-2, including expression of matrix metalloproteinase gelatinase B. J. Biol. Chem. 275, 10405–10412.

Oguchi, T., Ando, T., Matsunaga, K., Fujii, T., Yoshikumi, C., Nomoto, K., 1987. Effect of PK on cytotoxicity against sarcoma-180 in tumor-bearing mice. Anticancer Res. 7, 481–484.

Oikawa, T., Hirotani, K., Ogasawara, H., Katayama, T., Nakamura, O., Iwaguchi, T., Hiragun, A., 1990. Inhibition of angiogenesis by vitamin D_3 analogue. Eur. J. Pharmacol. 178, 247–250.

O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Folkman, J., 1994.

Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79, 315–328.

O'Reilly, M.S., Boehm, T., Shing, Y., Folkman, J., 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88, 277–285.

Passaniti, A., Taylor, R.W., Pili, R., Guo, Y., Long, P.V., Haney, J.A., Pauly, R.R., Grant, D.S., Martin, G.R., 1992. Simple and quantitative method for assessing antiangiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. Lab. Invest. 69, 519–528.

Sasisekharan, R., Shriver, Z., Venkataraman, G., Narayanasami, U., 2002. Roles of heparan sulfate glycosaminoglycans in cancer. Nat. Rev., Cancer 2, 521–528.

Suh, H.S., Jung, E.J., Kim, T.H., Lee, H.Y., Park, Y.H., Kim, K.W., 1997. Anti-angiogenic activity of ursodeoxycholic acid and its derivatives. Cancer Lett. 113, 117–122.

Tanaka, N.G., Sakamoto, A., Tohgo, Y., Nishiyama, T., Ogawa, H., 1986. Inhibitory effects of anti-angiogenic agents on neovascularization and growth of the choriollantoic membrane (CAM)—the possibility of a new CAM assay for angiogenesis inhibitor. Exp. Pathol. 30, 143–150.

Voest, E.E., Benyon, B.M., O'Reilly, M.S., Truitt, G., D'Amato, R.J., Folkman, J., 1995. Inhibition of angiogenesis in vivo by interleukin 12. J. Natl. Cancer Inst. 87, 581–586.

Wang, H., Toida, T., Kim, Y.S., Capila, I., Hileman, R.E., Bernfield, M., Linhardt, R.J., 1997. Glycosaminoglycans can influence fibroblast growth factor-2 mitogenicity without significant growth factor binding [published erratum appears in Biochem. Biophys. Res. Commun. 242, 248, 1998]. Biochem. Biophys. Res. Commun. 235, 369–373.

Zhang, N., Deuel, T.F., 1999. Pleiotrophin and midkine, a family of mitogenic and angiogenic heparin-binding growth and differentiation factors. Curr. Opin. Hematol. 6, 44–50.