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ORIGINAL ARTICLE

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Antiangiogenic and antitumoral properties of a polysaccharide isolated from the seaweed *Sargassum stenophyllum*

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Abstract The potential antiangiogenic and antitumoral properties of SargA, a polysaccharide extracted from the brown marine alga *Sargassum stenophyllum*, were studied in assays carried out in chick embryos and mice. Gelfoam plugs containing SargA (2–1500 μg/plug) implanted in vivo into fertilized 6-day-old chicken eggs induced dose-related antiangiogenic activity in the chorioallantoic membrane (CAM). By day 8, the highest dose of SargA alone decreased the vessel number in the CAM by 64%, but coadministered with hydrocortisone (156 μg/plug, which alone caused 30% inhibition) failed to potentiate its antiangiogenic effect. Combined with basic fibroblast growth factor (50 ng/plug), SargA (1500 μg/plug) abolished angiogenesis stimulated by this factor in both chick embryo CAM and in subcutaneous (s.c.) Gelfoam plugs implanted in the dorsal skin of Swiss mice (measured as plug hemoglobin content). Repeated s.c. injections of SargA (1.5 or 150 μg per animal per day for 3 days) close to B16F10 melanoma cell tumors in the dorsal skin of mice markedly decreased tumor growth in a dose-related fashion (by 40% and 80% at 2 weeks after the first injection, respectively), without evident signs of toxicity. SargA caused graded inhibitions of migration and viability of cultured B16F10 cells and also displayed antithrombotic activity in human plasma (5 mg/ml increased thrombin time 2.5-fold relative to saline). Thus, SargA exhibits pronounced antiangiogenic as well as antitumoral properties. Although the latter action of SargA might be related to the inhibition of angiogenesis, the polysaccharide also exerts cytotoxic effects on tumor cells. Because of its chemical characteristics and polyanionic constituents, we postulate that the polysaccharide SargA might modulate the activity of heparin-binding angiogenic growth factors.

Keywords Polysaccharide · Angiogenesis · Antitumoral activity · *Sargassum stenophyllum* · Angiostatic activity

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Introduction

During embryogenesis, blood vessel formation (i.e. vasculogenesis) proceeds at high rates to keep pace with the growth of the body, but this primordial process is usually negligible after adult stature is achieved [1, 2]. Defined as the generation of new capillaries from pre-

existing blood vessels, angiogenesis plays a fundamental role in a number of physiological processes, for example the reproductive cycle of fertile women and the healing of wounds in both soft tissue and bone [3, 4].

The formation of new blood vessels is a tightly controlled process that can be rapidly up- or downregulated [5]. However, during the angiogenic response, the normally quiescent endothelial cells become activated in response to various angiogenic mediators [6]. In various overlapping or parallel tissue events, activated endothelial cells show proteolytic activity in both the basement membrane and proximal extracellular matrix, detach from neighboring cells, migrate and proliferate to form tubes (tubulogenesis) and are engaged in forming a new basement membrane [7, 8].

Angiogenesis is regulated by the production of several angiogenic stimulators including basic fibroblast growth factor (b-FGF), a member of a large family of structurally related proteins, and the vascular endothelial growth factor family (VEGF) [9, 10]. These growth factors interact with glycosaminoglycans (GLG) and proteoglycans, such as heparan sulfate proteoglycan (HSPG) present in the extracellular matrix, basal lamina, and cell surface receptors, regulating the growth, proliferation, migration, differentiation, and survival of endothelial cells, among a variety of cell types [11, 12]. One current hypothesis is that heparan sulfates from HSPG serve to directly crosslink FGF to specific binding regions on competent FGF receptors (FGFRs) to form high-affinity activating complexes [13]. In this context, it is suggested that structural analog polysaccharides could bind growth factors to specific regions on receptors, modulating or antagonizing the related responses by forming complexes of higher affinity than those mediated by heparan sulfates.

In the adult organism, the usually stable and strictly regulated angiogenesis process when uncontrolled becomes critical for the progressive growth and metastasis of solid tumors [14]. These normally cannot grow larger than a few millimeters unless the development of new blood vessels is induced to invade and vascularize them [4, 15, 16]. Physiopathological angiogenesis (neovascularization) also is a feature of several non-malignant incapacitating diseases, including rheumatoid arthritis, endometriosis, psoriasis and proliferative retinopathies [3, 17, 18].

The intrinsic capacity of virtually all human solid malignant tumors to grow and metastasize results from their ability to produce substances that induce angiogenesis. Furthermore, it has been shown that inhibition of angiogenesis inhibits tumor growth, which has led to clinical interest in identifying factors that could inhibit neovascularization, or that induce regression of pathological vasculature [19–21]. From the preclinical and clinical position, the possibility of naturally occurring angiogenesis inhibitors that may be very effective without expressing significant toxicity, unlike conventional cytotoxic drugs, has motivated intensive current investigations in many laboratories [22–27].

In recent years, an increasing number of studies have shown the bioactive potential of compounds produced by marine organisms [28, 29]. Indeed, several species of algae (e.g. *Fucus* spp., *Laurencia* spp.) have been found to be sources of secondary metabolites, polysaccharides and glycoproteins with antitumoral, antiviral or immune-stimulant activity [30–35]. Among these macromolecules, polysaccharides from the *Sargassum* genus have been reported to have antitumoral activity [36]. Structural studies performed by Duarte et al. [37] on *S. stenophyllum*, a brown seaweed (Phaeophyceae) with a wide geographical range, have revealed the biosynthesis of two fucoidan groups (poly-L-fucopyranose with different levels of sulfation) in this algal species, compounds from which show a basic structure very similar to the heteropolysaccharides of extracellular matrix (ECM) from animal tissue.

Since in animal extracellular matrix GLG and HSPG molecules may act as a scaffold, regulating the activity of angiogenic factors such as bFGF by means of HSPG–bFGF–FGFR (FGF receptor) complexes [10, 38, 39], we hypothesized that compounds containing structural analog polysaccharides would be effective modulators of angiogenesis. This led us to carry out experiments to evaluate the capacity of a polysaccharide derivative obtained from *S. stenophyllum* (SargA) to inhibit blood vessel formation in embryonic and adult models: the in vivo chick embryo chorioallantoic (CAM) membranes and the Gelfoam implant assay in 2-month-old mice. Moreover, we also investigated the antitumoral activity against B16F10 murine melanoma cells, assayed in vivo and in vitro.

Materials and methods

Materials

Methylthiazol-2-yl-2,5-diphenyl-tetrazolum bromide (MTT), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), Coomasie brilliant blue G-250, Giemsa stain, and bovine thrombin, were purchased from Sigma (St. Louis, Mo.). Other materials were obtained as follows: methylcellulose (Aldrich, Milwaukee, Wis.); hydrocortisone (Biobrás, Brazil); phosphate-buffered saline (PBS; Purchase, N.Y.); sodium heparin (Cristália, Brazil); Drabkin reagent kit (Biodiagnostica, Brazil); trypsin-EDTA, penicillin/streptomycin, RPMI-1640 medium, bFGF, L-glutamine, sodium bicarbonate (Gibco, Auckland, New Zealand); Gelfoam (Pharmacia and Upjohn, Kalamazoo, Mich.); bacto-agar (Difco, USA); fetal bovine serum (FBS; Life Technologies, Grand Island, N.Y.); and HCl, ethanol, methanol, potassium bromide (KBr), acetic acid, sodium tetrahydroborate ($NaBH_4$), potassium hydroxide (KOH) (Reagen, Brazil).

Isolation of polysaccharide SargA from cell wall of *S. stenophyllum*

The brown alga *S. stenophyllum* was collected at Armação do Itapocoroy beach (Santa Catarina State, Brazil) in January 2001. About 815 g of fresh material was precleaned by removing residual matter and rinsing under tap water. Excess water was drained off and small fragments of plant and animal tissues were carefully removed. About 66.5 g (fresh weight) of the selected biomass were collected and washed with three volumes of deionized water, repeatedly, dried (65°C) to constant weight and then stored at −20°C.

For the purpose of cell wall polysaccharide isolation, algal biomass (66.5 g, dry weight) was sequentially treated in accordance with previously described procedures [40, 41]. Briefly, the extraction of the cell wall polysaccharides was carried out with 4 *M* KOH over 12 h at 25°C in a rotary shaker, with 10 mg $NaBH_4$ added to the medium. After treatment, the suspension was centrifuged (10,000 rpm/15 min/4°C), the supernatant collected, treated with AcOH to pH 5.2 and centrifuged. The polysaccharide A fraction was obtained as precipitate and the supernatant was treated with an excess of EtOH (approximately three volumes), yielding the polysaccharide B fraction. Residual potassium acetate was washed out through dialysis (molecular weight cut-off 3 kDa) against ten volumes of distilled water, overnight, with subsequent lyophilizing and storage of the polysaccharides at −20°C until analysis. The polysaccharide A and B fractions in powder from *S. stenophyllum* (Sarg) were initially diluted at 5 g/100 ml with deionized water and then further diluted to the required concentrations [41]. For further biological assays, only the polysaccharide A fraction was used.

Electrophoresis of the polysaccharide A fraction was performed on cellulose acetate paper (2.5×14 cm; Cellogel Chemetron, Milan, Italy) as follows: 0.1 *M* zinc acetate buffer (pH 6.6), 200 V, 1 h. Detection was carried out by staining with aqueous 0.5% toluidine blue solution. Infrared spectra were recorded for a KBr pellet of a test sample (3 mg, accurately weighed) with a BOMEM Michelson infrared spectrophotometer, operating with a laser frequency at 15,799.7 cm^{-1} and scanning between 4000 and 400 cm^{-1}. The recorded data were analyzed using Win-Bomem (version 3.01 C) software.

The protein content of polysaccharide A fraction was determined through dosage according to the method of Bradford [42], using bovine serum albumin for the preparation of a standard curve (0.5 µg to 100 µg/0.1 ml). Quantification of uronic acids was carried out by the method of Blumenkrantz and Asboe-Hansen [43] using D-glucuronic acid as standard. The absorbance was read at 520 nm using a spectrophotometer (Shimadzu HIS, UV/visible), quartz cuvettes and a reading cycle for samples with the automatic subtraction of control absorbance.

CAM assay

The ability of the compounds to inhibit angiogenesis was determined by assays essentially carried out in agreement with previous reports [19]. In brief, fertilized chicken eggs ($n = 8$), supplied by the poultry industry (Macedo Koerich, SC, Brazil) were kept in a humidified (33%) incubator at 37°C. After 48 h (E2), the eggs were removed from the incubator and a window (10 mm diameter) was opened in the egg shell at a position adjacent to the embryo (non-viable embryos were discarded). The treatment in ovo was performed by implanting a disk-shaped Gelfoam (methylcellulose) plug (10 µl volume, 3 mm diameter) impregnated with the substance on the outer one-third of the 6-day CAM where capillaries were still growing (CAM assay). After these manipulations the windows were closed with black binding Cellophane tape and the eggs returned to the incubator until the 8th day (E8 embryonic day).

The concentrations of SargA administered to the vascular membranes by means of the methylcellulose disk supports ranged from 2 to 1500 µg/disk. Additional experimental groups were either cotreated with the same doses of SargA together with hydrocortisone (156 µg/plug), or given hydrocortisone (HC, 156 µg/plug) or heparin sodium (HP, 50 IU/plug) alone. Blank methylcellulose plugs (ultrafiltered water as solvent, pH 7.2) and heparin sodium plus hydrocortisone (HP + HC) were used as negative and positive controls, respectively [44].

Each substance or combination of substances was adsorbed on methylcellulose and the solution (final concentration 0.45%) was air-dried on a Teflon-coated metal tray (3 mm diameter) and applied to the developing extra embryonic membrane for testing of angiogenesis inhibition by SargA.

Two days later (E8), the zone around the methylcellulose disk was examined under a dissecting microscope (Olympus, Tokyo, Japan). Inhibition of angiogenesis was determined by the decrease in the vessel number in the area surrounding the methylcellulose disk (appearance of an avascular zone exceeding 2 mm) and defined as the percentage reduction in vessels compared to the control.

Animals

Male specific pathogen-free Swiss-Tecpar 2-month-old mice were housed in a light-controlled room (light on from 7:00 A.M. to 7:00 P.M.) at a room temperature of 24 ± 1°C and fed on sterilized animal chow and water ad libitum. All animal studies were carried out in accordance with the procedures outlined in protocol number 256/CEUA, for the care and ethical use of animals in research (CEUA/UFSC, Florianópolis, SC, Brazil).

In vivo Gelfoam plug assay

Polysaccharide SargA (1500 µg; 35 mg/kg) plus 50 ng bFGF dissolved in PBS, bFGF only or HP + HC were used as negative and positive inhibition controls. Each compound or combination was adsorbed (50 µl) on a plug of Gelfoam, a gelatin sterile sponge (6 mm diameter x3 mm). Each Gelfoam plug was implanted subcutaneously (s.c.) into the rear right flank of a mouse ($n = 5–8$). The mice were killed 2 weeks later by ether overdose and the skin was carefully pulled away to expose the intact Gelfoam plug. The amount of hemoglobin (Hb) inside the Gelfoam was measured using the Drabkin reagent as a quantifiable index of blood vessel formation [45]. The concentration of Hb is expressed as milligrams per deciliter and calculated based on a Hb standard measured simultaneously using the following equation: sample absorbance/standard absorbance x10, as described by Lee et al. [46].

Cell culture

B16F10 murine melanoma cells were maintained in RPMI-1640 medium supplemented with heat-inactivated 10% FBS and 1% streptomycin antibiotic, L-glutamine and sodium bicarbonate. The cells were cultured in a humidified incubator at 37°C in air containing 5% CO_2.

In vivo antitumoral activity

B16F10 melanoma cells (1×10^6 in 200 µl/animal) were inoculated s.c. into the back of mice ($n = 7$) on day 0. When the tumor volume was at least 150 mm^3 the mice were injected s.c. as close as possible to the tumor site with a single daily dose of SargA (1.5 and 150 µg/animal; 35 mg/kg) for 3 days. Tumors of similar size were matched for use in control (receiving RPMI-1640 medium only) and experimental groups. Tumor volumes and animal body weight were measured every 3 days throughout the 15-day experiment, and the effect of SargA on tumor volume (cubic millimeters) was recorded when the experiment was over. The tumor volume was measured using a precision caliper and estimated according to the following standard formula: tumor volume (mm^3) = $width^2 \times length \times 0.52$, according to Ingber et al. [47].

Wound assay for B16F10 melanoma cell migration

Confluent monolayers of B16F10 melanoma cells cultured in six-well plates (Corning, Corning, N.Y.) were scratched with a razor blade to produce a cell-free zone on the bottom of the well, which was then washed with Ca^{2+}- and Mg^{+2}-free PBS. The cells were cultured in RPMI-1640 medium containing increasing concentrations of SargA from 50 to 200 µg/ml (2 ml well volume) for 12 h at 37°C. Control cells were cultured in medium alone. The experiments were performed in triplicate.

The cells were fixed with absolute methanol and stained by the Giemsa method. The central region of the monolayer adjacent to the wound into which cells had migrated was examined by transillumination light microscopy (×100). The cells that had migrated into the wound in ten randomized fields (1 mm^2) of the well were counted using a graduated glass and the results are presented as the average number of cells per field [48, 49].

Assay of cell viability

B16F10 melanoma cells were seeded (2.5×10^6) in RPMI-1640 medium in 96-well plates (Corning 3594, Corning, N.Y.). After 24 h of incubation, cells were treated with increasing concentrations of SargA (50–2000 µg/well; 200 µl well volume). The medium was removed 3 days later and the wells carefully washed several times with PBS (100 µl/well) to remove any remaining test compounds before performing the MTT assay [50]. Fresh RPMI-1640 medium was added to MTT solution (5 mg/ml in PBS) at a ratio of MTT solution to medium of 1:10. After incubation in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h, the solution was supplemented with 50 µl 20% SDS in 0.02 *M* HCl/well to dissolve formazan and the plates were incubated for a further 30 min. The absorbance at 570 nm was then determined on an automatic microplate reader (Wallac, model Victor 2, workstation 1420 multilabel counter). The results are expressed as percent of control, and the linearity of the relationship between the SargA concentration and the number of viable cells was examined. All the experiments were performed in triplicate.

Assay for anticoagulant activity

Thrombin time (TT) was measured using human plasma and three concentrations of SargA (0.05–5 mg/ml). Volumes of 170 µl human plasma and 30 µl test solution were mixed and the mixture was incubated at 37°C for 2 min. A volume of 100 µl bovine thrombin (7.5 U/ml) was added to the mixture and the time to clot formation was recorded [51].

Data analysis and statistics

Data are presented as means ± SEM from at least two independent experiments. The statistical analyses were performed using one-way analysis of variance (ANOVA) and the least squares difference method (LSD) to analyze deviations. The influences of substance, concentration, site and treatment period were tested. Effects were considered to be statistically significant at *P* values less than 0.05.

Results

Characterization of SargA isolated from cell wall of *S. stenophyllum*

Alkaline treatment (4 *M* KOH, 10 mg $NaBH_4$, at room temperature) of *S. stenophyllum* followed by neutralization (AcOH, pH 5.2), yielded a cell wall polysaccharide fraction which we named polysaccharide A fraction (SargA). The yield of SargA employing this extraction protocol was 4.92% relative to the initial algal biomass.

Paper electrophoresis revealed the existence of only one polysaccharide in the polysaccharide A fraction. The FT-IR spectrum of SargA showed characteristic signals for alcohol (C–O 1030 cm^{-1} and O–H 3400 cm^{-1}) and aldehyde (2929 cm^{-1}) functions, which are typical of that type of macromolecule. Signals in the 1715 cm^{-1} region, that are characteristic of C = O groups, indicated that the monosaccharide constituents of the SargA polysaccharide were aldoses. Other chemical groups, NH (1630 cm^{-1}) and carboxyl (1408 cm^{-1}), indicated the presence of acid groups in the primary chemical structure (Fig. 1). A typical signal (817.003 cm^{-1}) suggesting the occurrence of equatorial sulfate groups in the C-2 and C-3 positions or a sulfate group linked to the C-6 position was also found [52]. A low acidic content (0.34 mol%) and complete absence of protein content were found as structural features of SargA. Preliminary results (data not published) of the primary structure of SargA show a monosaccharide composition characterized by sugar units of α-L-fucose, β-D-mannose, β-D-galactose, β-D-xylose, α-D-glucose and glucuronic acid. These findings are similar to previous results [37] of studies on a set of fucoidans isolated from *S. stenophyllum* also originating from southern Brazil. A more detailed analysis of the fine chemical structure of SargA is in progress and will be published elsewhere.

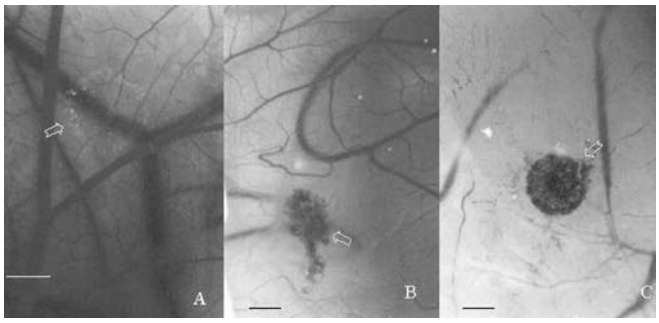

Fig. 2 Photographs of the inhibitory effect of SargA on vascularization of the 8-day CAM. In each egg, one control or one drug-containing disk-shaped support (*arrows*) was placed on the 6-day old CAM, where capillaries were still growing. **a** Negative control (water); **b** SargA 1500 μg/disk; **c** positive control, heparin plus hydrocortisone (50 IU and 156 μg/disk) (*bars* 1 mm)

Effect of SargA on vessel development in the CAM assay

The CAM assay was first carried out to determine whether SargA displayed antiangiogenic activity. Whereas control CAMs treated with vehicle developed an average of 103 vessels on the disk limits, those treated with SargA (1500 μg/disk) exhibited consistently fewer vessels around the disk, such that the avascular zone around the disk was similar to that of CAMs treated with heparin plus hydrocortisone (50 IU/disk and 156 μg/disk, respectively; Fig. 2). Moreover, as shown in Fig. 3, this antiangiogenic effect of 2–1500 μg/disk of SargA was clearly dose-related (5–64% inhibition), but the simultaneous application of hydrocortisone (156 μg/disk), which alone caused 30% inhibition, failed to potentiate its antiangiogenic effect. The data displayed in the inset of Fig. 3 show that the positive control performed with heparin plus hydrocortisone (50 IU/disk and 156 μg/disk, respectively) strongly inhibited angiogenesis in the CAM by 90%. Furthermore, when the effects of hydrocortisone

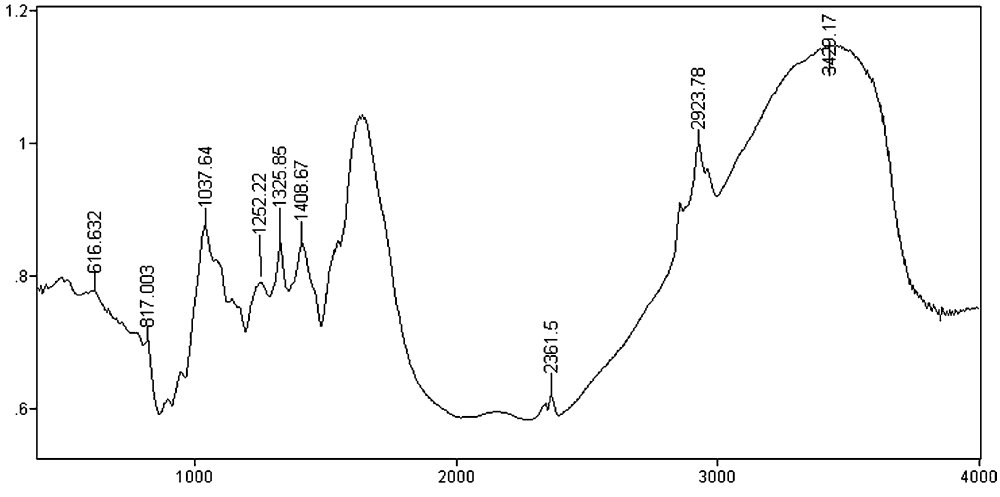

Fig. 1 Absorbance, expressed as wavelength (cm^{-1}), obtained by infrared spectroscopy of polysaccharide SargA derived from *S. stenophyllum* cell wall. Chemical group references: alcohol (C–O 1030 cm^{-1} and O–H 3400 cm^{-1}), aldehyde (2929 cm^{-1}), C = O (1715 cm^{-1}), NH (1630 cm^{-1}) and carboxyl (1408 cm^{-1})

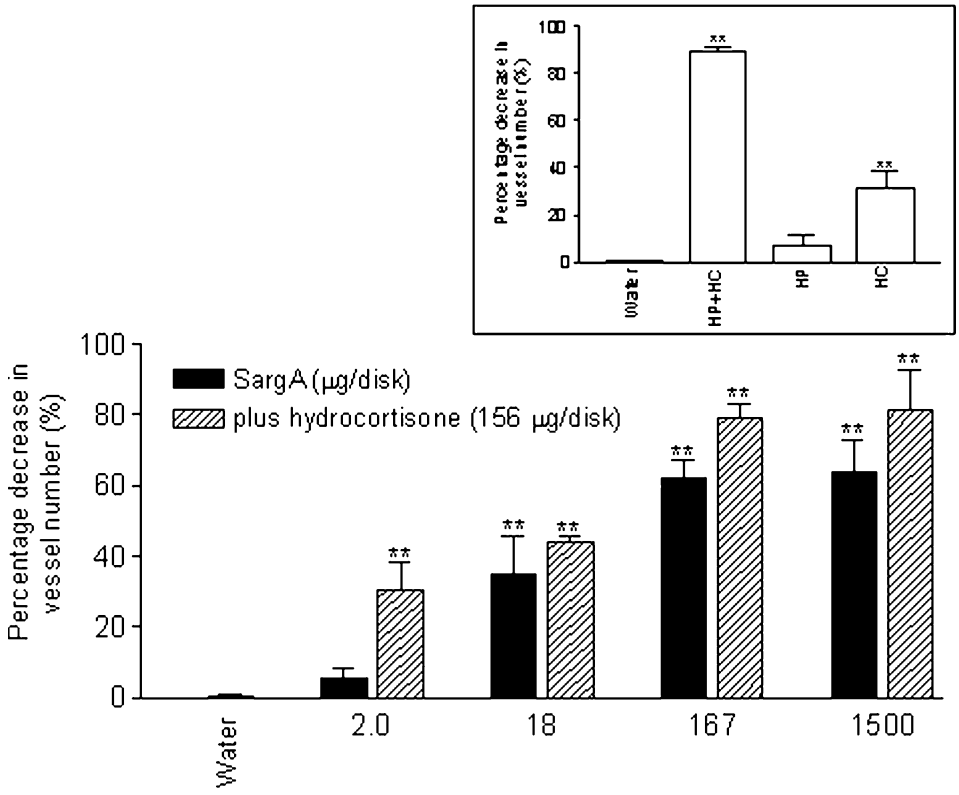

Fig. 3 Inhibitory effect of SargA on vascularization of the 8-day CAM. The controls (*inset*) were performed with vehicle (water, negative control) and heparin plus hydrocortisone (50 IU and 156 μg/disk, positive control). The CAMs were also treated with heparin (50 IU/disk) and hydrocortisone (156 μg/disk) alone. SargA was administered alone or together with hydrocortisone. Results are expressed as the percent reduction in the number of microvessels around the drug-containing disk compared to the number of vessels present in the controls. Each *bar* represents the mean ± SEM of eight eggs. $^{**}P < 0.01$

(156 μg/disk) and heparin (50 IU/disk) administered alone were compared the former reduced the number of vessels by 30%, whereas the latter did not significantly inhibit angiogenesis compared to control. No thrombi or apparent morphological changes in microvessel growth patterns were observed with any of the compounds tested, although SargA alone did decrease slightly the total body length of chick embryos on day 8 (data not shown).

On the other hand, the results shown in Fig. 4 show that when SargA was applied concomitantly with the angiogenic substance bFGF on the same disk, it fully prevented the angiogenic effect of bFGF, as compared to control (vehicle).

In vivo Gelfoam plug assay in mice

To investigate whether the inhibitory effects of SargA on embryonic angiogenesis were reproducible on adult vessels, we evaluated the ongoing angiogenic process in the mouse Gelfoam plug assay (Fig. 5). This consisted of the s.c. implantation of a plug of Gelfoam (sterile gelatin sponge) into which was injected 50 μl heparin (50 IU/plug) plus hydrocortisone (156 μg/plug), bFGF (50 ng/plug) with or without SargA (1.5 mg/plug), or PBS (vehicle) as control. By 15 days after implantation, PBS-treated Gelfoam plugs excised from controls clearly exhibited newly formed vessels, whereas those originally containing heparin plus hydrocortisone (positive control) had a significantly reduced vessel content. Treatment with bFGF alone gave rise to the growth of many vessels from the surrounding tissues into the Gelfoam plug, but this effect was effectively ablated by simultaneous application of SargA in the same plug (1500 μg/plug). The Hb content in the Gelfoam plugs from the bFGF-alone group was 128% greater than that in the PBS control group, and com-

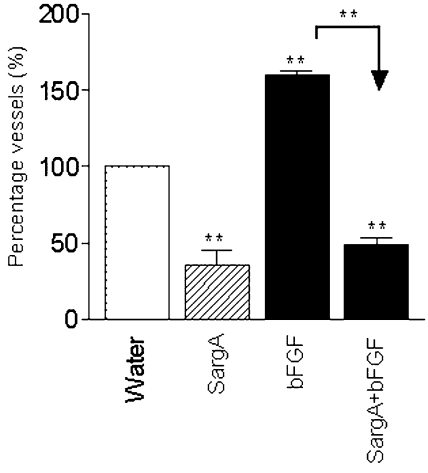

Fig. 4 Inhibitory effect of SargA on vascularization of the 8-day CAM. The controls were performed with vehicle (water, negative control). Drugs administered were SargA alone (1500 μg/disk), bFGF alone (50 ng/disk) and SargA plus bFGF (50 ng/disk). Results are expressed as the number of microvessels around the drug-containing disk as a percentage of control. Each *bar* represents the mean ± SEM of eight eggs. $^{**}P < 0.01$

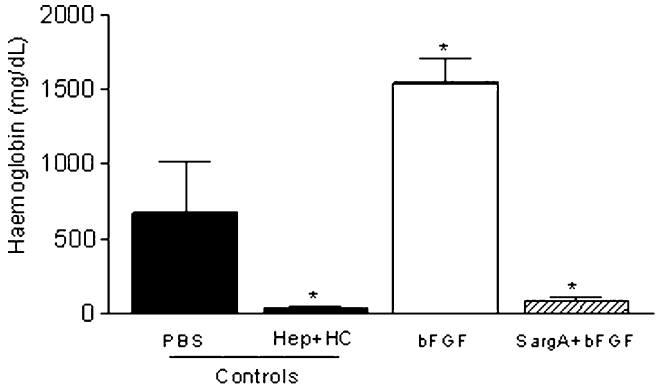

Fig. 5 Effect of SargA on bFGF-induced angiogenesis in the adult mouse model. Gelfoam plugs (gelatin sterile sponge) adsorbed with 50 µl of 50 ng/plug bFGF with or without 1500 µg/plug SargA were implanted s.c. into Swiss adult mice. PBS (vehicle) alone adsorbed in Gelfoam was used as a negative control and plugs containing heparin (50 IU/plug) plus hydrocortisone (156 µg/plug) were used as a positive control. The content of Hb inside the Gelfoam was measured by photometry using Drabkin's method. Each *bar* represents the mean ± SEM from at least five animals. $^{*}P < 0.05$

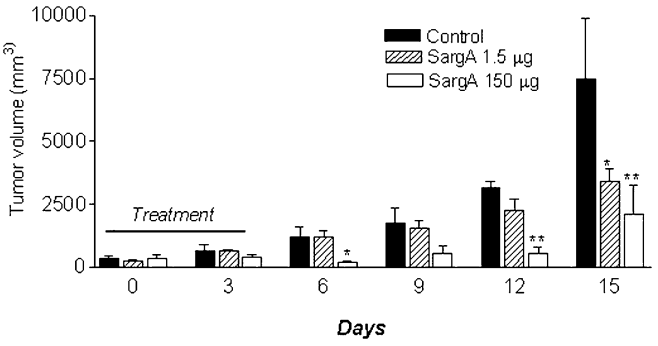

Fig. 6 B16F10 melanoma cells (1×10^6 in 200 µl/animal) were inoculated s.c. under the dorsal skin of mice ($n = 8$) on day 0. When the tumor volume was at least 150 mm^3 the mice were injected s.c. with a single daily dose of SargA. Control animals received RPMI-1640 medium only, and the experimental groups received SargA at 1.5 and 150 µg/day. Tumor volumes were measured every 3 days throughout the 15-days experiment. Each *bar* represents the mean ± SEM of at least eight animals. $^{*}P < 0.05$, $^{**}P < 0.01$

bination with SargA abolished this augmentation (Fig. 5). Indeed, SargA not only prevented the effect of bFGF, but actually inhibited Hb accumulation to values significantly below those seen on control plugs (94.8%; i.e. to the same extent as that observed in plugs treated with heparin plus hydrocortisone). The bFGF vehicle did not inhibit angiogenesis in this assay and none of the implant sites showed any signs of inflammation or hemorrhage (not shown).

Inhibition of tumor growth in mice inoculated with B16F10 melanoma cells

To evaluate the effect of SargA on tumor growth, Swiss mice were inoculated s.c. with B16F10 melanoma cells (1×10^6 in 200 µl/animal) and treated with the polysaccharide. The results of these experiments are summarized in Fig. 6. Daily s.c. injections (single dose each day) of 1.5 or 150 µg/animal (100 µl) during a consecutive 3-day treatment period decreased the growth rate of primary tumors throughout the remaining 12 days of the experiment. After the 3-day treatment period, SargA at a dose of 150 µg/animal suppressed the tumor growth for the next 3–9 days. By the end of the experiment, tumor growth was inhibited by about 55% (3385.0 mm^3) and 72% (2087.7 mm^3) at doses of 1.5 and 150 µg/animal, respectively, in relation to that in control mice treated with cell culture medium only (7486.6 mm^3). These results indicate that SargA at doses of 1.5 and 150 µg/animal has the ability to retard the increase in tumor volume by at least 2.5 and 5 days, respectively. Treatment with either dose of SargA did not induce any deaths or body weight loss (data not shown), suggesting little or no toxicity.

Wound assay for B16F10 melanoma cell migration

Confluent monolayers of murine melanoma cells were scratched with a razor blade and the cell migration into the wound from adjacent areas of the monolayer evaluated at the conclusion of the experiment. As shown in Fig. 7, SargA (50, 100 or 200 µg/ml) exerted marked and dose-related inhibitory effects on B16F10 cell migration (55–95% inhibition). However, the presence of a large number of rounded cells was also noted in the wells treated with the highest dose of SargA, which may suggest a toxic effect of this compound.

Effect of SargA on in vitro cell viability

We next examined the effect of increasing concentrations of SargA (50–2000 µg/well; 96 wells) on B16F10 melanoma cell viability by the MTT assay. As demonstrated in Fig. 8, SargA had a negative effect on cell viability. After a 3-day treatment, SargA at concentrations as high as 2 mg/well decreased the number of viable cells to about 21% in relation to the control (RPMI-1640 medium only).

Assay for anticoagulant activity

In order to assess the anticoagulant activity of SargA, we measured its effect on TT. The time to clot formation (TT value) of saline-treated blood was 17 s. SargA, at 0.5 and 5 mg/ml (but not 0.05 mg/ml) increased TT significantly to 23.34 and 44.25 s, respectively (Fig. 9). Despite these results, it should be noted that the anticoagulant activity of SargA was far less than that displayed by heparin (TT about 120 s at 5 µg/ml; data not shown).

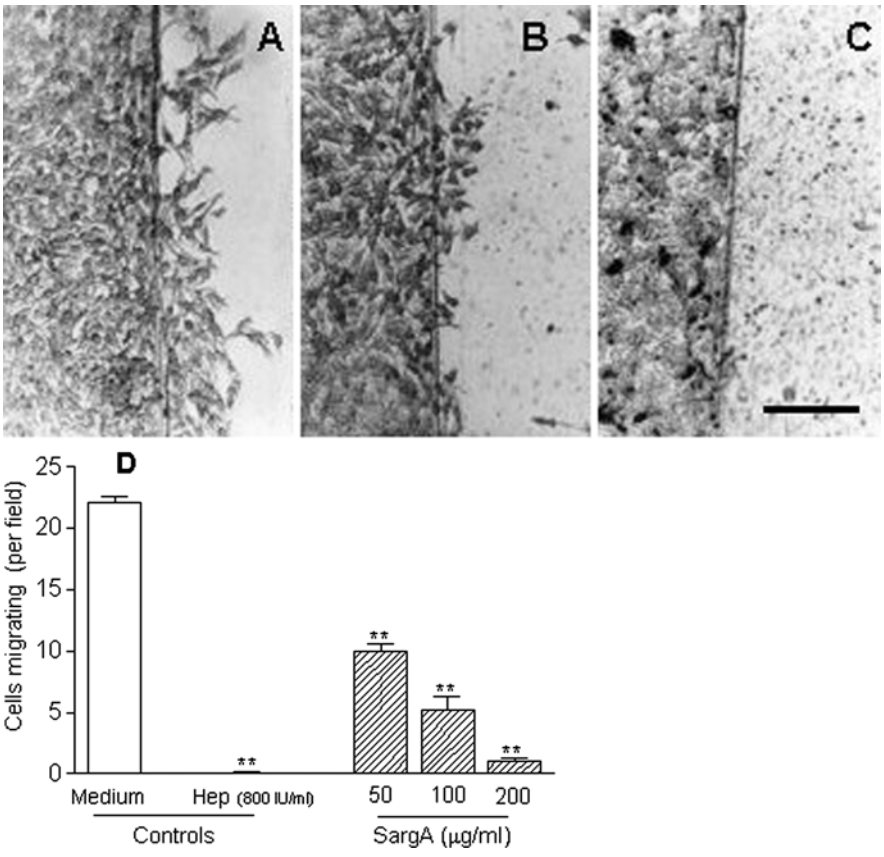

Fig. 7 Photographs of confluent monolayers of B16F10 melanoma cells cultured in six-well plates wounded with a razor blade. Plates were incubated for 12 h with RPMI-1640 medium containing increasing concentrations of SargA (50–200 μg/ml, 2 ml volume). **a** Cells cultured in medium alone (negative control); **b** cells cultured with 50 μg/ml SargA; **c** cells cultured with 200 μg/ml SargA; **d** Cells migrating per field. *Bars* represent mean ± SEM of the number of migrating cells in ten randomized fields of 1 mm^2 under a ×100 magnification (*bar* 127 μm). ** $P < 0.01$, vs the control group

Discussion

As dysregulated angiogenesis has been implicated in tumor growth and metastasis, as well as in the progression of a number of other angiogenic diseases [21], much attention has been directed to the development of new pharmacological strategies for targeting this process [24]. In this regard, the current study demonstrates that SargA, a polysaccharide isolated from *S. stenophyllum*, effectively inhibits developmental angiogenesis in chick embryos, as well as vascularization of Gelfoam skin implants and melanoma cell tumor growth in mice.

In the CAM assay in chick embryos, which is perhaps the most widely employed in vivo model for studying vessel development [10, 53], SargA markedly and dose-dependently inhibited the development of capillary networks. The inhibition afforded by the highest dose of SargA (1500 μg/plug) was roughly double that achieved by hydrocortisone (156 g/plug). The CAM model does

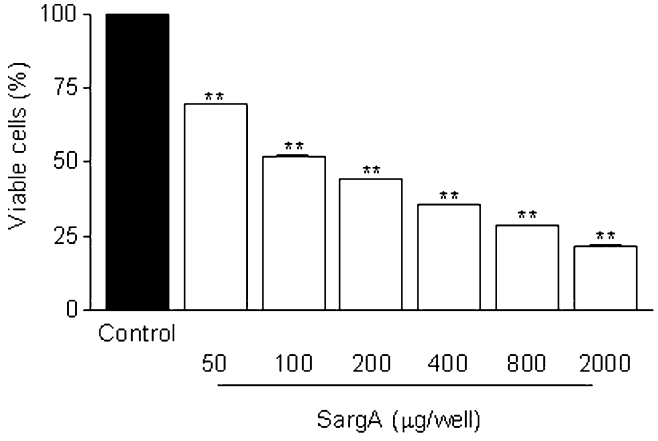

Fig. 8 Effect of SargA on B16F10 melanoma cell viability by the MTT assay. The cells were seeded at a density of 2.5×10^6 cells into each well and after 24 h the indicated concentrations of SargA were added. After 3 days, the percentage of viable cells was measured by the MTT assay. Experiments were performed in triplicate. ** $P < 0.01$, vs control

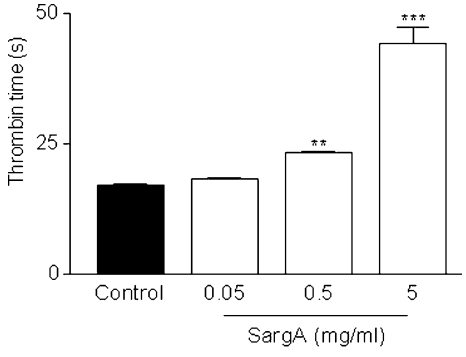

Fig. 9 TT was measured using human plasma and three concentrations of SargA (0.05–5.0 mg/ml). Volumes of 170 μl human plasma and 30 μl test solution were mixed and incubated. After addition of 100 μl bovine thrombin to the mixture, the time to clot formation was recorded. *Bars* represent mean ± SEM. ** $P < 0.01$, *** $P < 0.001$, vs control

not necessarily distinguish between newly formed microvessels (after application of drug-containing plugs on the CAM) and those already present at day 6, when plugs are implanted. Thus, it is especially noteworthy that the 64% reduction in vascular density around the plug limits on the CAM afforded by the highest dose of SargA actually indicated that no new microvessels were present. This view is further supported by the fact that SargA fully prevented the well-known angiogenic effect of exogenous bFGF when both agents were applied together on the same plug in the CAM, or in the back skin of mice (evaluated as reduction in plug Hb content).

Sulfated polysaccharides, such as pentosan polysulfate, heparin, heparan sulfate, dextran sulfate and chondroitin sulfate, stimulate vascular tube formation, whereas the nonsulfated polysaccharides such as xylan and non-sulfated dextran exhibit no effect [54]. Indeed, endogenous sulfated proteoglycans are essential for the transport of FGF and activation of its receptor due to stabilization of a complex between bFGF and its receptor with distinct chains of specific heparan sulfate species [10, 38, 39, 55–57]. Proteolytic degradation of HSPG triggers release of bFGF from extracellular matrix sites, reducing its availability for receptor activation [58]. However, depending on their particular structure, certain sulfated polysaccharides can sequester FGF, thus preventing activation of FGF receptors, leading to inhibition of angiogenesis. Whether a particular polysaccharide displays angiogenic or antiangiogenic properties appears to depend importantly on the degree of sulfation. Thus, the angiogenic activity of native fucoidans, which are sulfated to some degree [59], can be reversed into a clear-cut antiangiogenic profile of action if they are oversulfated [60]. It is also noteworthy that other highly polyanionic compounds such as the polysulfonated naphthylurea suramin and its analogs, can also markedly inhibit angiogenesis by preventing binding of bFGF, TGF-β and EGF to their respective receptors [22, 61–63].

On the other hand, during embryogenesis, heparan sulfate can also bind other growth factors in addition to bFGF, such as RA (retinoic acid—also an angiostatic factor) and VEGF [64], as well as the morphogens Wnt (Wint), SHH (sonic hedgehog) and BMP-4 (bone morphogenetic protein) [65, 66]. At present, it is unclear if these inhibitory effects of SargA on blood vessel development in CAM or skin implants are due to selective blockade of bFGF-mediated mechanisms or to interference with the actions of other endogenous growth factors and/or morphogens. Likewise, it remains to be determined if the reduction in total body length of chick embryos on day 8 caused by SargA is a result of similar mechanisms or reflects an impact on nutritional status. However, considering that preliminary structural analysis revealed the presence of sulfate (and also anionic carboxyl) moieties in SargA, inhibition of blood vessel formation by sequestration of such factors appears to be a feasible mechanism of action. This might be especially applicable to the results obtained in the CAM assay, as HSPG content in eggs at 6–10 days of age is reduced by about 25% [67], which encompasses the period of SargA treatment.

Although the extraction procedure employed in the current study yielded a homogeneous SargA polysaccharide from *S. stenophyllum*, another study detected the presence of two distinct fucoidan fractions in extracts of this alga [37]. It appears highly probable that the fucoidan fractions obtained in that study, using a slightly different extraction procedure, constitute breakdown products of SargA.

In contrast to polysaccharides or suramin and its analogs, glucocorticoids such as hydrocortisone appear to inhibit angiogenesis either through an angiostatic effect mediated via blockade of estrogen receptors and/or direct stimulation of protein kinase C [44]. Thus, the antiangiogenic effects of hydrocortisone are markedly potentiated by combined treatment with heparin (present study; [19, 68]) or suramin [22]. Surprisingly, however, the antiangiogenic effects in the CAM model produced by coadministration of SargA (18–1500 μg/disk) together with hydrocortisone were merely additive. This might suggest that the antiangiogenic mechanism(s) of action of SargA is distinct from that displayed by polyanionic compounds.

Another important finding of the current study was the pronounced antitumoral effect of SargA in mice bearing murine B16F10 melanoma tumors, without producing any overt signs of general toxicity (such as death or body weight loss) within the 2-week observation period. Several polysaccharides have been shown to display antitumoral properties, including heparin [1, 69], polysulfated pentosan [70], hyaluronan oligomers [71, 72] and especially oversulfated fucoidans [25, 60, 73, 74]. Moreover, the polyanionic compound suramin also suppresses tumor growth [75, 76]. Therefore, it is possible to expect that the mechanism of the antitumoral action of SargA is mediated, at least in part, via its antiangiogenic properties, as discussed above. Nonetheless, the finding that SargA also inhibited migration of cultured B16F10 melanoma cells and their viability points to an additional cytotoxic mechanism of antitumoral action of this polysaccharide.

The usefulness of suramin and its derivatives in chemotherapy is severely limited by neurologic toxicity [61, 76, 77]. In this regard, the absence of any obvious signs of toxicity of repeated SargA injections in mice is quite encouraging. Moreover, considering that thromboembolism is a common cause of death in cancer patients, due to dislodging of tumor cells [21], some degree of antithrombotic action is desirable in an antitumoral agent. The relatively modest antithrombotic effect of SargA at doses which are cytotoxic to tumor cells would seem to be an advantageous feature of this compound over other polysaccharides, such as heparin [33, 73, 78], providing an additional beneficial action with a low risk of provoking hemorrhage. Additional potential advantages in the use of SargA is the worldwide distribution of *S. stenophyllum* and the ease with which the polysaccharide can be extracted, which would enable it to be

obtained at relatively low cost and in useful quantities, when compared to other angiogenesis inhibitor substances such as ergosterol from the fungus *Agaricus blazei*, fucosylated chondroitin sulfates (from the sea cucumber *L. grisea*), acharan sulfate (glycosaminoglycan from the snail *Achatina fulica*) and plant-derived flavonoids [26, 46, 49, 69].

In conclusion, we demonstrated that the polysaccharide SargA from *S. stenophyllum* displays antiangiogenic and antitumoral properties which might by instrumental in providing alternative tools for the control of diseases associated with angiogenic dysfunction. Nonetheless, the full chemical structure of SargA, as well as the mechanisms underlying its actions, remain to be characterized.

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