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A polysaccharide isolated from the brown seaweed *Sargassum stenophyllum* exerts antivasculogenic effects evidenced by modified morphogenesis

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Abstract

A polysaccharide (Sarg) extracted from the brown marine alga *Sargassum stenophyllum* was studied for its antivasculogenic effects in both *in vivo* and *in vitro* assays, as well as for its capacity to modify embryonic morphogenetic processes endogenously regulated by bFGF, a well-known angiogenic stimulator. The antivasculogenic activity of Sarg (6–1500 μg/implant) was evaluated in a chick yolk sac membrane assay and the embryonic morphogenesis was measured as the percentage cephalic length. Sarg alone (96–1500 μg/implant) and co-administered with hydrocortisone (HC; 156 μg/implant) decreased the vitelline vessel number by 23–100% and 54–100% respectively. The polysaccharide potentiated the antivasculogenic effect of HC (42% inhibition). Basic fibroblast growth factor-stimulated vasculogenesis (141% of vessels as compared to control) was partially reversed by Sarg. The treatment with Sarg also decreased the percentage cephalic length of 3.5- and 4-day chick embryos (as cultured *in vivo* and *in vitro*, respectively), uncoupled from any impairment in the body shape or embryotoxic effect. Due to polyanionic characteristics of Sarg, which are similar to those seen in the heparin molecule, we suggest that this polysaccharide should modulate the activity of heparin-binding vascular growth factors (such as bFGF, which also acts as a morphogen) mimetically interfering with heparan sulfate proteoglycans during microvessel formation.

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Introduction

During early embryogenesis the microvasculature is laid down through vasculogenesis (Folkman, 1971). However, it has become evident that progenitors to vascular endothelial cells also exist in the adult, and they can promote de novo vessel formation (adult vasculogenesis) associated with some pathologies such as cancer and ischemia (Zammaretti and Zisch, 2005).

Vasculogenesis is defined as the development of blood vessels from in situ differentiation of mesodermal progenitor endothelial cells (angioblasts) to endothelial cells. These precursor cells are recruited from mesoderm areas adjacent to the embryo and/or originated by local cell division, organizing blood islands and afterwards establishing a primordial vascular plexus (Ruiter et al., 1992). A subsequent remodeling of the vascular network gives rise to a more refined microvasculature. The sprouting and proliferation of capillaries from preexisting vessels (angiogenesis) accompanies the growth and shaping of the body (Folkman, 1971). For example, development of the heart and the great vessels occurs by vasculogenesis, whereas organs that require invasion of blood vessels for their development (brain, lung, kidney) are subject to angiogenesis (Risau

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et al., 1988). According to Rafii et al. (2002) and Bergers and Benjamin (2003), factors that stimulate angiogenesis, such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (ANG1), have been also shown to stimulate the process of vasculogenesis.

From the point of view of embryogenesis, a growing set of evidences has established a functional connection involving vascular positive or negative modulators and inductors of the embryonic body plane (Princivalle and de Agostini, 2002). Molecules of proteoglycans and other ECM compounds present in vascular tissue, and others, bind multiple species of growth factors as well as morphogens, e.g., basic fibroblast growth factor (bFGF) (Burg et al., 1995). This growth factor (bFGF) is a well-known angiogenic molecule involved in maintaining the specification of the neural tube border in the chick, in the late step of neural crest induction, which accompanies embryo growth along the cephalic–caudal domains (Dhoot et al., 2001). NOTCH proteins have also been investigated for their ability to direct the differentiation of endothelial cells into vascular networks (Gridley, 2001).

From the pre-clinical or clinical position, the possibility of natural angiostatic agents that might act with efficacy and with lower toxicity than conventional cytotoxic drugs has motivated intensive investigations by many laboratories (Gagliardi et al., 1992; Leali et al., 2001; Matou et al., 2002; Koyanagi et al., 2003; Tan et al., 2003). With this in mind, brown seaweed polysaccharides from the *Sargassum* genus (Phaeophyceae), an alga species with a wide geographical range, have been related to antitumor, antioxidant and antiangiogenic activities (Noda et al., 1989; Lim et al., 2002; Dias et al., 2005).

Structural studies previously performed by Duarte et al. (2001) related to *Sargassum stenophyllum* revealed the biosynthesis of heteropolysaccharides with a basic structure very similar to the ECM constituents from animal tissue.

Since in animal extracellular matrix, molecules of glycosaminoglycans (GLGs) and proteoglycans such as heparan sulfate proteoglycan (HSPG) may work as scavengers of angiogenic factors (Grumet et al., 1993; Katz and Yamada, 1997; Dowd et al., 1999; Yancopoulos et al., 2000), we hypothesized that compounds containing structural analog polysaccharides would be effective modulators of vasculogenesis, also correlating with alterations in patterns of embryonic morphogenesis.

In this study we focused on the capacity of a cell wall polysaccharide obtained from *S. stenophyllum* (Sarg) to inhibit the formation of early vitelline blood vessels. Such an approach allowed us to investigate vasculogenesis as well as embryonic morphogenesis, with assays *in vivo* and *in vitro* in yolk sac membranes from 1.5- to 4-day chick embryos.

Materials and methods

Materials

The materials were purchased as follows: sodium dodecyl sulfate (SDS), phosphate-buffered saline (PBS), methylcellulose (Aldrich, Milwaukee, WI, USA); Bacto-Agar (Difco, USA); hydrocortisone (Biobras, São Paulo, SP, Brazil); penicillin–streptomycin (Gibco, Auckland, New Zealand); basic fibroblast growth factor (bFGF; Intergen, Purchase, NY, USA); trypsin–ethylenediaminetetraacetic acid (trypsin–EDTA), L-glutamine, silica-G 60® (Pharmacia and Upjohn, Kalamazoo, MI, USA), trifluoroacetic acid (TFA), *bis*(trimethylsilyl)trifluoroacetamide (BSTFA), Coomassie® brilliant blue G-250, pyridine, deuterated water (D_2O), tetramethyl-silane, 1-methylimidazole, phenyl-β-D-galactopyranoside (Sigma, St. Louis, MO, USA). Other materials: hydrochloric acid (HCl), ethanol, methanol, potassium bromide (KBr), solid paraffin, nitroethane, orcinol, phosphoric acid, sodium chloride (NaCl), acetic acid, sodium tetrahydridoborate (NaBH4), potassium hydroxide (KOH), formaldehyde, were obtained from Reagen (Rio de Janeiro, RJ, Brazil).

Stock solution of 150 mg/ml Sarg as well as 11.25 mg/ml methylcellulose was kept (−20 °C) in 500 μl aliquots and diluted to working concentrations in the same vehicle previously used. Hydrocortisone was diluted in sterile ultra-filtered water to the desired concentration immediately before use.

Isolation of the cell wall polysaccharide (Sarg) from Sargassum stenophyllum

About 1000 g (fresh weight) of biomass of the brown alga *S. stenophyllum*, collected at Armação do Itapocoroy beach (Santa Catarina State, Brazil), was pre-cleaned by removing residual matter and rinsing extensively under tap water. The excess of water was drained off and small fragments of plant and animal tissues were removed. About 800 g (fresh weight) of the selected biomass was collected and washed repeatedly with three volumes of de-ionized water, then dried (65 °C) to constant weight (340 g, dry weight). The cell wall polysaccharide isolation was performed sequentially in accordance with previously described procedures (Selvendran et al., 1985; Maraschin et al., 2000). In brief, alkaline treatment (4 M KOH, 10 mg NaBH₄, at room temperature) of *S. stenophyllum* followed by neutralization (AcOH, pH 5.2) yields a cell wall polysaccharide fraction further referred to as Sarg. The yield of the above protocol was 4.9% Sarg relative to the initial algal biomass. The polysaccharide was lyophilized, diluted to 5 g/100 ml with sterile de-ionized water and afterwards diluted to the required concentrations.

Chicken yolk sac membrane assay

The ability of the compounds to inhibit vasculogenesis was determined by the yolk sac membrane (YSM) assay. This method was adapted essentially from chorioallantoic membrane (CAM) assays, which were carried out to evaluate angiogenesis (Jakob et al., 1978; McCormick et al., 1984; Vu et al., 1985; Peek et al., 1988; Nguyen et al., 1994).

Pathogen-free fertilized chicken eggs ($n=8$), supplied by poultry producers (Agrovêneto, as well as Macedo Koerich, SC, Brazil), were previously kept in a humidified (33%) incubator at 37.5 °C for either 36 h (Stage 10 — HH; Hamburger and Hamilton, 1951) or 48 h (Stage 13 — HH), in accordance with the *in vitro* and *in vivo* embryo culture methods, respectively.

Chick embryo in vivo culture

After 48 h at 37.5 °C 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. The treatment in ovo was performed by implanting disk-shaped methylcellulose supports (10 μl volume, 3-mm diameter; one disk per embryo) impregnated with the polysaccharide under study (Sarg) and/or hydrocortisone (positive control) on the outer one-third of the 2-day yolk sac membrane (YSM), near the embryo, where blood islands were still present in the early establishing capillary network (YSM assay), as evidence of vasculogenesis. After these manipulations the windows were closed with black binding cellophane tape and the eggs were returned to the incubator until the 4th day (96 h, or E4 embryonic day; Stage 23 — HH).

The concentrations of Sarg administered to vascular membranes by means of the methylcellulose disk supports ranged from 6 to 1500 μg/disk. Additional experimental groups were either co-treated with the same doses of Sarg and hydrocortisone (156 μg/disk — positive control), or given hydrocortisone (HC; 156 μg/disk) alone. Basic fibroblast growth factor (bFGF; 50 ng/disk) was also administered alone or associated with Sarg (1500 μg/disk). Blank methylcellulose disks (ultrafiltered water as solvent; pH 7.2) and disks containing hydrocortisone were used as negative and positive controls, respectively (Gagliardi and Collins, 1993).

Each substance or combination of substances under investigation was adsorbed on methylcellulose and the solution (final concentration = 0.45%) was air dried on a Teflon®-coated metal tray before being applied to the developing extraembryonic membrane for testing of vasculogenesis inhibition by Sarg.

Two days after implantation (E4), the zone around the methylcellulose disk was examined under a stereoscopic microscope (Olympus, Tokyo, Japan). Inhibition of vasculogenesis was determined by the decrease in vessel number in the area surrounding the methylcellulose disk (appearance of an avascular zone exceeding 2 mm) and defined as the percentage of vitelline vessels compared to the control. All animal studies were carried out in accordance with the procedures outlined in protocol number 256/proc.23080.028649/2003-07/CEUA/UFSC, approved by the local committee for the care and ethical use of animals in research (CEUA/UFSC, Florianópolis, SC, Brazil).

Chick whole-embryo in vitro culture

After a preliminary period of 36 h of incubation (E1.5), the eggs were removed from the incubator and handled according to the procedures previously described by Chapman et al. (2001). The treatment was performed by implanting disk-shaped methylcellulose supports (5 μl volume, 2 mm diameter; one disk per embryo) in the outer one-third surface of the 1.5-day yolk sac membrane (YSM), near the embryo, where blood islands were present together with early capillaries (YSM *in vitro* assay).

The whole embryos were cultured for 48 h, with the ventral side placed up, in Petri dishes containing solid agar-albumen medium (0.72 g Bacto-Agar; 120 ml egg thin albumen and 120 ml of 7.19 g/l NaCl simple saline) in an incubator (at 37 °C; saturated humidity) containing an atmosphere of 5% CO_2 in air. After that period had elapsed, the plates containing 3.5-day embryos (E3.5; Stage 21 — HH) were removed from the incubator and the vascular area surrounding the supports was analyzed by transillumination microscopy (25×), following the same criteria described above for the in ovo culture system.

Percentage cephalic length (PCL) as embryonic morphogenesis parameter

At the end of the total incubation period, the embryos of all experimental groups referred to above in the *in vivo* and *in vitro* procedures of yolk sac culture were also examined with regard to morphogenesis by comparison of the percentage cephalic length among the treatments. The embryos were quickly desensitized in a liquid N_2 atmosphere, dissected from ovular membranes, and fixed in 10% formaldehyde. The percentage cephalic length was determined through measures of total body length based on the segments defined as head flexure–cervical flexure–curled tail axis, and calculated by means of the following expression: [length axis from head to cervical flexures ÷ (length axis from cephalic to cervical flexures + length axis from cervical to caudal flexures)] × 100.

When the percent cephalic length value was either significantly higher or lower than the negative control value, the rate of growth of the cephalic segment was relatively altered and this was considered as indicative of a change in the embryonic morphogenesis pattern.

Data analysis and statistics

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

Results

Characterization of polysaccharide isolated from cell wall of Sargassum stenophyllum (Sarg)

Paper electrophoresis of the cell wall polysaccharide fraction revealed the existence of only one polysaccharide, which after acidic hydrolysis showed a monosaccharide composition cha-

racterized by units of α-L-fucose, β-D-manose, β-D-galactose, β-D-xylose, α-D-glucose and D-glucuronic acid as revealed by TLC. Further FTIR spectroscopy analysis of Sarg detected characteristic signals for alcohol (C–O 1030 cm^{-1} and O–H 3400 cm^{-1}) and aldehyde (2929 cm^{-1}) functions, typical of that type of macromolecule. A signal at 1715 cm^{-1}, characteristic of C=O groups, indicated that the monosaccharide constituents of the Sarg polysaccharide are aldoses. A typical signal (817 cm^{-1}) suggesting the occurrence of equatorial sulfate groups in the C2 and C3 positions or a sulfate group linked to the C6 position was also found (Orr 1954). Other chemical groups, NH (1630 cm^{-1}) and carboxyl (1408 cm^{-1}), indicated the presence of acid groups in the primary chemical structure. In fact, a low uronic acid content (0.34 mol%) was detected and its occurrence was further confirmed by ^{13}C NMR (δ 175.3 ppm). Interestingly, the more prominent resonances detected in the ^{13}C NMR spectrum of Sarg at δ 175.3 ($C6_{L\text{-iduronic acid}}$), 100.7 ($C1_{L\text{-iduronic acid}}$), 97.7($C1_{\beta\text{-D-Glc}}$), 77.7 (C4 linked), 70.9 ($C3_{L\text{-iduronic acid}/\beta\text{-D-Glc}}$), 67.6 ($C6_{\beta\text{-D-Glc}}$), and 59.8 ppm ($C2_{\beta\text{-D-Glc}}$) were also present in the ^{13}C NMR spectrum of heparin (standard compound, data not shown), as well as absorptions corresponding to acetyl groups (173.3 ppm and 21.9 ppm). Taken together, the spectroscopic data reveal a close structural similarity between Sarg and heparan sulfate. These findings are in agreement with previous results of studies on a set of fucoidans isolated from *S. stenophyllum* also originating from southern Brazil (Duarte et al., 2001).

Effect of Sarg on vessel development in yolk sac assay

Chick embryo in vivo culture

The yolk sac vascular membrane (YSM) *in vivo* assay was first carried out to determine whether Sarg displayed anti-vasculogenic activity. Whereas control YSMs treated with vehicle developed an average of 92 vessels on the disk limits, those treated with Sarg (1500 μg/disk) exhibited consistently fewer vessels around the disk (Fig. 1). In addition, the effect promoted by Sarg was higher than that observed on YSMs treated with hydrocortisone (156 μg/disk) which resulted in 54 vessels on the disk limit, a finding that demonstrates an antivasculogenic effect. However, the angiostatic action elicited by hydrocortisone in the YSM is not photographically depicted in this work (which would have provided a qualitative demonstration of vessel number) because until that level of inhibition it was only feasible to consider this by means of a resolving power performed through the quantitative analysis.

Furthermore, as shown in Fig. 2, as 6–1500 μg/disk of Sarg was simultaneously applied with hydrocortisone, which alone caused 42% inhibition, the polysaccharide clearly potentiated the antivasculogenic effect of HC. It can be seen from Table 1 that summation of effects distinctively obtained in each treatment (polysaccharide and glucocorticoid) was about 70–90% (over the range of 6–375 μg/disk of Sarg) of the effects observed with their association. On the other hand, the results shown in Fig. 3 indicate that as Sarg was concomitantly applied with the proangiogenic substance bFGF on the same disk, it fully prevented the marked pro-vascular effects
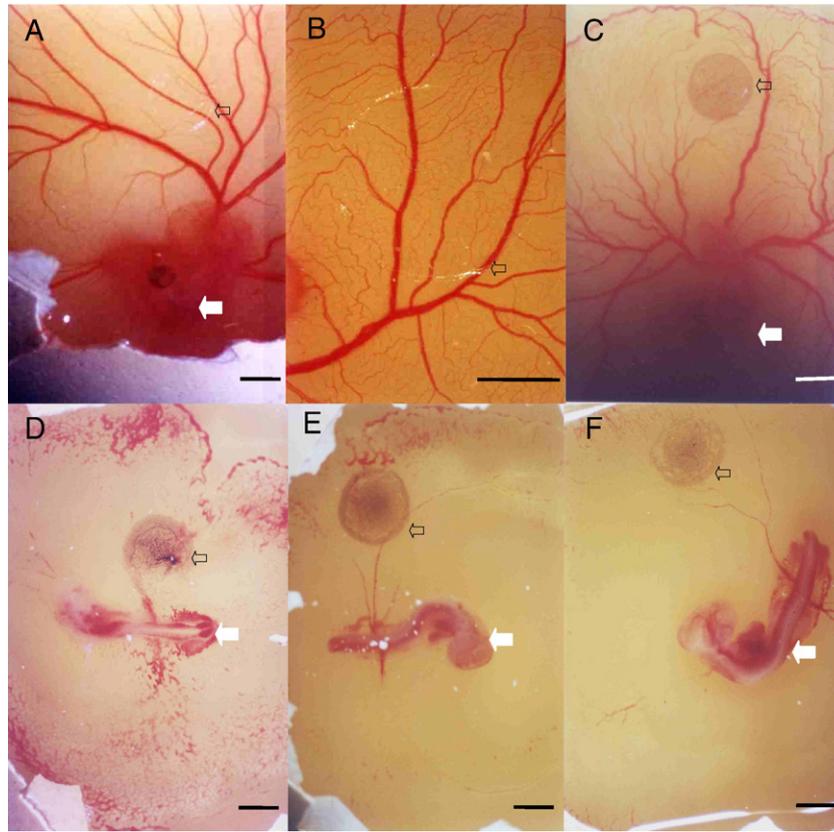
Fig. 1. Photographs of the inhibitory effect of Sarg on vascularization of 4-day yolk sac membranes (YSM) of chick embryos (white arrows). In each egg, one control or one drug-containing disk-shaped support (open arrows) was placed on the 2-day-old YSM, where blood islands where still present; (A) negative control (water); (B) detail of microvessels surrounding the disk (control); (C) Sarg 94 μg/disk; (D) Sarg 375 μg/disk; (E) Sarg 1500 μg/disk; (F) Sarg plus hydrocortisone (1500 μg/disk and 156 μg/disk) (bars 1 mm).

exhibited by that growth factor alone, as compared to control (vehicle).

Chick whole-embryo in vitro culture

To investigate whether the inhibitory effects of Sarg on embryonic vessel formation were being influenced by egg yolk compounds (such as insulin-like growth factors) or were in fact reproducible in the absence of the yolk, we determined the vasculogenesis in the 3.5-day chick embryos cultured *in vitro* together with yolk-free membranes. By 2 days after implantation (performed after 1.5 days of egg incubation), the control YSMs clearly exhibited primary, newly formed vitelline vessels. Whereas the control treated with vehicle developed an average of 26 early vitelline vessels on the disk limits, those
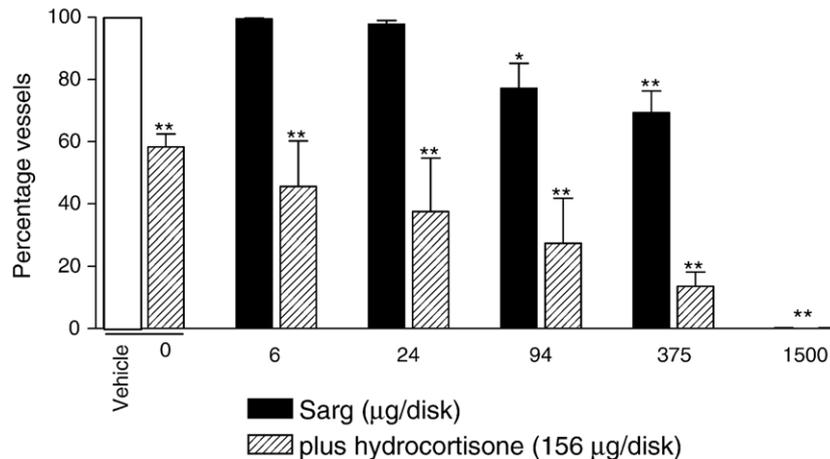

Fig. 2. Inhibitory effect of Sarg on vascularization of 4-day yolk sac membranes (YSMs). The controls were performed with vehicle (water, negative control) and hydrocortisone (156 μg/disk, positive control). Sarg was administered alone or together with hydrocortisone. Results are expressed as the number of microvessels around the drug-containing disk as a percentage of microvessels of control. Each bar represents the mean±SEM of eight eggs. **$P<0.01$, vs. control.

Table 1
Percentage vasculogenesis inhibition in 4-day yolk sac membranes of chick embryos ($n=8$), in comparison to control (water)

Effect of treatment				
Sarg (µg) dose	Sarg alone	(a) Sarg plus HC	(b) $\sum$[HC[a]]+ [Sarg alone]	(b/a) %
%Vasculogenesis inhibition				
6	0.5	4154.4	42.2	78
24	2.2	4162.5	43.9	70
94	22.8	4172.6	64.5	89
375	30.7	4186.4	72.4	83
1500	100.0	100.0	141.7	142

Sum of inhibitory effect of treatment with 5 concentrations of Sarg and 156 µg hydrocortisone (HC) separately administered (b) as compared to treatments performed by combination of the substances (a). Values obtained by relation (b/a) that result in less than 100% in sum of effects of Sarg and HC (alone) indicate that Sarg potentiated the HC inhibitory action.

[a] 41.7% Vasculogenesis inhibition in the treatment with 156 µg/disk HC (54 vessels) compared to control (92 vessels).

groups treated with Sarg (125–500 µg/disk) exhibited about 50% fewer vessels around the disk, which confirms the antivasculogenic effect of Sarg, independent of yolk factors (Fig. 4).

It may be assumed that Sarg was a little more effective in inhibiting vessel formation in the yolkless YSM of 3.5-day chick embryos cultured in vitro than in the in vivo culture of 4-day embryos, due to the distinct ages (developmental times of 1.5 and 2 days, respectively) of eggs used in the two assays.

Effect of Sarg on embryonic morphogenesis

In order to assess the antivasculogenic activity of Sarg on measurements of embryonic morphogenesis, we first determined the effect of Sarg on the total body length of 4-day chick embryos. As demonstrated in the inset in Fig. 5, the polysaccharide at a concentration of 1500 µg/disk, which blocked the process of vasculogenesis, concomitantly and significantly decreased the body length by 40% as compared to control (vehicle). We next examined the effect of increasing concentrations of Sarg (6–1500 µg/disk) on embryonic morphogenesis related to percentage cephalic length (PCL) in 4-day chick embryos, calculating this in accordance with the described methodology (scheme and photograph of body axes on 4-day chick embryo in Fig. 5). Whereas control 4-day embryos treated with vehicle presented a PCL of 45%, the treatment with Sarg (exclusively at the concentration of 1500 µg/disk) modified that pattern of embryonic morphogenesis, significantly decreasing the PCL to 38% (Fig. 5). The concomitant application of hydrocortisone (156 µg/disk) and Sarg (6–1500 µg/disk) markedly decreased the embryonic PCL to 41–22%, respectively (Fig. 5). Moreover, the isolated application of FGF (50 ng/disk) did not change the PCL or any other apparent embryonic morphological feature. However, the treatment with FGF (50 ng/disk) in association with Sarg (1500 µg/disk) in the same implant effectively abolished the previously recorded effect of decreas-

ing PCL exerted by the polysaccharide on morphogenesis (data not shown), suggesting a common molecular target for the two agents.

The effects of treatment with Sarg alone (1500 µg/disk) on the morphogenesis of 4-day embryos were corroborated by the results obtained with the administration of Sarg (250–500 µg/disk) in 1.5-day chick embryos cultured in vitro together with their yolk-free membranes (and analyzed after 3.5 days of incubation). As displayed in Fig. 6A, this treatment caused a significant decrease in the embryonic >PCL to 34–32%, res<?A3B2 show pectively, as compared to control (PCL=40%). Furthermore, when the effects of increasing concentrations of Sarg (125–500 µg/disk) were compared between cephalic (head) lengths and trunk lengths of 3.5-day chick embryos cultured in vitro, only the former measure (head length) was significantly changed in comparison to the control group (Fig. 6B).

In spite of the changes in the percentage cephalic length observed in the Sarg-treated group, as well as the reported developmental delay, those effects afforded by Sarg were uncoupled from apparent dimorphic features or any detectable embryotoxic effect. Further, embryos which had been exposed early to the highest concentration of the polysaccharide (1500 µg/disk) during the 4 days of the experiment, and then additionally incubated for at least 8 days (data not shown), still kept their viability features.

Discussion

Processes of vessel formation, such as angiogenesis, play a pivotal role in physiological processes in adults, e.g.,
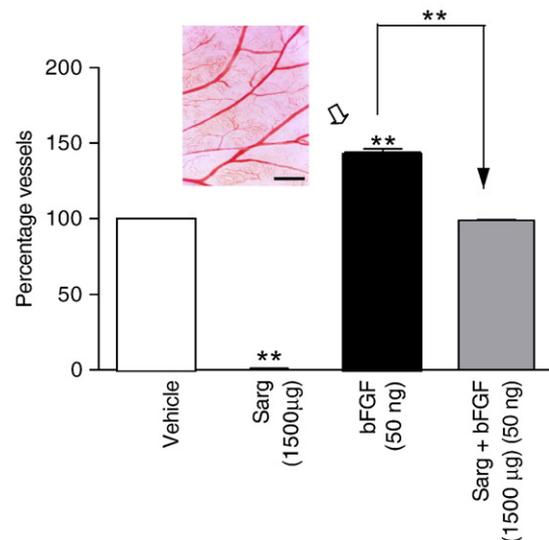

Fig. 3. Inhibitory effect of Sarg on vascularization of 4-day yolk sac membranes (YSMs). The controls were performed with vehicle (water, negative control). Drugs administered were Sarg alone (1500 µg/disk), bFGF alone (50 ng/disk) and Sarg plus bFGF (50 ng/disk). Results are expressed as the number of microvessels around the drug-containing disk as a percentage of microvessels of control. Each bar represents the mean±SEM of eight eggs. bFGF alone (50 ng/disk) **$P<0.01$, vs. control. Photography: detail of microvessels in YSM in the group treated with bFGF (bar 1 mm).
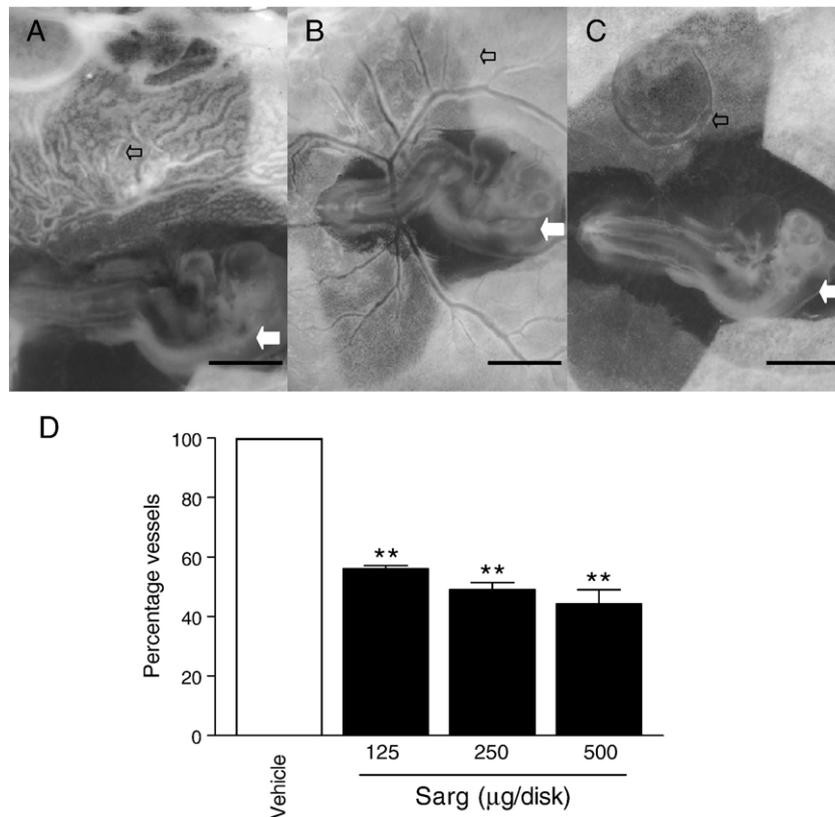
Fig. 4. Antivasculogenic effects of Sarg on 3.5-day yolkless YSMs of chick embryos (white arrows) cultured *in vitro*. Photographs: the control group (A) was implanted with disks (open arrows) containing vehicle (water, negative control) and the treated groups (B and C) received doses of 125 and 500 µg/disk of Sarg, respectively (scale bars 1.5 mm). Graphic (D) shows the number of microvessels around the drug-containing disk as a percentage of microvessels of control. Each bar represents the mean±SEM of eight eggs. **$P<0.01$, vs. control.

reproduction, tissue repair and inflammation. In particular, the implication of deregulated angiogenesis in the progression of tumors, besides a number of other diseases, has motivated sustained attention to well-structured pre-clinical investigations concerning the implications of that process (Zhou et al., 1997). In spite of the concept that pathophysiological vascularization appears as a rule to occur via angiogenesis (Folkman and Shing, 1992), the vasculogenic process that works in the early embryo, forming primary vessels at high rates to keep pace with the growth of the body, has been adapted, under certain situations, in the adult (Hanahan and Folkman, 1996; Hendrix et al., 2003; Zammaretti and Zisch, 2005).

Notwithstanding, in some tumor conditions (tumor neovascularization), for example, endothelial cell precursors can be mobilized from the bone marrow and transported through the bloodstream to become incorporated into the walls of growing blood vessels (Rafii and Lyden, 2003). Moreover, the intrinsic capacity of malignant human tumors to proliferate and to undergo metastasis because they produce substances that induce formation of vascular networks in a form of vasculogenesis recapitulation or vasculogenic "mimicry" (Maniotis et al., 1999; Folberg et al., 2000; Shubik and Warren, 2000) supports the clinical interest in identifying factors that could either inhibit the growth or induce early regression of pathologic vasculature (Gullino, 1978; Carmeliet and Jain, 2000; Hlatky and Folkman, 2002;

Carmeliet, 2003; Fidler, 2003). In this regard, the current study shows that Sarg, a sulfated heteropolysaccharide or sargassan (Abdel-Fattah et al., 1974), isolated from *S. stenophyllum*, effectively inhibits vasculogenesis as well as developmental angiogenesis in chick embryos and triggers concomitantly with vasculogenesis a specific change in the morphogenetic pattern. The chorioallantoic membrane (CAM) assay had been previously used to determine whether this polysaccharide has antiangiogenic activity, which was demonstrated on the ongoing vascularity of chick embryos and mice (Dias et al., 2005).

Differently from the CAM assay, which is perhaps the most widely employed *in vivo* model for studying vessel development (Nguyen et al., 1994), the yolk sac membrane (YSM) assay (Chapman et al., 2001) used in the current work may distinguish between newly formed microvessels (after application of drug-containing disks to the YSM) and those early vitelline vessels already present on the 2nd embryonic day of chick development. In the *in vivo* YSM assay Sarg markedly and dose dependently inhibited the development of primordial capillary networks. The inhibition afforded by the highest dose of Sarg (1500 µg/disk) was roughly double that achieved by hydrocortisone (156 µg/disk). Thus, it is especially noteworthy that the 100% reduction in vascular density and/or the eventual permanence of some late blood islands around the disk limit on the YSM afforded by the highest dose of Sarg
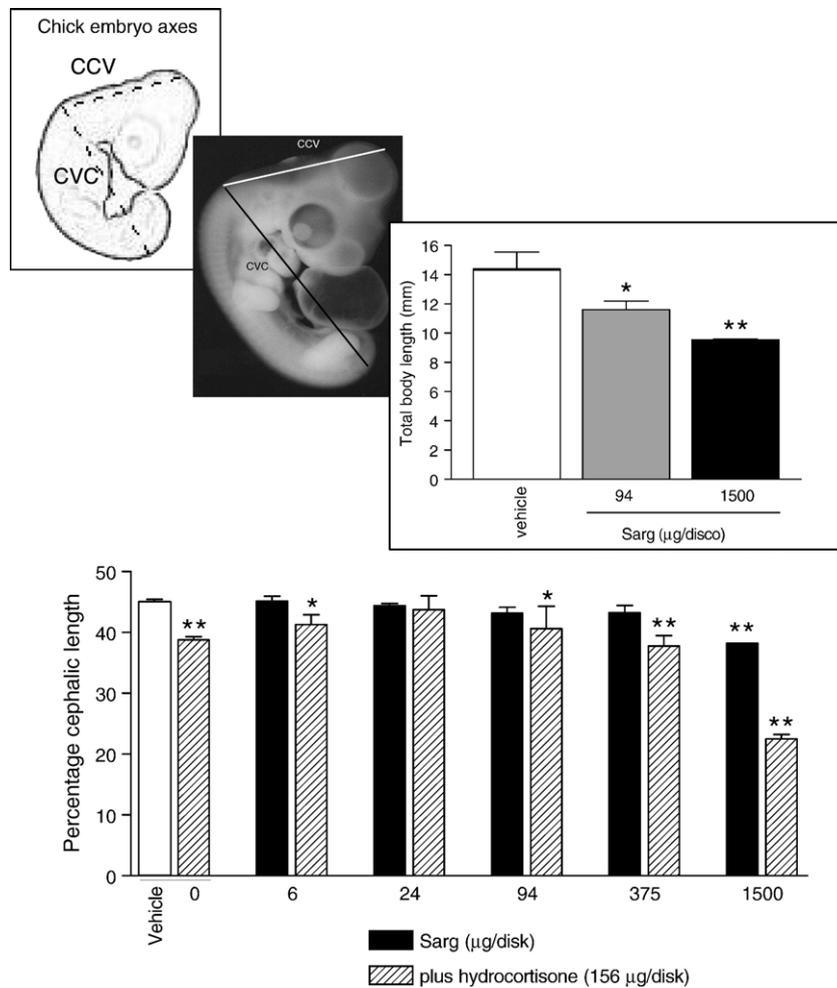
Fig. 5. Activity of Sarg (6–1500 μg/disk) on morphogenesis of 4-day chick embryos. Results are expressed as the percentage cephalic length. In the inset: body axes on chick embryo in scheme and photograph illustration, with biometry results (body length) of 4-day chick embryos (96–1500 μg/disk). Each bar represents the mean± SEM of eight embryos. *$P < 0.05$, **$P < 0.01$, vs. control.

actually indicated that no new microvessels were present. This evidence is further supported by the fact that Sarg inhibited vessel formation in the area vasculosa of yolkless YSMs of chick embryos cultured *in vitro* and fully prevented the well-known pro-vascular effect of exogenous bFGF, when both agents were applied together on the same disk in the YSM. Moreover, the absence of newly formed vessels already present on day 2, when disks were implanted, suggests that Sarg may also have induced regression of early vasculature during the process of angiogenic remodeling.

Basic FGF stimulates vascular endothelial cell mitogenesis through a mechanism which involves arachidonic acid release as well as eicosanoid formation (Dethlefsen et al., 1994) and it is probably mediated by lipoxygenase metabolites (Fafeur et al., 1991). 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 (Jackson et al., 1994). Indeed, endogenous sulfated proteoglycans are essential for the transport of FGF and activation of its receptor, due to the stabilization of a complex between bFGF and its receptor with distinct chains of specific heparan sulfate species (Katz and Yamada, 1997; Gallagher, 1994; Miao et al., 1997). Proteolytic degradation of HSPG triggers the release of bFGF from extracellular matrix sites, reducing its availability for receptor activation (Mundhenke et al., 2002). However, depending on their particular structure, certain sulfated polysaccharides can sequester FGF, thus preventing activation of FGF receptors and leading to inhibition of vessel formation. The ability of a particular polysaccharide to display pro- or antivascular properties appears to depend significantly on the degree of sulfation. For example, the angiogenic activity of native fucoidans, which are sulfated to some degree (Matou et al., 2002), can be reversed into a clear-cut antiangiogenic profile of action if they are oversulfated (Soeda et al., 2000). It is also relevant that other highly polyanionic compounds, such as the polysulfonated naphtylurea suramin and its analogs, can also markedly inhibit vessel formation by preventing binding of bFGF, TGF-β and EGF to their respective receptors (Coffey et al., 1987; Yayon and Klagsbrun, 1990; Pesenti et al., 1992).

On the other hand, during embryogenesis, heparan sulfate can also bind other growth factors in addition to bFGF and VEGF (Fairbrother et al., 1998), as well as morphogens such
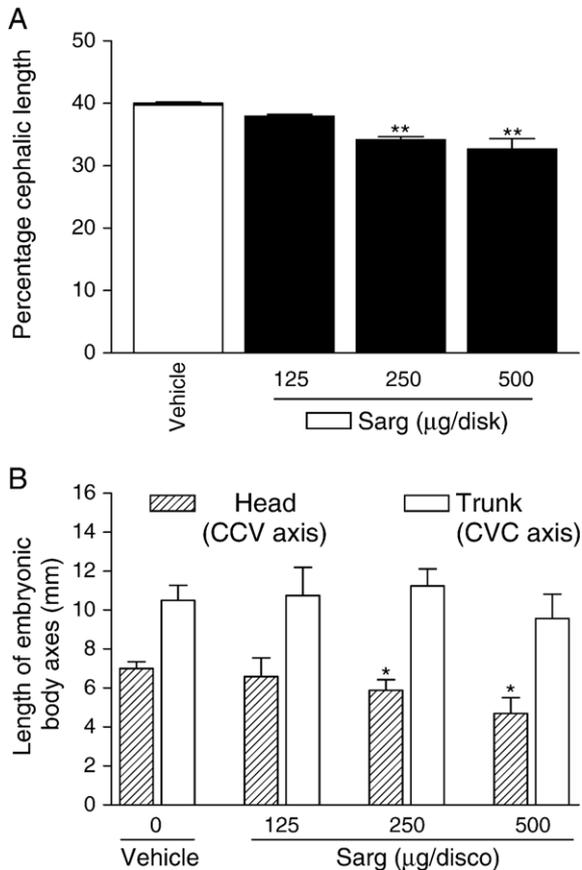
Fig. 6. (A) Activity of Sarg (125–500 μg/disk) on morphogenesis of 3.5-day chick embryos cultured *in vitro*. Results are expressed as the percentage cephalic length. (B) Comparison of effects of increasing concentrations of Sarg (125–500 μg/disk) between the measures of cephalic (head) lengths and trunk lengths (mm) of 3.5-day chick embryos cultured *in vitro*. Each bar represents the mean± SEM of eight embryos. $*P<0.05$, $**P<0.01$, vs. control.

as retinoic acid (RA — also an angiostatic factor), Wnt (*Wint*), SHH (Sonic Hedgehog) and BMP-4 (bone morphogenetic protein) (Dhoot et al., 2001; Princivalle and de Agostini, 2002). At present, it is unclear if the inhibitory effects of Sarg on blood vessel development in the YSM are due to selective blockade of bFGF-mediated mechanisms and/or interference with the actions of other endogenous growth factors. However, as the preliminary structural analysis revealed the presence of sulfate (and also anionic carboxyl) moieties in Sarg, the inhibition of blood vessel formation by sequestration of such factors appears to be a feasible mechanism of action.

Although the extraction procedure employed in the current study yielded a homogeneous Sarg polysaccharide from *S. stenophyllum*, another study detected the presence of two distinct fucoidan fractions in extracts of this algal species (Duarte et al., 2001). It appears highly probable that the fucoidan fractions obtained in that study, using a slightly different extraction procedure, constitute breakdown products of Sarg. In contrast to polysaccharides or the polyanionic compound suramin and its analogs, glucocorticoids such as hydrocortisone appear to inhibit angiogenesis through an

angiostatic effect mediated via blockade of estrogen receptors and/or direct stimulation of protein kinase C (Gagliardi and Collins, 1993). However, the antivasculogenic effects in the YSM model produced by treatment with hydrocortisone were markedly potentiated in the order of 10% to 30% by co-administration of Sarg, an effect very similar to that exerted by association of hydrocortisone with another polysaccharide — heparin (Crum et al., 1985; Tobelem, 1990). As described above, in this study previous comparative analyses of the spectral profile (infrared and ^{13}C NMR) of Sarg indicated that this polysaccharide presents a basic structure consisting of a main chain (backbone) and an α-L-fucose (Fucan), as well as sulfate chemical groups linked to carbon atoms in the C2, C3 and/or C6 positions. This might suggest that the antivasculogenic mechanism(s) of action of Sarg may be, in part, due to its (poly) anionic character.

Another important finding of the current study was the change in the pattern of embryonic morphogenesis in response to treatments. The pronounced growth decrease in the body segment extending from the cephalic flexure to the cervical flexure (CCV axis), in relation to the whole embryo (CCV plus CVC, which is the total distance between the cephalic–cervical–caudal flexures), was expressed as the percentage cephalic length (PCL). According to details given in the methodology, when the percentage cephalic length value is either significantly lower or higher than the control value, the growth rate on the anterior body axis was altered, which was considered as indicative of a change to the standard timing of embryonic morphogenesis.

A previous study on inductive signaling in the embryonic body plane (Aybar and Mayor, 2002) shows that bFGF, which induces pluripotent cells from the blastodisk of the chick to undergo vasculogenesis, is in fact also a signal involved in mediating the specification of the neural tube in chick embryos. This role is related to the late step of neural crest induction, which follows the embryonic growth along the cephalic–caudal domains. Thus, the neural tube flexures of early chick embryos might be considered as positional landmarks as well as structural products of the homeobox genes operating in the developing central nervous system (Hamburger and Hamilton, 1951; Takamatsu and Fujita, 1987; Männer et al., 1995; Simon et al., 1995). We have hypothesized that changes in the inductive signaling for embryonic body shaping, such as that involving bFGF signal transduction, in fact arose from the blockade caused by Sarg of this growth factor as well as other morphogens. In this regard, we consider that morphogenesis should be a relevant factor to consider in the design of antivasculogenic agents and vascular therapies. Likewise, it remains to be determined if the decrease in the PCL of chick embryos brought about by Sarg, isolated or in association with hydrocortisone, and underlying the reduction in cephalic length during the developmental period of 1.5–4 days, is due only to selective blockade of bFGF (e.g., sequestration), or if it also reflects an impact on nutritional status in the morphogenetic process, due to a decrease in vessel number.

It is remarkable that no thrombus was observed with any of the antivasculogenic compounds tested, which is in accordance

with previous findings concerning the antithrombin property of *S. stenophyllum* polysaccharides (Duarte et al., 2001). Furthermore, the absence of any obvious dimorphic features or apparent signs of toxicity from repeated Sarg administration by means of implants in early chick embryos is quite encouraging. An additional potential advantage in the use of Sarg is the world-wide 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 angiostatic or vasculogenic inhibitor substances such as ergosterol from the fungus *Agaricus blazei* (Takaku et al., 2001), acharan sulfate (glycosaminoglycan) from the snail *Achatina fulica* (Lee et al., 2003) or some plant-derived flavonoids (Tan et al., 2003).

While the inhibition of vessel formation from preexisting vessels is a relevant feature of useful drugs for the treatment of angiogenic diseases such as cancer, the efficacy of a specific antiangiogenic agent may be potentiated/enhanced if it is concomitantly capable of blocking early vessel formation, such as those capillaries formed in the yolk sac by means of vasculogenesis. Due to this process of initiating events related to tissue differentiation and vascular remodeling (for instance the recruitment of endothelial progenitor cells), the blockade of vasculogenesis should represent an additional target for neo-angiogenesis inhibitors. It thus becomes clear that the anti-vascular effect of Sarg application, also corroborated by our findings on embryonic morphogenesis, suggests a new strategy for the therapy of vascular diseases. This hypothesis is also supported by the evidence that melanoma cells, for example, are themselves capable of expressing endothelium-related genes that produce capillary networks with a high concentration of extracellular matrix (an event typical of vasculogenesis). Moreover, it is currently recognized that the vascularization observed in some tumors found in cancer patients appears to recapitulate events in the initial vitelline vessel network (Carmeliet, 2003; Zammaretti and Zisch, 2005).

In addition, from a clinical point of view some degree of antithrombotic action is a desirable effect of antitumoral agents, considering that thromboembolism is a common cause of death in cancer patients due to dislodging of tumor cells in the vasculature (Carmeliet, 2003). The action of thrombin is central to the process of thrombosis and, through the cleavage of fibrinogen, thrombin is also a primary promoter of coagulation (for a review, see Trento et al., 2001). In a previous report we demonstrated that Sarg at 5 mg/ml enhanced the thrombin time (TT) significantly to 44.25 s. It should be noted that this anticoagulant activity of Sarg was about 37% lower than that exhibited by heparin (TT of about 120 s at 5 µg/ml; data not shown). Otherwise, a potent anticoagulant activity, in some conditions, can overlap efficacy with detrimental side effects (Sinaÿ, 1999). Thus, considering the relatively modest anticoagulant effect exhibited by Sarg, as determined by TT properties, this last response could be considered an (therapeutic) advantage of polysaccharides, which would produce a mild anticoagulant effect without undesired side effects as compared to standard heparin. Nonetheless, definition of the full chemical structure of Sarg

is currently been followed up and the mechanisms underlying its actions remain to be characterized.

In conclusion, we have demonstrated that the polysaccharide Sarg, from *S. stenophyllum*, displays antivasculogenic properties that may also be usefully evidenced by change in the embryonic morphogenesis pattern. These properties might by instrumental in providing alternative tools to control diseases associated with vascular dysfunction.

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