Angiogenic inhibitor protein fractions derived from shark cartilage

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Synopsis
Development of therapies based on the growth inhibition of new blood vessels is among the most intensively studied approaches to the treatment of cancer and other angiogenesis-related diseases. Shark cartilage has been proven to have inhibitory effects on the endothelial cell angiogenesis, metastasis, cell adhesion and MMP (matrix metalloprotease) activity. In the present study, we have used a chromatography-based procedure for the isolation and partial purification of a shark cartilage protein fraction containing anti-angiogenesis activity. Proteins were extracted in 4 M guanidinium chloride, followed by sequential anion- and cation-exchange column chromatography. Angiogenesis assays were performed using the rat aortic ring and chick CAM (chorioallantoic membrane) assay models. The results show that the final fraction contains two proteins with molecular masses of 14.7 and 16 kDa. The protein fraction is able to block microvessel sprouting in the collagen-embedded rat aortic ring assay in vitro and inhibition of capillary sprouting in the CAM assay in vivo. It is suggested that these are partially purified anti-angiogenesis proteins, which have further biotechnological or biomedical applications.

Key words: angiogenesis inhibitor, chorioallantoic membrane (CAM) assay, chromatography, protein purification, rat aorta ring assay, shark cartilage

INTRODUCTION
Shark cartilage has been regarded as a natural source of anti-angiogenic compounds and has been used in the treatment of cancer, psoriasis, osteoarthritis, progressive systemic sclerosis, neurovascular glaucoma and other angiogenesis-related diseases for more than 30 years [1–3]. Previous results suggest that shark cartilage also contains anti-inflammatory, immunostimulatory and wound healing agents [4]. Shark cartilage extract exerts its angiostatic properties through inhibition of MMP (matrix metalloprotease) activity of endothelial cells [5] and VEGF (vascular endothelial growth factor)-mediated signalling events [6], and activation of endothelial cell-specific pro-apoptotic activities [7]. Chen et al. [8] have shown that shark cartilage extract can inhibit endothelial cell growth and attachment in vitro. Also, Liang et al. [9] have obtained a shark cartilage fraction, SCF2 (shark cartilage factor 2), that blocks endothelial cell proliferation in vitro and the development of new blood vessels in the CAM (chorioallantoic membrane) of chicken embryos and tumour-induced angiogenesis in the rabbit cornea [10]. Two separate proteins with anti-angiogenic properties have also been identified by Oikawa et al. [11].

In view of the popular interest in shark cartilage as an anticancer agent and anti-angiogenesis effects, in the present study we have attempted to purify a protein fraction with potent anti-angiogenesis activity from shark cartilage using both the rat aorta ring sprouting assay and the CAM assay. The resulting fraction was identified as containing low-molecular-mass proteins with high anti-angiogenesis activity.

MATERIALS AND METHODS

Materials
Benazidine hydrochloride, guanidinium chloride, \(N\)-ethylmaleimide, PMSF and EDTA were purchased from Sigma. Iodoacetic acid, acrylamide, bisacrylamide, Bromophenol Blue, Coomassie Blue R250, sodium cacodylate and glutaraldehyde were purchased from Merck. 6-Aminohexanoic acid was purchased from Fluka. Q-Sepharose fast flow and Sp-Sepharose fast flow were from Amersham Biosciences. Methylcellulose was from Shin-ETSU Chemical Company (Tokyo, Japan). DMEM (Dulbecco’s modified Eagle’s medium), penicillin and streptomycin were from Gibco BRL.

Abbreviations used: CAM, chorioallantoic membrane; DMEM, Dulbecco’s modified Eagle’s medium; MMP, matrix metalloprotease; SCF2, shark cartilage factor 2; VEGF, vascular endothelial growth factor.

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Shark cartilage (obtained from the Persian Gulf, Bushehr, Iran) was washed with PBS and 70% (v/v) ethanol, cut into small pieces, freeze-dried and pulverized. The cartilage powder was then stored at −70°C until use.

Type-1 rat tail collagen was prepared using the method of Strom et al. [12]. Male Sprague–Dawley rats (8–10 weeks old) were obtained from the Institute of Razi (Karaj, Iran). The work was undertaken with the permission of institutional animal care committees.

Fertilized pathogen-free Lohman chick eggs were obtained from the Iran-German Poultry Company (Tehran, Iran) and handled according to institutional animal care procedures.

**Protein extraction**

The cartilage powder was extracted using seven volumes of 0.1 M sodium acetate buffer (pH 5.8) containing 4 M guanidinium chloride, which was stirred at 4°C for 48 h in the presence of a protease inhibitor cocktail (6.25 mM EDTA, 1 mM PMSF, 0.25 mM benzamidine hydrochloride, 0.25 mM 6-aminohexanoic acid, 2 mM iodoacetic acid and 10 mM N-ethylmaleimide). The extract was centrifuged at 60000 g for 60 min at 4°C, and the clear supernatant was concentrated using an Amicon Stirred Ultrafiltration Cell (Millipore) equipped with a YM-10 membrane and dialysed against 50 volumes of 6 M urea in 0.05 M sodium acetate buffer (pH 8.0) containing protease inhibitors for 48 h at 4°C then stored at −20°C until use. The protein concentration was determined by using a modified method described previously [13], with BSA used as a protein standard.

**Anion-exchange chromatography**

The dialysed fraction was applied to a Q-Sepharose fast flow anion-exchange column (14 × 2.5 cm) which had been pre-equilibrated with 50 mM Tris/HCl (pH 8.0) containing 6 M urea at 4°C. A flow rate of 1 ml/min was maintained and 2 ml fractions were collected. After elution of the nonabsorbed peak, the bound materials were eluted with a salt gradient of 0–1 M NaCl and A230 (absorbance) was measured using a UV-260 spectrophotometer (Shimadzu, Kyoto, Japan). The two peaks (fractions A and B) were subsequently concentrated using an Amicon YM-10 ultrafiltration membrane and their protein concentration was determined. The proteins were then subjected to bioassays for anti-angiogenesis activity (see below), and the bioactive fraction (Peak A) was dialysed against 6 M urea in 0.05 M sodium acetate (pH 5.8) containing protease inhibitors for 48 h at 4°C.

**Cation-exchange chromatography**

Fraction A isolated from Q-Sepharose chromatography was applied on to a Sp-Sepharose column (15 × 1.8 cm) pre-equilibrated with 0.05 M sodium acetate (pH 5.8) containing 6 M urea at 4°C. After elution of nonbound fractions, the column was eluted stepwise with an increasing salt gradient (0.05, 0.1, 0.2 and 0.5 M NaCl) in the same buffer at a flow rate of 1 ml/min and 2 ml fractions were collected by measuring A230 (absorbance).

**Gel electrophoresis**

SDS/PAGE was carried out as described by Laemmli [14]. The samples were dissolved in sample buffer [2% (w/v) SDS, 0.125 M Tris/HCl (pH 6.8), 0.002 M Bromophenol Blue and 20% (v/v) glycerol] and loaded on to SDS/12% PAGE mini gel. Electrophoresis was performed at 100 V for 2 h at 23°C. The gels were then stained with Coomassie Blue R250 and destained in 10% (v/v) acetic acid and 10% (v/v) methanol and photographed.

**Rat aortic ring assay**

This procedure was performed using the method in Montesano et al. [15], with some modifications. Briefly, the gels of collagen fibers (1.5 mg/ml) were prepared by mixing 7 volumes of cold collagen solution (prepared as described in [12] and maintained at 4°C) with 1 volume of 10 × DMEM and 2 volumes of NaHCO3 (11.76 mg/ml) on ice. Rat thoracic aortas were cut and transferred to a sterile Petri dish containing ice-cold Hanks balanced salt solution (pH 7.4; Gibco). After removing peri-aortic fibroadipose tissue, aortic rings (1 mm long) were sectioned and washed with serum-free DMEM supplemented with 100 units/ml of penicillin and 100 µg/ml streptomycin.

For the lower gel layer, 800 µl of ice cold type-1 collagen solution was dispensed into 35 mm tissue-culture Petri dishes (Nunc) and incubated at 37°C for 10 min to polymerize. The aorta explants were then placed on the top of the lower collagen gel layer and covered with another 800 µl gel layer and incubated at 37°C in a fully humidified 5% CO2 incubator for 15 min. After polymerization, 1.8 ml of DMEM supplemented with 25 mM NaHCO3, 2.5% (v/v) autologous rat serum (prepared in the laboratory), 1% (v/v) glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma) was added and incubated at 37°C in a humidified 5% CO2 incubator. In order to test the isolated proteins, after 24 h incubation, the medium on gel cultures was replaced by 1.8 ml DMEM supplemented with 25 mM NaHCO3, 2.5% (v/v) autologous rat serum, 1% (v/v) glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, along with various concentrations of the test proteins, and the incubation continued for a further 7 days. For each group 3 petri dishes, each containing 3 aorta rings, were used.

The number of aorta cell sprouting tips were counted using the blinded observer method [16] and photographed using a stereomicroscope (Axiovert 25; Zeiss) equipped with the KP-D20BP camera system (Hitachi).

The sprouts were scored by comparing the number and density of aorta sprout tips in the presence of the test proteins with that observed with medium alone (Control) as follows: M, maximum cell sprouting (++++); S, significant cell sprouting, but lower than observed in M (+++) and L, low levels of cell sprouting (+).
the second day of incubation, approx. 2 ml albumin was aspirated through the narrow end of the eggs with a hypodermic needle (18-gauge), allowing the CAM to drop away from the shell membrane. After sealing the holes, the eggs were returned to the incubator for the next 8 days. After 8 days of incubation, a 2 × 2 cm² zone on the shell, above the air sac, was punched out with a handed disc drill and the CAM was exposed.

The protein samples and 1% methylcellulose gel were prepared separately in sterile 50 mM phosphate buffer (pH 7.4). The methylcellulose gel was mixed with the protein samples (1:1 ratio) and 50 μl of this gel mixture containing 2 μg/μl of the test proteins was pipetted on to the surface of a sterile Teflon film and allowed to air-dry in a sterile laminar-flow hood. The resulting 2 mm methylcellulose discs were peeled away from the film surface and implanted on to the CAM surface. The windows were sealed with cellophane tape and incubated for 48 h at 37°C (55–60% humidity). The CAMs were subsequently fixed in place for 2 h by depositing 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and photographed in vivo using a stereomicroscope equipped with the MPS 52 camera system (Leica Wild M8; Leica, Germany).

The anti-angiogenesis effect was assayed by considering parameters such as a decrease in vessel density, branching number, bends and other angiogenesis-related morphology in the area of the CAM near the methylcellulose discs compared with untreated control (no test proteins). For each group, five eggs were used. Capillary density was scored qualitatively with a double-blinded statistical analysis.

Statistical differences between groups were evaluated using Student’s t test. The results are expressed as the means ± S.D. for at least 3 (duplicate or triplicate) independent experiments with similar results (where P < 0.05 is significant).

RESULTS AND DISCUSSION

As a result of the opinion that sharks rarely get cancer [8], many efforts have been made to determine the purity, bioactivity and clinical efficacy of shark cartilage. The isolation, purification, analysis and standardization of the active materials in shark cartilage is still extremely challenging. But most medical research on shark cartilage proteins (crude extract, fractions A and B) on angiogenesis and microvessel sprouting was tested in vitro using the rat aorta ring assay. In this system, aortic rings cultured in collagen generate a self-limited angiogenic response which, under appropriate culture conditions, can be monitored and quantified. The anti-angiogenic activity was quantified by counting the cell-sprouting tips around the aorta fragments in the samples exposed to the test proteins and the control samples as described in the Materials and methods section. The anti-angiogenesis effect of the isolated protein samples on rat aorta cell sprouting, as shown in Figure 2, is dose-dependent, and fraction A contained the most anti-angiogenesis activity. Therefore peak A (the bioactive fraction) was then applied on to a Sp-Sepharose column and the proteins were eluted as a function of a salt gradient. Fraction A resolved into five major peaks, designated A0, A1, A2, A3 and A4 (Figure 1B).

Figure 3 shows a typical image of the anti-angiogenic activity of the Q- and Sp-Sepharose fractions as determined using the rat aorta ring assay. For simplicity, only the results for 0.1 mg/ml protein are shown. As can be seen in Figure 3(A), fraction A shows the highest inhibitory effect on aorta ring sprouting, whereas fraction B showed a similar pattern of cell sprouting and migration to that of the control group, i.e. migrating cells were observed further away from the aorta fragment and the number of sprouts was greater than that of the crude extract or fraction A. But in the samples treated with fraction A, sprouting cells were not observed except in areas close to the aorta fragments. This observation supports the theory that fraction A has a potent inhibitory effect on cell migration and sprouting in the rat aorta angiogenesis model. The results obtained from analysis of the fractions from Sp-Sepharose chromatography using the rat aortic ring assay are shown in Figure 3(B). The results indicate that fraction A1 has the strongest anti-angiogenic effect. Fractions A3 and A4 displayed a similar pattern to that of the control group (results not shown).

Although the rat aortic ring sprouting assay is an effective in vitro method of identifying and screening substances that influence angiogenesis, findings must be confirmed using in vivo angiogenesis tests. Therefore the samples were also evaluated using the in vivo CAM assay model. As shown in Figure 4, the quantification and microscopic observation of CAMs treated with cartilage proteins demonstrated that the microvessel density and the normal vessel branching of methylcellulose discs containing protein fraction A1 was decreased considerably compared with the control group or the other cartilage-derived fractions examined.

Electrophoresis of the fractions by SDS/PAGE (Figure 5) shows that bioactive fraction A1 consists of two closely related proteins (lane 3) which migrate with apparent molecular masses of approx. 14.7 and 16 kDa. The other fractions were composed of a diverse wide range of high and low molecular mass proteins (lanes 4–6). The two bands present in lane 3 are possibly isomers of the same protein or are the result of some sort of modification, e.g. carbohydrate content, although no carbohydrate was detected in these fractions when the gel was stained with ammoniacal silver to detect glycoproteins.
In summary, shark cartilage contains low-molecular-mass proteins with potent anti-angiogenesis activity, and this protein fraction has not been reported previously. There is extensive interest in elucidating the cellular and molecular mechanism involved in the angiogenesis process. As a result, different chromatographic procedures, together with in vitro and in vivo assays, have been employed in order to obtain and define bioactive anti-angiogenic materials. Partially purified proteins from shark cartilage with anti-angiogenesis-related activities extracts has been the subject of many reports. These proteins, including U-995, SCAF-1 and SCF2, have different molecular masses {10–14 kDa for U-995 [19], 18 kDa for SCAF-1 (SR-related CTD-associated factor 1) [21] and 10 kDa for SCF2 [9]}, but their real identity is still obscure. Shark cartilage extract also contains anti-MMP [5], anti-VEGF [6], pro-apoptotic [7] and tPA stimulatory [22] activities, which have distinct size characteristics compared with the proteins identified in the present study.

We used two-step fast flow chromatographic methods to fractionate the crude extract of shark cartilage. The fractionation steps yielded two closely-related low-molecular-mass proteins which elute at the same position in the SP-Sepharose chromatogram. In our purification procedure, the anti-angiogenesis activity of all cartilage fractions were screened simultaneously with two models of in vitro and in vivo angiogenesis (rat aorta ring assay and CAM assay respectively) for certification of anti-angiogenesis activity of bioactive cartilage proteins, and only active fractions were submitted to further purification steps. The results

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**Figure 1**  Ion-exchange chromatography of proteins extracted from shark cartilage:

(A) Chromatogram of cartilage crude extract fractioned using a Q-Sepharose fast flow column (14 × 2.5 cm). The eluent was 0.05 M Tris/HCl and 6 M urea (pH 8.0). The flow rate was 1 ml/min and the salt gradient of 0–1 M NaCl in the same buffer was applied (dashed line). A_{230} (absorbance) was measured (♦). (B) Fractionation of fraction A from Q-Sepharose separation on a Sp-Sepharose column (15 × 1.8 cm). The eluent was 0.1 M sodium acetate and 7 M urea (pH 5.4). The flow rate was 1 ml/min and a stepwise salt gradient of 0.05, 0.1, 0.2 and 0.5 M of NaCl was used (dashed line) and A_{230} (absorbance) was measured (♦).
suggest that, unlike the other fractions detected, fraction A1 blocks *in vivo* and *in vitro* angiogenesis in the assay systems used in this study. Since the next steps in the separation of these two proteins from each other requires advanced procedures and will demand extensive work, at this stage it remains to be resolved whether one or both of the low-molecular-mass proteins in lane 3 is responsible for the angiogenesis inhibition detected in fraction A1.

Finally, it has already been reported that angiogenesis, the formation of new capillaries from pre-existing vessels, is essential
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Figure 4 CAM assay of the fractions A0, A1, A2, A3 and A4

In this assay, 100 μg of each of the isolated fractions (A0, A1, A2, A3 or A4) was used, and this was replaced in the control sample with 50 mM phosphate buffer only. Each photograph is representative of 3 experiments each performed in replicates of three to five eggs. Solid circles represent the location of methylcellulose implant.

Figure 5 SDS/PAGE of purified shark cartilage proteins

Fractions were resolved by SDS/12% PAGE. Lane 1, fraction A from Q-Sepharose separation. Lanes 2–6, fractions A0–A4 from Sp-Sepharose chromatography respectively. Molecular-mass marker (M) consisted of lysozyme (14.3 kDa), bovine β-lactoglobulin (18.4 kDa), bovine pancreas trypsinogen (24 kDa), chicken egg ovalbumin (45 kDa) and BSA (66 kDa).

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REFERENCES

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