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Short communication

Identification of low-molecular-weight protein (SCP1) from shark cartilage with anti-angiogenesis activity and sequence similarity to parvalbumin

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Abstract

Cartilage was considered as a possible natural source of anti-angiogenesis compounds due to its known avascular nature. In this study, a low-molecular-weight protein with an anti-angiogenesis activity was isolated from shark cartilage using a mild extraction procedure. The protein was purified to homogeneity by gel filtration and electroelution techniques and its N-terminal amino acid sequence was determined. The purified protein, designated as SCP1, represented a molecular weight of 13.7 kDa, pI of 6.9–7 and its N-terminal sequence revealed sequence similarity to alpha parvalbumin family. The protein inhibited angiogenesis when subjected to microvessel sprouting of collagen-embedded rat aortic ring assay. It is suggested that SCP1 could be considered as a new angiogenesis inhibitor derived from shark cartilage.

Keywords: Angiogenesis; Anticancer drugs; Shark cartilage; Aorta ring assay; Parvalbumin

1. Introduction

The idea that any increase in tumor cells population is preceded by an increase in new capillaries opened a new approach in the field of cancer treatment based on the fact that the inhibition of new vascularization induced by tumors would lead to arrest tumor growth [1]. Angiogenesis is fundamental process by which new blood vessels are formed and is one of the crucial steps facilitating tumor growth [2,3].

Cartilage is normally avascular tissue and rarely develops malignant tumors. It was the first normal tissue to be regarded as a natural source of anti-angiogenesis compounds [4]. Moses et al. reported a protein with angiogenesis inhibitory activity from calf cartilage and called cartilage-derived inhibitor (CDI) [5]. Subsequently, McGuire et al. found that an extract prepared from shark cartilage had anti-angiogenesis activity. This activity was observed only in the fraction having a molecular weight less than 10 kDa [6]. Also, two purified fractions have been obtained from shark cartilage, U-995 and Neovastat (AE-941) [7,8] and shown to have a potent anti-angiogenesis effect in the CAM model. A novel cytokine of the TNF superfamily (VEGI) has also been identified as a highly effective inhibitor of angiogenesis [9].

In the present study we have attempted to isolate and purify a protein with an anti-angiogenesis activity from shark cartilage. The protein provides a potent inhibitory effect on capillary growth when analyzed on rat aortic ring assay.

2. Material and methods

2.1. Materials

DMEM (Gibco) supplemented with 30 mg/l of asparagines, 120 mg/ml penicillin and 200 mg/ml streptomycin, pH 7.4 was prepared and after sterilization by 0.2 μm Millipore filter, kept at 4 °C until use. Rat tail collagen (Type I and 1.5 mg/ml) was prepared according to the method of Strom and Michalopoulos [10]. Sp-Sepharose fast flow was from Pharmacia-Amershame and protein molecular weight marker was from Sigma. Sprague–Dawley rats weighting of 150 g were obtained from RAZI Institute, Karaj, Tehran, Iran.

Abbreviations: PMSF, phenylmethylsulfunylfluoride; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CAM, chorioallantoic membrane; DTT, dithiotritol; IEF, isoelectric focusing; CD, circular dichroism.

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2.2. Protein extraction and purification

Shark cartilage was obtained from Persian Golf, Bosheh, Iran, cut into small pieces, lyophilized and pulverized. The pulverized cartilage powder was homogenized in five volumes of extraction buffer (50 mM Tris–HCl (pH 6.2), 25 mM NaCl, 0.1 mM EDTA and 1 mM PMSF) for 1.5 min at half speed and stirred overnight at 4 °C. The tissue suspension was then centrifuged at 6000 × *g* for 30 min at 4 °C. The extraction from the pellet was repeated twice as above except that three volumes of buffer were used and stirring time was 2 h. The combined supernatants were dialyzed against the extraction buffer by changing the buffer every 6 h and after brief centrifugation the clear supernatant was applied onto a Sephadex G50 column (1.5 cm × 70 cm) preequilibrated with the same buffer. The protein fractions were eluted with 50 mM Tris–HCl pH 6.2, 25 mM NaCl with a constant flow rate of 7 ml/h. The absorbances were monitored at 220 nm (multi-λ system). Fraction A was passed through Sp-Sepharose column (35 cm × 2 cm) pre-equilibrated with 10 mM Tris–HCl pH 5.8 using flow rates of 0.8 ml/min. The fraction eluted at the beginning of the column was collected, dialyzed (cut off 3500, Sigma) against Tris–HCl buffer and stored at −70 °C.

2.3. Preparative gel electrophoresis

The fraction A was concentrated using Viva Spin filter (5000 MW cut off) and applied onto a 15% (w/v) SDS-polyacrylamide slab gel (15 cm × 18 cm, 5 mm thickness) described essentially by Laemmli [11]. The desired protein was cut carefully and squashed in Tris buffer. The sample was electroeluted using dialysis bag (3500 cut off, Sigma) and 10 mM Tris–HCl pH 8.0, as an electrode buffer. Elution was carried out for 5 h at 100 V at 4 °C. The sample was then centrifuged for 15 min at 3500 × *g* to remove gel particles. The protein solution was dialyzed extensively against Tris buffer for 96 h by changing the buffer every 6 h and stored at −70 °C for further use. The protein concentration was determined by the method of Bradford [12], using bovine serum albumin as a standard.

2.4. SDS-PAGE and IEF

The purity of the protein was checked on SDS-PAGE. Electrophoresis was carried out for 2 h at room temperature running at 100 V. The gel was stained with coomassie blue R-250, destained in 10% acetic acid, 10% methanol and photographed.

Analytical isoelectric focusing was performed according to Dieter et al. [13] with some modifications using 6% polyacrylamide containing 40% (v/v) ampholine ranging pH 3–10 and16% sucrose. The purified protein was dissolved in sample solvent (50% sucrose, 2% (v/v) ampholine, 1% (w/v) DTT) and layered onto the gel. The starting voltage was 200 V for 30 min and then increased to 400 V. The electrofocusing was continued for 2 h then the gel was run at 700 V for 2 h (4 °C). After electrophoresis, the gel was immersed in 20% (w/v) TCA for 1 h, then in distillated water to eliminate the excess of ampholine. The gel was stained with coomassie blue and destained with acetic acid/methanol/water (1:1:8, v/v/v). Carbonic anhydrase B (Sigma) with pI of 6.5 was used as a pI standard.

2.5. N-terminal sequence analysis

The protein was electrophoresed on 15% SDS gel as described above and blotted on PVDF (Bio-Rad) membrane. The membrane was briefly stained with 0.1% coomassie blue R-250 in 50% methanol and then rapidly destained in 50% methanol–10% acetic acid. Membrane was finally rinsed in deionized H_2O and air dried. The protein was then applied to a Procise cLC or a Procise HT sequencer (Applied Biosystem) for Edman degradation and N-terminal microsequencing, which was carried out at the PAC, Karolinska Institute, Sweden. Sequence similarity was determined using http://www.expasy.org/BLAST.

2.6. CD analysis

Circular dichroism (CD) experiment was performed using CD spectrometer model 215 (AVIV Instruments Inc.). The Far-UV CD spectrum of 150 μg/ml of SCP1 solution in 10 mM Tris–HCl, pH 6.2 was recorded in the range of 190–260 nm with a spectral resolution of 1 nm. The scan speed was 20 nm/min and the response time was 0.3330 s with a bandwidth of 1 nm. Quartz cells with a path length of 10 mm were used and the measurement was carried out at 25 °C. Tris–HCl was also run as a blank buffer and subtracted from the raw data. Results are expressed as molar ellipticity, $[\theta]$, in degree cm^2 $dmol^{-1}$ assuming a mean molecular weight of 13.7 kDa. The spectrum was analyzed for the secondary structural elements of the protein using cdnn program version2 software (http://bioinformatik.biochemtech.uni-halle.de/cdnn).

2.7. Rat aortic ring assay

The procedure of Nicosia and Ottinetti [14] was used with some modifications. Thoracic aortas were removed from rat and after removing the pre-aortic fiboadipose tissue; 1 mm long aortic rings were sectioned. The rings were extensively rinsed in five consecutive washes of DMEM and then embedded in collagen gel prepared by mixing 7.5 volumes of cold collagen solution with one volume of 10× DMEM and two volumes of $NaHCO_3$ (11.76 mg/ml) and approximately 0.2 volumes of 1 M NaOH to adjust the pH at 7.4. The collagen gels was dispensed in 35 mm culture Petri dishes (NUNC) and incubated at 37 °C for 10 min to be polymerized. The aorta rings were then placed on the top of the lower collagen gel layer and covered with another 800 μl of gel layer and incubated. After polymerization to each Petri dish 1800 μl of DMEM, supplemented with 25 mM NaHCO3, 2.5% autologous rat serum, 1% glutamine, 100 unit/ml penicillin and 100 μg/ml streptomycin were added and incubated at 37 °C, 5% CO_2 and fully humidified incubator. After 24 h of incubation, the medium of test groups were replaced by above medium but containing test proteins and incubation was further continued for 7 days. Some dishes were received the test material after 52 h.

For each sample three Petri-dishes each containing three aorta rings was used.

The sprouts were scored by comparing the number and density of aorta sprout tips of test groups to that observed with medium alone (control) and photographed using light stereomicroscope (Zeiss, Axiovert 25, Germany) equipped with Camera System KP-D20BP (Hitachi Kokusai electronic Inc).

3. Results and discussion

To date many natural and synthetic compounds with anti-angiogenic properties have been reported. Guanidium HCl and urea have been widely used to extract these bioactive materials from cartilage. Water soluble extracts from shark cartilage has also provided a potent anti-angiogenic materials which are potentially used in cancer chemotherapy [15].

3.1. SCP1 is a low-molecular-weight protein with a neutral pI

In this study we employed a mild buffering system to extract the shark cartilage proteins. The crude extract was fractionated on Sephadex G-50 which resolved into three main peaks designated A–C (Fig. 1). Fig. 2A shows the SDS gel pattern of the crude extract and fraction A. Crude extract represents several low- and high-molecular weight proteins (lane 1), whereas, fraction A shows two bands at the molecular weight position of 11–14 kDa (Fig. 2A, lane 2). As this fraction represented anti-angiogenesis activity when tested on aortic ring assay (see below), fractions B and C (with no biological activity) where not considered for further analysis.

As ion-exchange chromatography failed to separate the two bands of fraction A, this protein fraction was further purified by preparative gel electrophoresis as described in the method section. The purified protein, designated as SCP1 (shark cartilage protein 1), was appeared as a single protein band on the SDS gel focusing at the molecular weight position of about 13.7 kDa

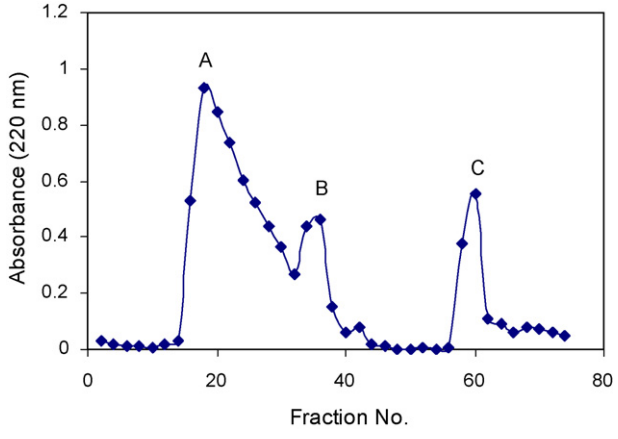

Fig. 1. Fractionation of the crude extract obtained from shark cartilage on Sephadex G-50 column (1.5 cm × 70 cm). Elution buffer was 50 mM Tris–HCl pH 6.2, 25 mM NaCl with a flow rate of 7 ml/l. Absorbances were read at 220 nm.

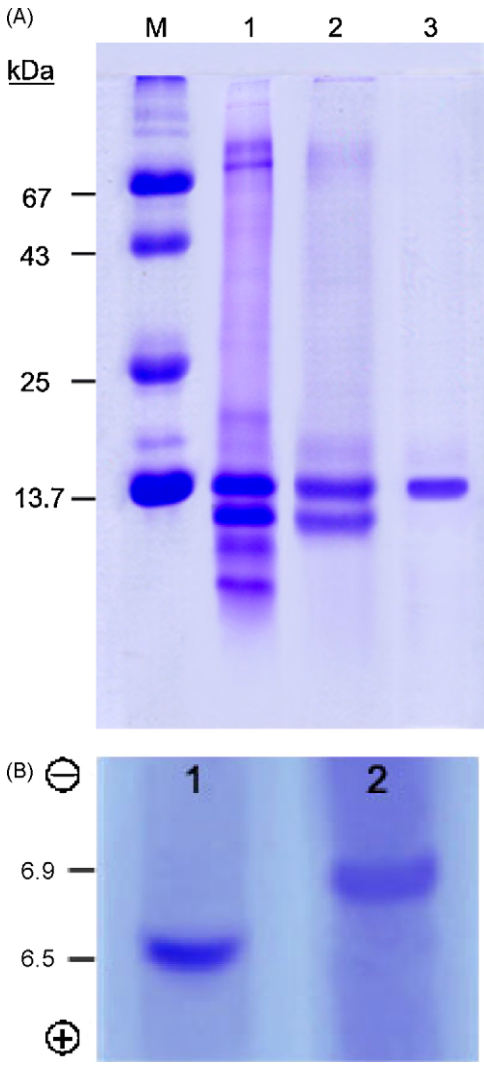

Fig. 2. (A) SDS-PAGE (15%) of the crude extract of shark cartilage (lane 1), fraction A obtained from Sephadex G50 (lane 2) and the purified protein through electroelusion (SCP1). M, molecular weight marker. (B) Isoelectric focusing gel of the purified SCP1 (lane 2) against carbonic anhydrase B as a standard pI (lane 1).

(Fig. 2A, lane 3). Isoelectric focusing gel of the protein is given in Fig. 2B. The protein was run in parallel to carbonic anhydrase B with a known pI of 6.5 and represented pI of about 6.9–7.

3.2. Secondary structure analysis

To obtain further information about the structure of the purified protein, SCP1, it was subjected to CD analysis and the spectrum is shown in Fig. 3. As is seen, the protein represents a main negative band at 220 nm which is characteristic of helix structure in the molecule. The percentage of the four secondary structures which were estimated by the use of the CD analyzer software between 205 and 260 nm in the advanced mode and the result is given as an insert in Fig. 3. As is obvious, the molecule shows 28.1% helix, nearly 20% β-sheet and 40% of the molecule is random coil.

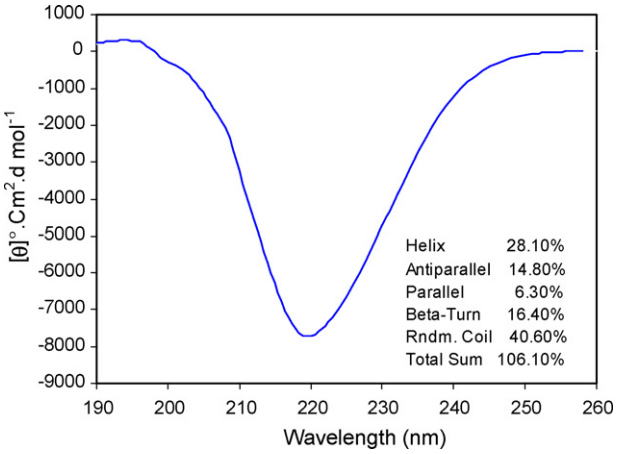

Fig. 3. Far UV circular dichroism spectrum of purified SCP1 protein in 10 mM Tris–HCl pH 6.2. The spectrum was obtained at 25 °C with 10 mm path length cell. All necessary corrections were made for background absorption.

3.3. SCP1 inhibits angiogenesis on aortic ring assay

Rat aorta ring model has proven to be among the most useful and sensitive assay for the evaluation of the angiogenesis process [14]. In this assay, rings of rat aorta embedded in collagen gel to generate an angiogenic response, can be blocked by angiogenesis inhibitors or amplified with angiogenic activators. To determine the effect of the purified protein, SCP1, on angiogenesis, the aortic ring assay was carried out in the absence and presence of SCP1 and the results is shows in Fig. 4. Microvessel outgrowth from rat aorta in the absence of SCP1 demonstrate a normal pattern as migrating cells are observed even at more distance away from the aorta fragment (panel a in Fig. 4). In contrast, sprouting cells can not be observed in the sample treated with the purified protein, SCP1 (panel b). Panel c is the same as panel b except that the protein sample (SCP1) was added to the culture after 52 h of aorta ring culture. As is seen, the sprouting cells has occurred but could not be continue in the presence of SCP1 which clearly demonstrates the inhibitory effect of SCP1 on angiogenesis.

3.4. SCP1 shows sequence similarity to parvalbumin family

In order to identify and further characterize SCP1, the protein was subjected to conventional N-terminal sequence analysis. Determination of 23 amino acids from the N-terminal revealed sequence similarity to alpha parvalbumin (Fig. 5). The sequence of SCP1 differs only in five positions compared to Leopad shark muscle parvalbumin, in nine positions from cat shark muscle parvalbumin [16], 12 positions with chicken muscle [17] and in nine positions with mouse muscle parvalbumin [18]. But the SCP1

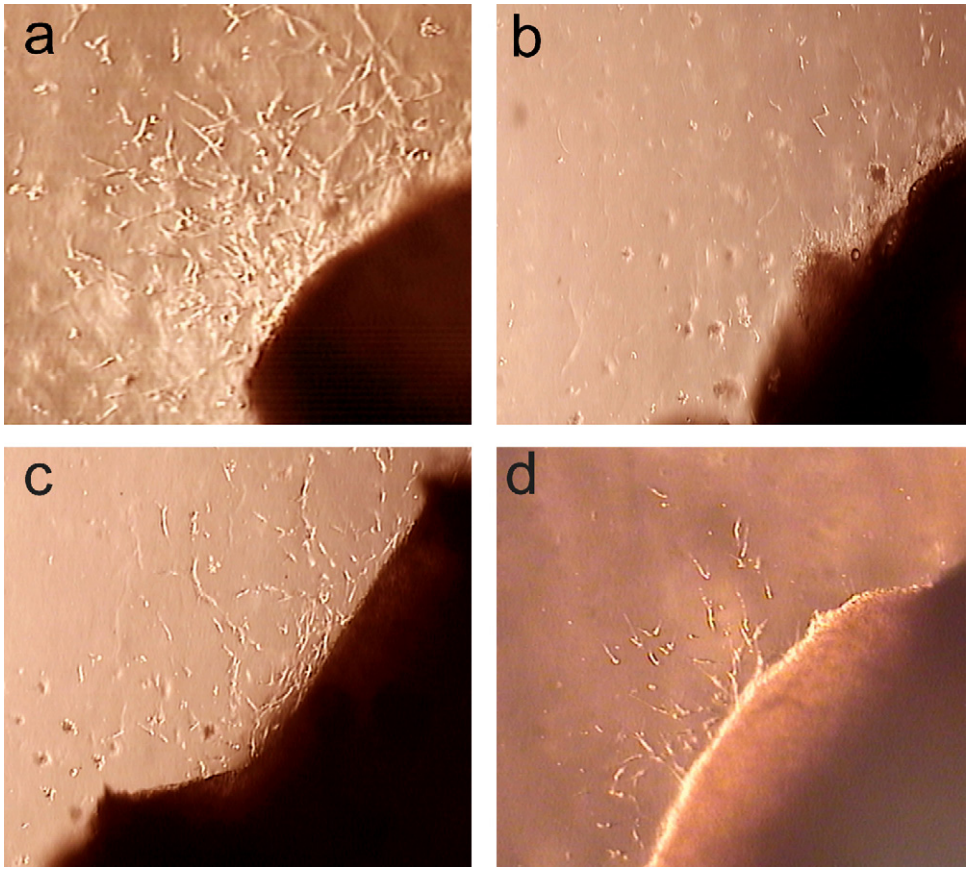

Fig. 4. Aortic ring assay: the aorta rings were cultured in full growth medium in the absence (panel a) and presence of the purified SCP1 protein after 7 days of culture (panel b). Panel c, the ring were treated with SCP1 after 52 h of culture and the incubation was continued till 7 days. Panel d shows the assay of the crude extract. Number of individual experiments was three (duplicate).

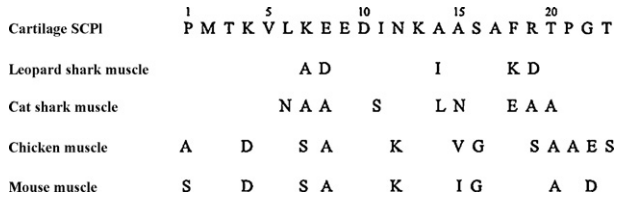

Fig. 5. The N-terminal amino acid sequence of the SCP1 obtained from shark cartilage. Alignment with the similar proteins is shown and for the simplicity, only the differed positions have been indicated.

sequence differs completely from chicken thymus parvalbumin [17,19].

Parvalbumins are calcium binding proteins first isolated from muscle of vertebrates. Two subclasses of parvalbumin have been described, alpha parvalbumin with pI of above 5.0 and beta parvalbumin with pI below 4.5 [20]. The pI of SCP1 purified from shark cartilage was neutral as indicated in Fig. 2B suggesting that this protein can be qualified as an alpha parvalbumin family. Parvalbumins have also been identified in non-muscle tissues such as chicken thymus, rat skin, liver, kidney and also brain cells [21,22]. Parvalbumin from neoplastic liver cells of rat has a very similar amino acid composition compared to rat muscle parvalbumin but can be distinguished from the later by its immunological behavior [23]. The herein isolated and purified SCP1 protein from shark cartilage can be considered as parvalbumin like protein but is distinguished from it by its anti-angiogenesis activity.

4. Conclusion

In summary, the results presented above demonstrate that shark cartilage contains low-molecular-weight proteins with potent anti-angiogenesis activity. One of these proteins, with a molecular weight of 13.7 kDa and pI 6.9, has been purified and its N-terminal sequence was determined. The protein shows high anti-angiogenesis activity and its 23 residues from the N-terminal resembles to parvalbumin family. The protein is highly structured thus more than 50% of the molecule is α-helix and β-sheet. Partially purified proteins with anti-angiogenesis related activities from shark cartilage extracts, has been the subject of many reports. These proteins including 10–14 kDa U-995 [7]; 18 kDa, SCAF-1 [24] and 10 kDa, SCF2 [25] and Neovastat (Æ-941) [26,27] which show distinct size characteristics compared to our protein and their real identity is still obscure. Although herein reported SCP1 protein from shark cartilage shows sequence similarity to parvalbumin but it differs in its molecular weigh, pI and secondary structure content. Therefore our SCP1 protein could be considered as a new angiogenesis inhibitor; however the complete characterization of the protein demands further investigation to reach the final decision.

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