Demonstration of Inhibitory Effect of Oral Shark Cartilage on Basic Fibroblast Growth Factor-Induced Angiogenesis in the Rabbit Cornea

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Several angiogenic inhibitors have been obtained from shark cartilage, some of these are currently in clinical trials for assessment of safety and therapeutic efficacy in humans. Still, shark cartilage taken orally is commonly used in alternative and complimentary medicine for various ailments including serious diseases such as cancer. However, only few studies of oral shark cartilage have demonstrated pharmacological effects in experimental animals or patients, to indicate safe doses with sufficient bioavailability. In the present study we demonstrated the antiangiogenic properties of oral shark cartilage in the rabbit cornea model. Slow-release, polymethylmethacrylate pellets containing basic fibroblast growth factor (bFGF) were surgically implanted in the rabbit cornea to stimulate neovascularization scored by stereo microscopy. Powdered shark cartilage (PSC; commercial product) was tested orally along with a water-soluble fraction (WSF) of this cartilage product which was tested by local application. Animals were treated with oral dosages of 100 mg/kg PSC or 200 mg/kg thalidomide as positive control. Pellets containing WSF (50, 100 or 200 µg/pellet) or bFGF-inhibitor pentosan polysulfate were implanted adjacent to the bFGF pellet. Oral shark cartilage inhibited bFGF-induced angiogenesis, as did oral thalidomide, in this in vivo model. WSF and pentosan polysulfate was shown to block neovascularization in the cornea when applied locally. This study demonstrates that in the rabbit, oral shark cartilage appears to produce systemic levels of antiangiogenesis inhibitors that can exert their effect at the cornea.

Key words angiogenesis; basic fibroblast growth factor; shark cartilage; rabbit cornea

Angiogenesis is a multistep and redundant process crucial in several physiological events and pathophysiological developments such as cancer. Since the early findings by Folkman et al. that tumor growth is dependent on new vascularization, it is now broadly accepted that solid tumors cannot grow beyond 1—2 mm without a vascular supply of oxygen and nutrients. Further, there is a substantial body of evidence indicating that attacking tumor neovascularization is a promising approach in the treatment of cancer.2

The acquisition of an angiogenic phenotype in endothelial cells requires that angiogenic factors be overexpressed and released by cancer cells and host cells. These factors include fibroblastic growth factors and vascular endothelial growth factors that bind to specific receptors on the endothelial cell surface stimulating their migration and proliferation.5,4 On the other hand, the migration and organization of endothelial cells in capillary structures depend on the activity of the pericellular fibrinolytic system and the overexpression of different cellular adhesion molecules.5 All these events in the angiogenic process afford potential targets for an antiangiogenic therapy.

Many compounds derived from various natural sources have been found to have antiangiogenic effects using both in vitro and in vivo models.6 These compounds include extracts and fractions from cartilage.7 Cartilage is an avascular tissue, and for this reason was believed to contain compounds with antiangiogenic activity. This hypothesis was first tested in 1973 by Eisenstein et al., who reported that cartilage extracted with guanidine 1 M did not resist to becoming vascularized when placed on the chick chorioallantoic membrane (CAM).9 It was later demonstrated by Folkman and Ingber that cartilage could inhibit tumor-induced angiogenesis in the CAM model.9 Based on those works, recent studies have shown that substances isolated from shark cartilage, for example U-995 and AE-941, inhibit angiogenesis and tumor growth in vivo.10,11 Fontenele et al. recently described analgesic and antiinflammatory properties of a water soluble fraction (WSF) of shark cartilage that was attributed principally to a small peptide with a molecular weight of 2.3 KDa.12 In the latter work these in vivo pharmacological effects were observed with oral as well as intraperitoneally administered.

The aims of the present study were a) to demonstrate the ability of oral shark cartilage to exert an inhibitory effect on rabbit cornea neovascularization induced by basic fibroblast growth factor (bFGF), and b) to examine in the same model a newly described bioactive fraction of shark cartilage for antiangiogenic activity.

MATERIAL AND METHODS

Chemicals Powdered shark cartilage was provided by Selacchii Produtos Marinhos Ind., Ltd., Fortaleza, Brazil. This product was a mixture of spinal column cartilage of Galeocerdo cuvier, Ginglymostoma cirratum, Carcharhinus faliformes, C. porosus, Prionace glauca, and Sphyra mokarran, shark species native to the north and northeast coastal waters of Brazil. A water soluble fraction of the same shark cartilage, comprising 17% protein, was isolated and provided by the Laboratory of Neuropharmacology in our department. bFGF was purchased from Sigma Co., St. Louis, MO, U.S.A. Pentosan polysulfate (PPS) was from Gene, Munich, Germany. Polymethylmethacrylate (PMMA) and methylmetacrylate (solvent) were from Classicco Ltd., São Paulo, Brazil, and kindly donated by Dr. Marcus A. Rabelo Lima-Verde from the Odontology Faculty of Federal University of Ceará.

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Water Soluble Fraction Isolation WSF was isolated from 10 g of shark cartilage mixture from several species (see MATERIALS AND METHODS) by first extracting with 250 ml of hexane. After filtration the remaining solvent was evaporated away at room temperature and the residue re-suspended in 10 ml DMSO. The insoluble material retained on the filter paper was dried at room temperature and extracted with 0.1 M ammonium bicarbonate, pH 8.0, for 2 h. After centrifugation, the supernatant was collected and regarded as the water-soluble fraction. The protein content of the fraction was 17.0 ± 1.5% determined by the method of Lowry, using bovine serum albumin as the standard.

Rabbit Cornea Assay To assess the antiangiogenic activity of shark cartilage, we used the micropocket rabbit cornea assay as described originally by Gimbrone et al., and according to the modifications made by Ziche et al. Briefly, New Zealand white rabbits weighing 1.5—2.5 kg (Federal University of Ceará, Fortaleza) were anesthetized with sodium pentobarbital (30 mg/kg) followed by retrobulbar anesthesia using 2% xylocaine. A superficial incision 1.5—2 mm wide was made in the lower half of the cornea, and a micropocket (≥2 mm in length) was made inside the transparent avascular corneal stroma and in the direction of the limbus using a malleable iris spatula. Slow-release pellets 1.5 mm (diameter) × 0.5 mm (width) were prepared under sterile conditions using a plastic cast. The polymer was first mixed with test substance, suspended in solvent, and then allowed to dry at room temperature.

Angiogenic Activity Angiogenic activity was evaluated using a microsurgical microscope (D.F. Vasconcellos MP—90, magnification ×40) at 3-d intervals after the implant of the pellet, (i.e. days 3, 6, 9, 12 and 15). Angiogenic activity was calculated according to Ziche et al., by counting the number of vessels in the corneal vascularized area (vascular density), and by measuring their length in mm from the corneal-scleral limbus to the edge of capillary growth. The final results were then expressed as an angiogenic score (AS) calculated as: vascular density (1—5) × vessel length (mm). Vascular density was based on the following scale: 1 for 0 to 25 vessels; 2 for 25 to 50; 3 for 51 to 75; 4 for 76 to 100; and 5 for > 100 vessels.

Experimental Design New Zealand rabbits were maintained in individual cages and given chow and water ad libitum. They were randomly assigned to one of three groups. One group (n = 5) received by gavage a daily dose of 10 ml powdered shark cartilage suspension equivalent to a dose of 100 mg/kg body weight, for 12 d before and for 15 d after pellet implantation. The second group received daily 200 mg/kg thalidomide by gavage starting 1 d before pellet implantation. The second group received daily 200 mg/kg body weight, for 12 d before and for 15 d after pellet implantation. In contralateral corneas with only blank pellets without bFGF, in all cases for control or treated animals, AS ranged from 0.0 to 0.7. PSC administered orally inhibited the development of new blood vessels from the vascularized corneal-scleral limbus to the corneal stroma (Figs 1 and 3). Statistically significant differences in the antiangiogenic effect with PSC were observed at the second time point on day 6, where the AS was 0.9 ± 0.6 versus 2.2 ± 0.4 for the group treated with vehicle (p < 0.05; Mann–Whitney test). At the last time point, day 15, the difference between these two groups was markedly greater (p < 0.01) whereby the maximum inhibitory effect achieved with oral PSC treatment was 96%. These results can be clearly seen in the microphotograph where the vascular density in the cornea of animals treated with PSC is almost null when compared with that for the control group (Fig 3A). Thalidomide, 200 mg/kg also administered orally, produced a partial antiangiogenic effect, statistically significant on day 15 after pellet implantation and reaching maximal decrease in angiogenic score of 55% (Fig 1).

Effect of Oral Shark Cartilage on bFGF-Induced Angiogenesis Figure 1 shows the angiogenic scores (AS) in rabbit cornea stimulated with bFGF-bearing pellets for rabbits treated shark cartilage or thalidomide. In control rabbits receiving only vehicle, bFGF produced a time-dependent angiogenic response with a maximum on day 15 after pellet implantation (AS = 10 ± 1.3). There was however a delay in vessel growth during the first 3 d, while a strong angiogenic development was observed beginning on day 6 after pellet implantation. In contralateral corneas with only blank pellets without bFGF, in all cases for control or treated animals, AS ranged from 0.0 to 0.7 ± 0.2. PSC administered orally inhibited the development of new blood vessels from the vascularized corneal-scleral limbus to the corneal stroma (Figs 1 and 3). Statistically significant differences in the antiangiogenic effect with PSC were observed at the second time point on day 6, where the AS was 0.9 ± 0.6 versus 2.2 ± 0.4 for the group treated with vehicle (p < 0.05; Mann–Whitney test). At the last time point, day 15, the difference between these two groups was markedly greater (p < 0.01) whereby the maximum inhibitory effect achieved with oral PSC treatment was 96%. These results can be clearly seen in the microphotograph where the vascular density in the cornea of animals treated with PSC is almost null when compared with that for the control group (Fig 3A). Thalidomide, 200 mg/kg also administered orally, produced a partial antiangiogenic effect, statistically significant on day 15 after pellet implantation and reaching maximal decrease in angiogenic score of 55% (Fig 1).

Effect of Water-Soluble Fraction of Shark Cartilage on bFGF-Induced Angiogenesis Figure 2 shows dose-depen-
dent antiangiogenic effects with WSF-pellets at doses of 50 and 100 μg. Not shown are the data for 200 μg which were similar to those for 100 μg. The AS values obtained show that there was a decrease in the angiogenic response to bFGF with WSF beginning on the second measurement day (day 6). A statistically significant difference was obtained by the third measurement day (day 9) where the AS values in the treated groups were 5.6±0.4 (50 μg/pellet), 4.1±1.2 (100 μg/pellet) and 4.9±0.6 (200 μg/pellet), p<0.05 (Mann–Whitney test), in relation to the control group on the same day, 6.2±0.2. Moreover, in the subsequent time-points there was an increase in the difference between the AS values obtained with WSF-bearing pellets compared with blank pellets. The results show that WSF produced an inhibition on the angiogenic response to bFGF that was more apparent after 9 d. PPS at the single dose tested produced a decrease in AS similar to that by WSF at 100 or 200 μg/pellet, the relative antiangiogenic potency for WFS and PPS being 71.0 to 77.0% inhibition. PPS also showed an apparently time-dependent inhibition of angiogenic response that required 9 d after pellet implantation before evidence of statistically significant difference compared to control. Figures 3C and 3D give visual evidence of the difference in the corneal neovascularization between the group with tandem pellets bearing bFGF and PMMA alone, and the group with tandem pellets bearing bFGF and WSF (100 μg/pellet).

DISCUSSION

The present study demonstrated the antiangiogenic activity of oral shark cartilage in the rabbit cornea model whereby bFGF was used as the angiogenic stimulus. It thus appears that in the rabbit, bioavailability of angiogenesis inhibitor(s) present in shark cartilage does occur with oral administration. Here, bioavailability was not determined by the measure of blood or tissue drug levels, but was rather implied by the measure of a pharmacological effect at the cornea. This was also reproduced with local application at the cornea of a water soluble fraction of shark cartilage, previously shown to have in vivo analgesic and antiinflammatory activities. The inhibitory effects of WSF and PPS were statically significant (p<0.05) at days 6, 9, 12 and 15 compared with the control. Comparisons between groups were performed using the nonparametric Mann–Whitney test.

Fig. 2. Inhibition of bFGF-Induced Angiogenesis in the Rabbit Cornea by Local Application with Blank Pellet (■), Pellets with Water Soluble Fraction of Shark Cartilage at 50 (○) and 100 μg/Pellet (▲), and Pellet with PPS at 200 μg/Pellet (▼).

The antiangiogenesis effects of oral shark cartilage observed were similar to those for oral thalidomide, a known angiogenesis inhibitor with well established bioavailability characteristics. We also demonstrated that local application of pentosan polysulfate blocked angiogenesis in a manner similar to that of the water soluble fraction of shark cartilage. For both test substances, there appeared to be delayed inhibitory action that may have been due to their slow release from the polymer pellets requiring time to attain sufficient concentrations at the initiation site of angiogenesis. Pentosan polysulfate is a highly negative charged polysaccharide which has been shown to inhibit angiogenesis and tumor growth by binding to heparin-binding growth factors. These results suggest that one or more components of shark cartilage may have inhibited rabbit cornea angiogenesis through the blocking of the stimulatory action of bFGF, possibly through binding with the growth factor. bFGF is only one of the various angiogenesis stimulators, having a potent effect on the induction of endothelial cell migration and proliferation. It has been shown that bFGF stimulates new blood vessel formation through a pathway, different from other growth-promoting factors such as vascular endothelial growth factors or platelet derived growth factor. Since it was established that cancers and other diseases are angiogenesis-dependent, many compounds have been developed as antiangiogenic drugs and some of these seem to exert their effects blocking some step in growth factor signal pathways as recently reviewed by Malonne et al. and Paper. Effects of shark cartilage on angiogenesis factors other than bFGF cannot be ruled out.

In the present study of PSC, we used a relatively low oral dose of 100 mg/kg to demonstrate in vivo inhibition of angiogenesis in the rabbit cornea model whereby bFGF was used as the angiogenic stimulus. It thus appears that in the rabbit, bioavailability of angiogenesis inhibitor(s) present in shark cartilage does occur with oral administration. Here, bioavailability was not determined by the measure of blood or tissue drug levels, but was rather implied by the measure of a pharmacological effect at the cornea. This was also reproduced with local application at the cornea of a water soluble fraction of shark cartilage, previously shown to have in vivo analgesic and antiinflammatory activities. The inhibitory effects of WSF and PPS were statically significant (p<0.05) at days 6, 9, 12 and 15 compared with the control. Comparisons between groups were performed using the nonparametric Mann–Whitney test.

Fig. 3. Photomicrographs Showing the Effect of Powdered Shark Cartilage (PSC) and Its Water-Soluble Fraction (WSF) on bFGF-Induced Angiogenesis in the Rabbit Cornea

Corneal neovascularization elicited by bFGF (0.1 μg/pellet) in animals treated with A) vehicle or B) oral PSC (100 mg/kg) and in animals treated with C) a PMMA pellet without WSF or D) local application of WSF. All photographs were taken 15 d after pellet implantation. Arrows indicate the position of the bFGF-bearing pellets. Magnification, X40.
genesis induced by bFGF in the rabbit cornea. Recently, Davis et al. showed that oral doses of PSC could inhibit angiogenesis in a rat model, whereby angiogenesis observed through a mesenteric window was induced by mast cell stimulation. However, these authors required a higher oral dose of 600 mg/kg, suggesting some differences in the intestinal absorption of bioactive substances of shark cartilage between these two species. Moreover, the dose we used produced almost complete inhibition of angiogenesis, yet was 10-fold less than the 1 g/kg dose used by Miller et al. in a clinical trial to assess the safety and efficacy of shark cartilage in cancer treatment.

The antiangiogenic potential of shark cartilage has been attributed to different compounds or fractions such as U-995 and AE-941. These latter compounds have shown antiangiogenic activity in the chick embryo chorioallantoic membrane (CAM) model. Here, we tested a WSF preparation that was previously obtained and examined for bioactivity by Fontenele et al. This group demonstrated that WSF has two major chromatographic peaks with 103 kDa and 2.3 kDa respectively. Both constituents of WSF administered orally showed analgesic and anti-inflammatory activities in rat models. It was suggested that the smaller peptide could be responsible for these pharmacological properties of WSF.

There is some evidence supporting the idea that small quantities of intact proteins can be absorbed in the intestinal wall of animals and humans. For instance, it was shown that albumin and β-lactoglobulin are transported through the intestinal mucosal barrier using luminal and intercellular routes.

Notably, the maximal antiangiogenic effect achieved with WSF was less than that with orally administered shark cartilage, which may have been due to a delayed effect as noted above. Oral PSC was given prior to, as well as after, bFGF pellet implantation, that resulted in an earlier antiangiogenic effect. This schedule for oral shark cartilage was similar to that of Davis et al. with the aim of enhancing the bioavailability of the angiogenesis inhibiting components, likely high molecular weight substances. On the other hand, WSF may not be the only principal antiangiogenic component of PSC. It could be hypothesized that the mechanism of action of WSF involves the inhibition of some step in the pathway of angiogenesis induced by basic fibroblastic growth factor. In a separate study we found that WSF at concentrations as low as 30 μg/ml inhibited the growth of human umbilical vein endothelial cells (HUVEC) in bFGF-supplemented medium (data not shown). Also, WSF was found to differ from the other cartilage products studied, in that WSF show no inhibitory effect on collagenase activity (data not shown).

Finally, it cannot be ruled out that metabolic alteration of shark cartilage components including WSF leads to enhanced antiangiogenic activity. Further studies are warranted to examine this quantitative discrepancy in antiangiogenic potency of crude shark cartilage and WSF.

In conclusion, these results demonstrate the apparent bioavailability of oral shark cartilage in the rabbit resulting in a potent antiangiogenic activity on corneal neovascularization induced by bFGF, and that this activity is due, at least in part, the inhibition of endothelial cell growth by the water-soluble fraction isolated by the group of Fontenele.

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