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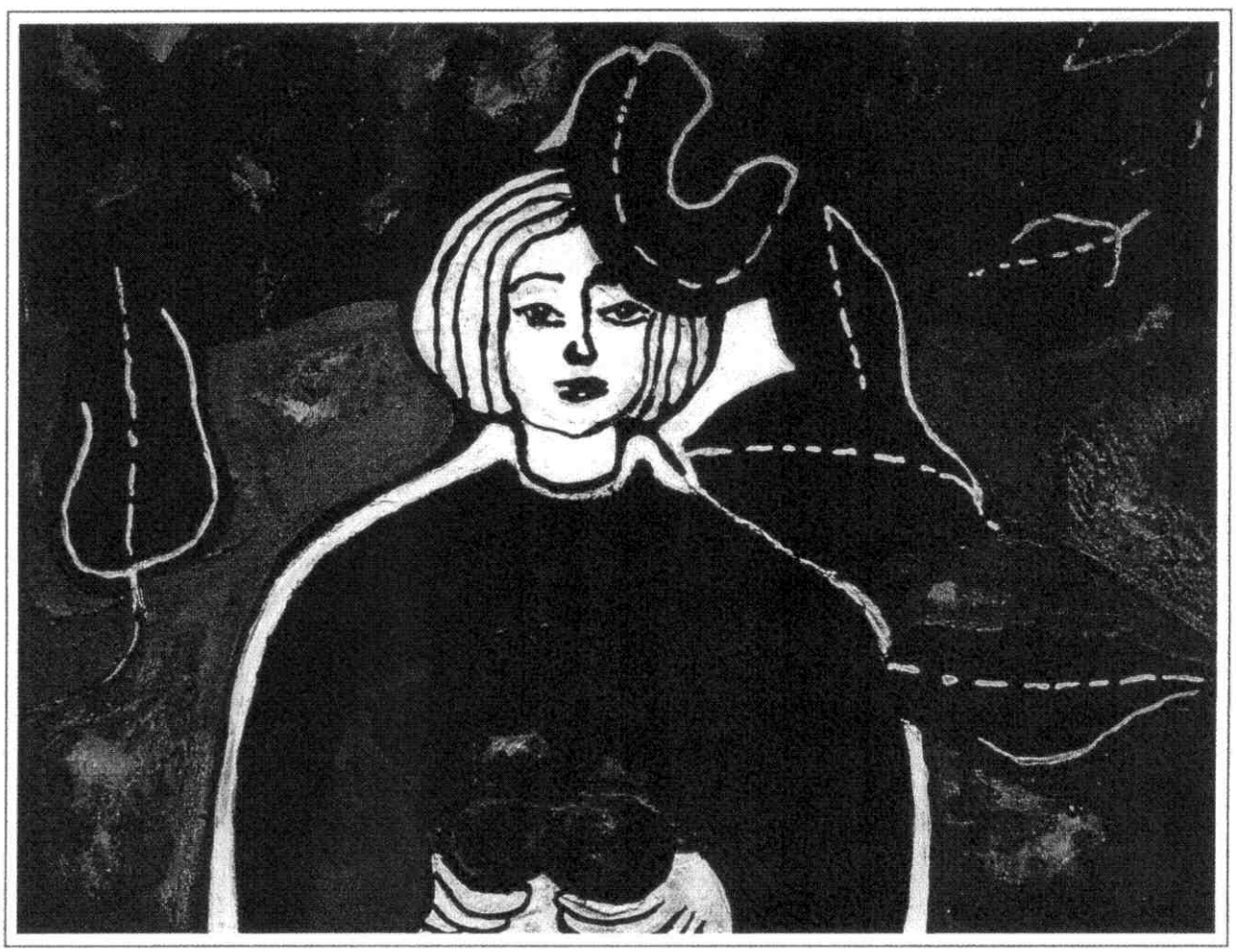

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Inhibition of Pro-Angiogenic Factors by a Lipid-Rich Shark Extract

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ABSTRACT This study aimed to determine whether a shark muscle oil–olive oil mixture influences activators of human angiogenesis. The mixture completely abolished the stimulation induced by vascular endothelial growth factor (VEGF), fibroblast growth factor-2, transforming growth factor-β, and platelet-derived growth factor. This suggests that it may compete with these growth factors for their binding sites on the endothelial cell surface either by binding to the growth factor or by blocking the actual receptor. The possibility of the oil binding to the VEGF receptor was studied through the use of soluble forms of the receptors (VEGF-R1 and VEGF-R2). It was found that the shark oil–olive oil inhibited the formation of the complexes of VEGF with both of the receptors. This could have been because the oil bound to either the VEGF or the receptor or both. To determine which is possible, the shark oil–olive oil was mixed with the receptors. The molecular size of the receptors increased, and these larger forms of the receptor had reduced capacity for complexing with VEGF. Therefore, one mode of potential anti-angiogenic action of the shark muscle oil–olive oil is the inhibition of the activity of a number of stimulatory molecules, including VEGF. This study demonstrates that the blend of shark and olive oils antagonizes VEGF activity by binding to at least two receptors for the factor, thereby inhibiting the activation by the growth factor.

KEY WORDS: • ***angiogenesis*** • ***receptor*** • ***shark lipid*** • ***vascular endothelial growth factor***

INTRODUCTION

SINCE AROUND 1990 SHARK CARTILAGE has been widely promoted as a possible therapeutic preparation for the treatment of solid tumors through its proposed anti-angiogenic action. However, it was not until 1997 that there was empirical evidence that the oral administration of shark cartilage was anti-angiogenic.[1] Preliminary observations suggested that shark lipids may be partially responsible for some of the anti-angiogenic properties of shark tissues (data not shown). Accordingly, we investigated the effect of shark lipid on activators of angiogenesis. Because of the potency of the lipid extract from shark muscle (macolipin), it needed to be diluted, so various vegetable/cooking oils were evaluated as the diluent. Most oils tested were inert, but when the macolipin was mixed with olive oil it was found that there was a synergistic action of the two oils.

This report presents the results of preliminary experiments to characterize the mechanisms of the anti-angiogenic activity of shark lipid–olive oil mixture. We evaluated the effect of the lipid blend on the pro-angiogenic activity of cytokines and growth factors and their receptors, including fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF).

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MATERIALS AND METHODS

Materials

Unless indicated otherwise, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). The growth factors used in this experiment were human VEGF (VEGF-121) (recombinant), FGF-2, platelet-derived growth factor (PDGF)-AB, and transforming growth factor-β (TGF-β). Soluble receptors were human recombinant VEGF-receptor-1 (FLT-1) and VEGF-receptor-2 (Flk-1, KDR). The primary antibodies used in this experiment were monoclonal anti-human VEGF antibody [immunoglobulin (Ig) G fraction] raised in goats and anti-human VEGF-receptor-1 antibody and anti-human VEGF-receptor-2 antibody, both raised as monoclonal antibodies in mice. The secondary antibodies were anti-goat IgG (peroxidase conjugated) and anti-mouse IgG (peroxidase conjugated) that had been raised in rabbits.

The reagents and equipment used for the polyacrylamide gel electrophoresis were purchased from BioRad Laboratories (Hercules, CA). The Immobilon membranes were obtained from Millipore Corp. (Bedford, MA). 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Sigma Chemical Co.

The shark muscle oil–olive oil used in this study was supplied by Aotea Pacific Ltd. (Auckland, NZ). The ratio of shark muscle oil to olive oil was 1:9.

Methods

In vitro *assay of angiogenesis (aortic ring assay).* This assay was based on the protocols described by Nicosia and Ottinetti[2] and Brown *et al.*[3] A length of aorta was removed from a rat and cleaned of adhering fatty and connective tissues before being cut into rings of approximately 3 mm in size. Fibrinogen was layered in the bottoms of wells of 24-well culture plates and allowed to gel by thrombin action. A ring was then layered on the top of each gel, and a further layer of fibrin was placed on this. The fibrinogen was prepared in MCDB131 medium supplemented with antibiotics. The double layer of fibrin was then overlaid with MCDB131 containing the test materials.

The gels were incubated at 37°C in an atmosphere of 3% CO_2/97% air. The rings were examined using an inverted microscope, and the growth of microvessels from their perimeters was observed. Digital pictures were taken of these after 7 days, and the extent of microvessel growth relative to the size of the ring was determined using NIH Image software. From this the growth of microvessels was determined for each well.

Each test substance was assayed in triplicate, and the mean growth rate was calculated. Because all the lipid-rich materials were dissolved in ethanol (final concentration was 0.2%), 0.2% ethanol was therefore used as the control in all of the assays. The calculation of angiogenesis in the control was 100% growth. The growth in samples that was more than 100% was defined as stimulation, and the growth that was less than 100% was defined as inhibition.

Preparation of immune complexes. The reaction samples were prepared as indicated in Table 1, reacted at room temperature with shaking for 2 hours (for No. 5 and No. 9, VEGF were added 1 hour after the reaction began).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Aliquots (20 μL) of the incubation mixtures were loaded onto the lanes of polyacrylamide gels (5% for VEGF-R1 and VEGF-R2 and 10% for VEGF) and electrophoresed at 200 V for 1 hour. The separated proteins were transferred electrophoretically from the gel to a PVDF membrane using a BioRad semidry transfer apparatus. Following the transfer, the membrane was blocked by incubating the membrane in blocking buffer (0.05% Tween-20 in phosphate-buffered saline containing 3% non-fat dry milk). After washing with phosphate-buffered saline containing 0.05% Tween-20 (washing buffer), the membrane was incubated with the primary antibody by shaking for 2 hours at room temperature and then washed three times (5 min for each wash) with the washing buffer. The membrane was incubated with shaking with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The membrane was again washed three times with washing buffer. The membrane was then developed using the ready-to-use TMB solution by incubating it in this solution and stopping the color development by rinsing with deionized water.

RESULTS

The effect of shark muscle oil–olive oil on angiogenesis was tested using the aortic ring culture model using olive oil as a control. As shown in Table 2, the results suggested that shark muscle oil–olive oil, but not olive oil alone, inhibits aortic ring angiogenesis in a dose-dependent manner.

The effect of shark muscle oil–olive oil on the actions of a number of positive effectors of angiogenesis was tested

TABLE 1. PREPARATION OF IMMUNE COMPLEXES OF VEGF AND THEIR RECEPTORS IN THE PRESENCE/ABSENCE OF SHARK MUSCLE OIL–OLIVE OIL

Sample number	*VEGF (5 μg/mL) (μL)*	*VEGF-R1 (10 μg/mL) (μL)*	*VEGF-R2 (10 μg/mL) (μL)*	*Shark muscle oil–olive oil (100 μg/mL) (μL)*	*Olive oil (100 μg/mL) (μL)*	*Binding buffer (μL)*	*Water (μL)*
1	20				20	50	110
2		100			20	50	30
3	20	100			20	50	10
4	20	100		20		50	10
5	20[b]	100		20		50	10
6			100		20	50	30
7	20		100		20	50	10
8	20		100	20		50	10
9	20[b]		100	20		50	10

[a]Consisting of 4 m*M* $CaCl_2$, 0.4 m*M* $CuSO_4$, and 0.5% Tryptone in Dulbecco's Modified Eagle's Medium.
[b]Add 1 hour later.

TABLE 2. EFFECT OF SHARK MUSCLE OIL ON ANGIOGENESIS

	% of control	*% of inhibition*
Olive oil (30.0 μg/mL)	100.0%	0.0%
Shark muscle oil–olive oil (10 μg/mL)	81.03%	18.97%
Shark muscle oil–olive oil (30 μg/mL)	44.06%	55.94%

using the aortic ring culture model. The effectors tested were VEGF, FGF-2, PDGF, and TGF-β.

VEGF

VEGF is one of the most potent stimulators of angiogenesis,[4–7] and so we undertook several experiments to determine whether shark muscle oil–olive oil had any influence on this factor. A summary of the results is presented in Table 3.

VEGF stimulated angiogenesis and the shark muscle oil–olive oil inhibited it as expected, but when the two were combined, the shark muscle oil–olive oil inhibited the 34% stimulation that VEGF produced. The inhibition by the combination was exactly the same as for the shark muscle oil–olive oil (at the same concentration) on its own. This indicates that shark muscle oil–olive oil strongly interferes with VEGF and its role in the angiogenesis process.

Basic FGF-2

FGF-2 is also a well-characterized stimulator of angiogenesis.[8–10] While FGF-2 is not as potent as VEGF, there are often higher concentrations of FGF-2 in tissues where angiogenesis is active, and so its role is important. We investigated the interaction of shark muscle oil–olive oil on FGF-2 stimulation (Table 4).

As expected, the FGF-2 stimulated angiogenesis and the shark muscle oil–olive oil inhibited it. The combination of these two effectors resulted in an overall inhibition that was similar to that produced by the shark muscle oil–olive oil alone. This would suggest that shark muscle oil–olive oil can abolish completely the effect of the FGF-2. This indicates that it interferes with the action of FGF-2. This is analogous to the interaction between shark muscle oil–olive oil and VEGF reported above.

PDGF

PDGF has been frequently reported to activate and promote angiogenesis.[11,12] This growth factor comes in several isoforms,[13–17] and the form investigated in this study was a mixture of the three characterized isomers (PDGF-AA, PDGF-BB, and PDGF-AB). The results are summarized in Table 5.

TABLE 3. EFFECT OF SHARK MUSCLE OIL ON VEGF STIMULATION OF ANGIOGENESIS

	% of control	*% effect*
VEGF (32.6 ng/mL)	134.14%	34.14% stimulation
Shark muscle oil–olive oil (30 μg/mL)	45.18%	54.82% inhibition
VEGF (32.6 ng/mL) + shark muscle oil–olive oil (30 μg/mL)	46.00%	54.00%

TABLE 4. EFFECT OF SHARK MUSCLE OIL–OLIVE OIL ON FGF-2 STIMULATION OF ANGIOGENESIS

	% of control	*% effect*
FGF-2 (10 ng/mL)	141.22%	41.22% stimulation
Shark muscle oil–olive oil (10 μg/mL)	89.46%	10.54% inhibition
FGF-2 (10 ng/mL) + shark muscle oil–olive oil (10 μg/mL)	84.18%	15.82% inhibition

TABLE 5. EFFECT OF SHARK MUSCLE OIL–OLIVE OIL ON PDGF STIMULATION OF ANGIOGENESIS

	% of control	*% effect*
PDGF (6 ng/mL)	130.23%	30.23% stimulation
Shark muscle oil–olive oil (30 μg/mL)	64.25%	35.75% inhibition
PDGF (6 ng/mL) + shark muscle oil–olive oil (30 μg/mL)	115.99%	15.99% stimulation

TABLE 6. EFFECT OF SHARK MUSCLE OIL–OLIVE OIL ON TGF-β STIMULATION OF ANGIOGENESIS

	% of control	*% effect*
TGF-β (0.08 ng/mL)	121.09%	21.09% stimulation
Shark muscle oil–olive oil (10 μg/mL)	75.69%	24.31% inhibition
TGF-β (0.08 ng/mL) + shark muscle oil–olive oil (10 μg/mL)	81.27%	18.73% inhibition

At the relatively low concentration of 6 ng/mL, PDGF induced a 30% stimulation of angiogenesis, and, in this particular assay, a somewhat higher concentration of shark muscle oil–olive oil was needed to produce a measurable inhibition. However, the shark muscle oil–olive oil was only able to reduce this stimulation marginally. An alternative way of viewing the outcome was that PDGF can largely overcome the inhibition resulting from shark muscle oil–olive oil. Thus it would seem that shark muscle oil–olive oil is not able to interfere with PDGF activity to any significant extent.

TGF-β

TGF-β is a pleiotropic growth factor capable of inducing angiogenesis.[18–20] The effects of TGF-β and the shark muscle oil–olive oil on the angiogenesis are presented in Table 6.

TGF-β produced a moderate elevation of the angiogenic growth rate at quite low concentrations, and the inhibition by shark muscle oil–olive oil was in the range expected. When the two factors were combined there was a net inhibition that was slightly less than that produced by the shark muscle oil–olive oil alone. That is, the shark muscle oil–olive oil would appear to be able to partially but significantly block the angiogenic stimulatory activity of TGF-β. To that extent it seems to have a similar effect to what has been observed with VEGF and FGF-2. This outcome with TGF-β was confirmed in a total of three experiments.

Further studies of shark muscle oil–olive oil inhibition of VEGF modulation

Since VEGF is recognized as a very important promoter of angiogenesis, it was used as a model for studying further

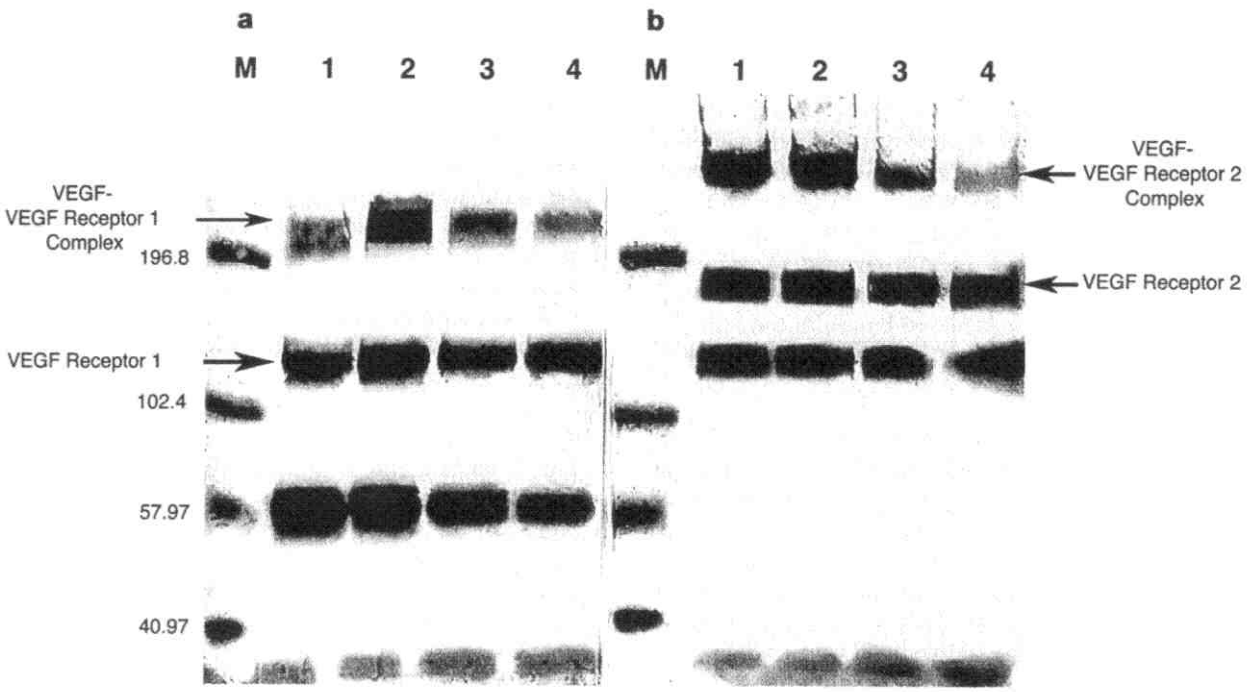

FIG. 1. Immunoblotting of VEGF receptors and VEGF. Incubation was performed with VEGF-R1 (**a**, lanes 1–4) and with VEGF-R2 (**b**, lanes 1–4). Immunoblotting was performed with anti-VEGF-R1 (a, lanes 1–4) and with anti-VEGF-R2 (b, lanes 1–4). Lane M, molecular weight markers (the actual molecular weights are indicated on left side of the blot). The following incubation mixtures were loaded onto the gel: (a) lane 1, VEGF-R1; lane 2, VEGF-R1 + olive oil + VEGF; lane 3, VEGF-R1 + shark muscle oil–olive oil + VEGF; lane 4, VEGF-R1 pretreated with shark muscle oil–olive oil before VEGF is added; (b) lane 1, VEGF-R2; lane 2, VEGF-R2 + olive oil + VEGF; lane 3, VEGF-R2 + shark muscle oil–olive oil + VEGF; lane 4, VEGF-R2 pretreated with shark muscle oil–olive oil before VEGF is added.