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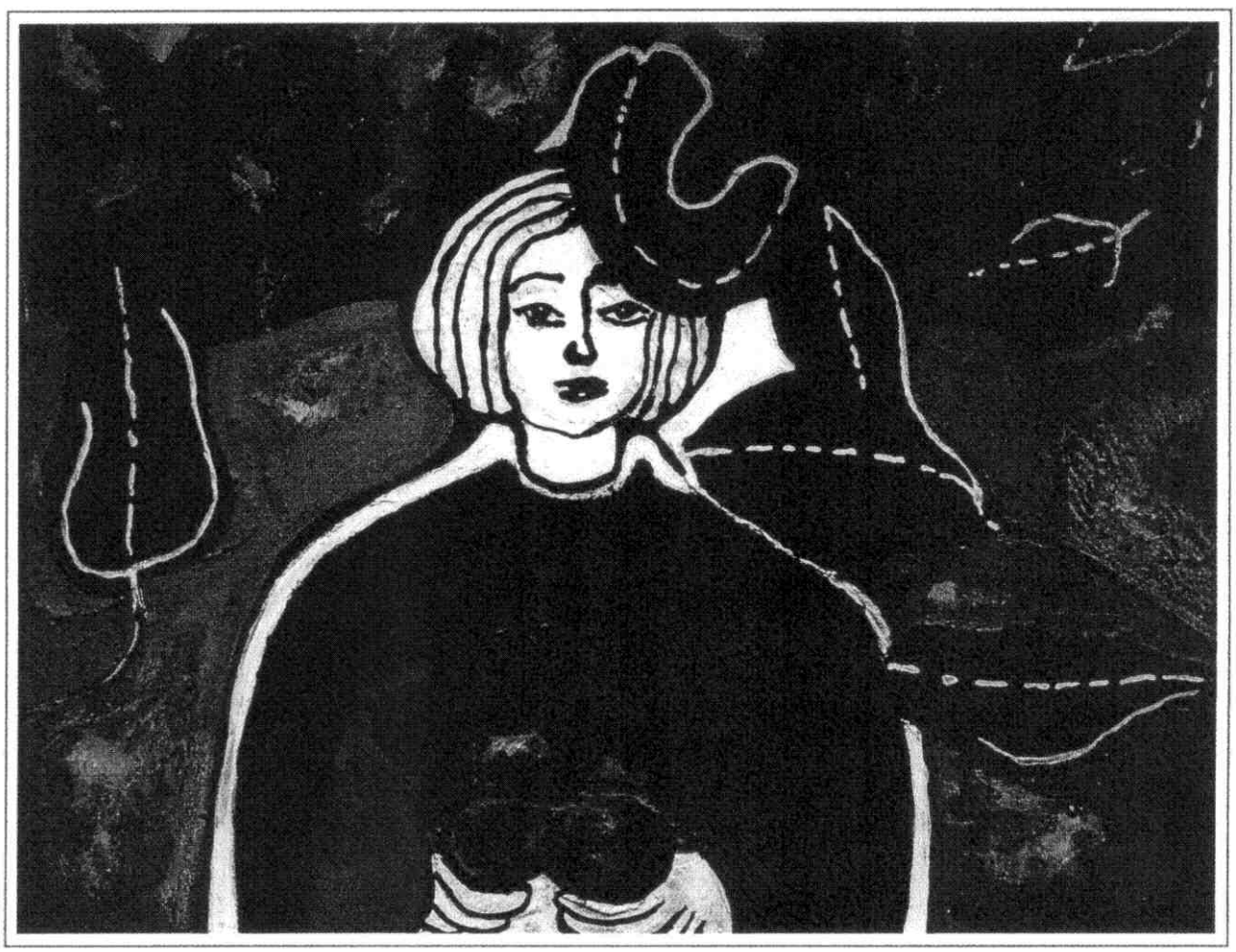

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Inhibition of Pro-Angiogenic Factors by a Lipid-Rich Shark Extract

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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

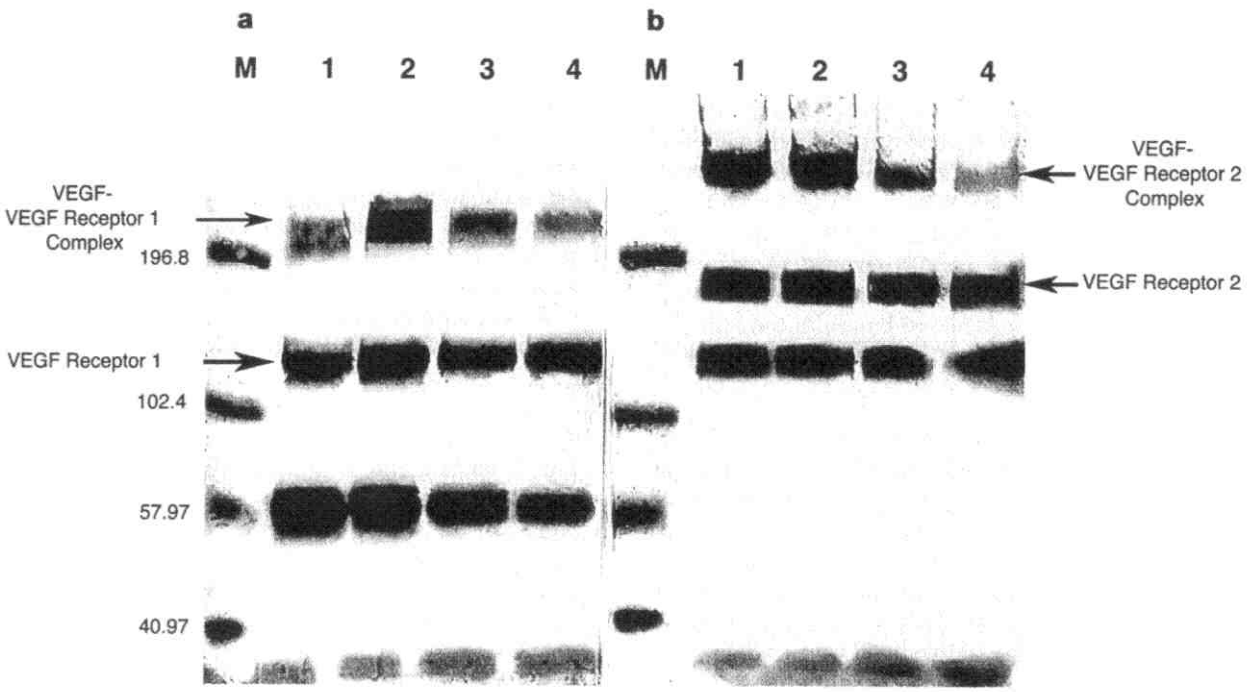

FIG. 1. Immunoblotting of VEGF receptors and VEGF. Incubation was performed with VEGF-R1 (**a**, lanes 1–4) and with VEGF-R2 (**b**, lanes 1–4). Immunoblotting was performed with anti-VEGF-R1 (a, lanes 1–4) and with anti-VEGF-R2 (b, lanes 1–4). Lane M, molecular weight markers (the actual molecular weights 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.

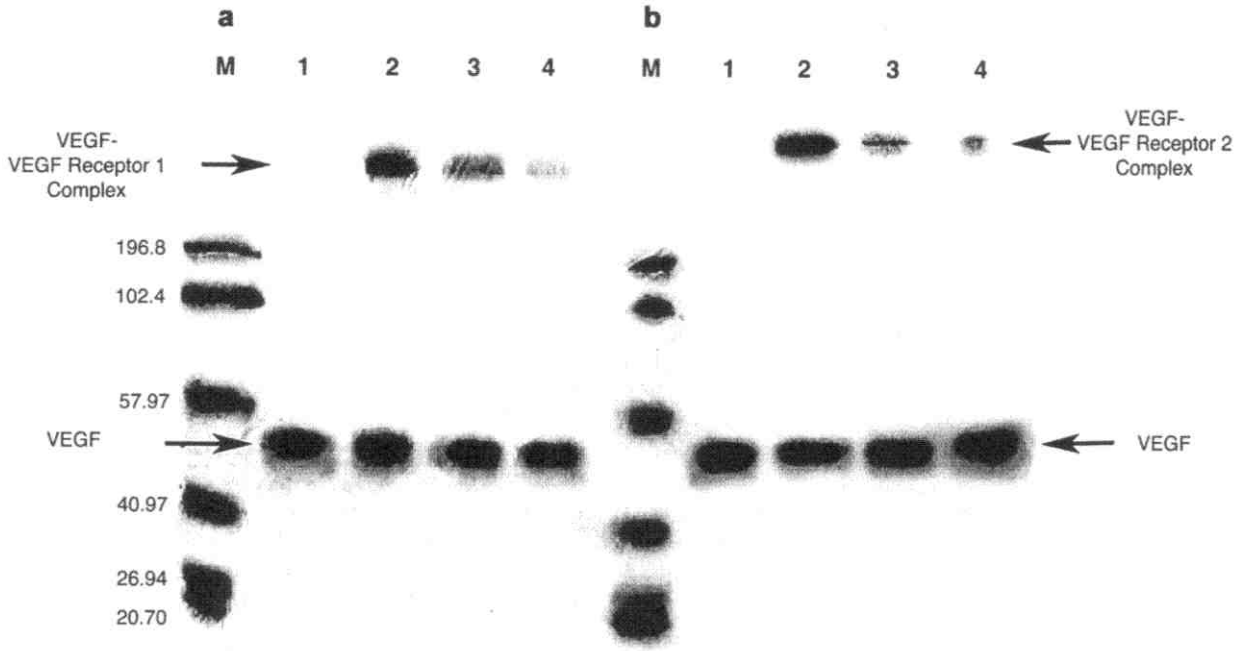

FIG. 2. Immunoblotting of VEGF receptors and VEGF. Incubation was performed with VEGF-R1 (**a**, lanes 1–4) and with VEGF-R2 (**b**, lanes 1–4). Immunoblotting was performed with anti-VEGF (a and b, lanes 1–4). Lane M, molecular weight markers (the actual molecular weights are indicated on left side 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.

how the action of a positive modulator might be affected by shark muscle oil–olive oil.

It is possible that the shark muscle oil–olive oil can bind to the VEGF receptors and thereby block or inhibit the binding of the VEGF to the cell surface.

Incubation of VEGF, VEGF receptors, and shark muscle oil–olive oil

When soluble forms of the two primary receptors for VEGF were incubated with shark muscle oil–olive oil and VEGF simultaneously, the extent of VEGF–VEGF(receptor) complex formation was reduced. This occurred with both VEGF receptors (VEGF-R1 and VEGF-R2) (Fig. 1).

The impeded formation of the complex was confirmed using antibodies to both VEGF and VEGF receptors in the analyses of the binding interactions. When VEGF is incubated with VEGF-R1, a high-molecular-weight band appears that stains positively for VEGF-R1 (indicated by the arrow in Fig. 1). The intensity of this band is less when shark muscle oil–olive oil is added to the incubation mixture (Fig. 1, lane 3), implying that the shark muscle oil–olive oil may have inhibited the binding of the growth factor to the receptor.

This reduction in staining intensity is also seen when anti-VEGF is used as the primary detecting antibody, thereby confirming the result (compare high-molecular-weight bands in Fig. 2, lanes 2 and 3). These results indicate that shark muscle oil–olive oil can bind to VEGF-R1 and so inhibit the binding of VEGF to the receptor.

There is also shown to be a decrease in the staining intensity of the lower-molecular-weight VEGF-R1 band for the incubation mixture that contained the shark muscle oil–olive oil compared with the mixture that did not have shark muscle oil–olive oil (compare the low-molecular-weight band in Fig. 1, lanes 2 and 3).

A very similar outcome is observed when the experiment is repeated with VEGF-R2 substituted for VEGF-R1. That is, it is apparent that shark muscle oil–olive oil can bind to this receptor also, and so inhibit the binding of VEGF to the R2 receptor (compare the high-molecular-weight band in Fig. 1, lanes 6 and 7, indicated by an arrow).

Based on the changes in staining intensity when the antibodies to the VEGF-R1 and VEGF-R2 are used as the detecting agents, these results suggest that shark muscle oil–olive oil can bind to both receptors for VEGF and as a consequence reduce the binding of VEGF to both of them. This interpretation is also supported when the same gel is stained with anti-VEGF (Fig. 2, lanes 6 and 7), although the differences in intensity are not striking for the high-molecular-weight band.

Preincubation of VEGF receptors with shark muscle oil–olive oil

When shark muscle oil–olive oil is preincubated with either of the receptors before the VEGF is added, the shark

muscle oil–olive oil was again able to inhibit the binding of VEGF to both of the receptors (Fig. 1).

The outcome is similar to when the VEGF, the receptor, and the shark muscle oil–olive oil are mixed simultaneously. The high-molecular-weight band is less intense when VEGF-R1 and VEGF are co-incubated in the presence of shark muscle oil–olive oil and stained for VEGF-R1 (compare Fig. 1, lanes 2 and 4). The intensity of this band is about the same in lane 4 and lane 3, suggesting that the preincubation with shark muscle oil–olive oil has no different effect than just co-incubating. The pattern and intensities of these bands are very similar when anti-VEGF is used as the primary staining antibody (Fig. 2, lanes 2–4).

However, it appears that there is considerably less of the high molecular VEGF–VEGF-R complex when incubation is with shark muscle oil–olive oil and the R2 receptor is used. That is, the intensity of the VEGF–VEGF-R2 complex is less when the R2 receptor is preincubated with shark muscle oil–olive oil (compare Fig. 1, lanes 7 and 8). This would suggest that during the preincubation period there is stronger binding of shark muscle oil–olive oil to the R2 receptor than to the R1 receptor, suggesting a higher affinity for the former. Again this interpretation is supported by the intensity of staining of the same high-molecular-weight complexes that are detected with anti-VEGF (compare Fig. 2, lanes 7 and 8).

DISCUSSION

Angiogenesis is a multistep process,[21,22] and modulators can act at any one of these to produce an effect. Therefore an inhibitor could act at steps such as the proliferation of endothelial cells, the enzymatic activity of the matrix metalloproteinases, and the cytokines and growth factors that regulate the process or the formation of intact, patent neovessels. As well, shark muscle oil–olive oil is a multicomponent preparation, and, as such, it is quite possible that different components could act at different phases or stages of the angiogenesis process.

In an attempt to better understand the cellular and molecular aspects of shark muscle oil–olive oil's action, experiments were designed to determine whether it affected the activity of known positive regulators of angiogenesis. There are numerous compounds that can stimulate angiogenesis. In this study it was found that shark muscle oil–olive oil can completely abolish the effects of VEGF, FGF-2, and TGF-β. That is, if shark muscle oil–olive oil is mixed with any of these, the outcome is that the stimulatory effects are lost, and there is inhibition to about the same level as for shark muscle oil–olive oil alone. For the other agonist evaluated (PDGF), it appears to have some inhibitory effect. So shark muscle oil–olive oil can, in effect, antagonize the known stimulators of angiogenesis. This pleiotropic effect suggests that it can interfere with a multiple number of steps and may, therefore, be a potent antagonist of the angiogenic process.

VEGF is believed to be one of the most important promoters of angiogenesis.[4–6] Since this experiment indicates that shark muscle oil–olive oil can completely abolish its effect, an investigation of how it affects VEGF activity was undertaken. There are a number of possible ways that this can be achieved. For example, it could act on the gene responsible for VEGF production, suppressing its transcription; it could bind to VEGF and so inhibit its affinity to bind to the specific VEGF receptors on the cell surface; or it could block the receptor on the cell surface and thereby prevent the binding of VEGF to the cell. The investigation reported here utilizes the fact that there are several receptors known for VEGF, and that soluble (as opposed to cell-bound) forms of them are available. The results here quite clearly show that the shark muscle oil–olive oil can bind to both of the receptors for VEGF, inhibiting their binding of VEGF and hence its ability to promote angiogenesis. That it binds to both receptors is not too surprising considering the multicomponent nature of shark muscle oil–olive oil. There may be different constituents responsible for binding to each of them.

Interestingly, if the VEGF and the shark muscle oil–olive oil are both added at the same time to the receptor, the effect would appear to be rather similar for each of the receptors. It could be that there is some binding of shark muscle oil–olive oil to VEGF as well as to the receptor that is partially responsible for the inhibition. However, preincubating the R2 receptor with shark muscle oil–olive oil, allowing it to bind to the receptor (if that is what it does), before there is any interaction with the growth factor produces an increased antagonistic effect. This would confirm that the shark muscle oil–olive oil has the ability to bind strongly to the receptor. It would also appear to have a stronger affinity for the R2 isoform than to the R1.

One possible method for the inhibitory action of shark muscle oil–olive oil is for it to bind to the receptor for the VEGF, rather than to the VEGF directly, and so antagonize the ability of VEGF to bind to the endothelial cells and promote angiogenesis. There are several other cytokines and growth factors known to promote angiogenesis (*e.g.*, FGF-2) that are inhibited by shark muscle oil–olive oil. Whether a similar mechanism of action occurs with them is not known. It is possible that it does, but it needs to be verified experimentally.

As noted above, this effect of the blocking of the receptors for the angiogenic promoter, VEGF, is possibly only one of a number of mechanisms that the shark muscle oil–olive oil uses for inhibiting angiogenesis. It is quite likely that there are other mechanisms of action as well. There may be other unidentified modes of actions such as the inhibition of the transcription of the genes that code for promoters such as VEGF. The fact that shark muscle oil–olive oil is not a single compound but contains many constituents makes this quite possible.

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