viroids. In homogenates from tobacco mosaic virus–infected tobacco, brome mosaic virus–infected barley, and cowpea mosaic virus–infected cowpeas ^{32}P-incorporation from [γ-^{32}P]ATP into two bands in the region of M_r 68,000 was significantly elevated compared to extracts from the respective mock-inoculated tissues. Nucleotide photoaffinity labeling and immunoprecipitation experiments showed that the M_r 68,000 protein in these systems has an ATP-binding site and is immunologically related to the P_1 dsRNA-dependent protein kinase. Nucleotide-protein interactions that involve nucleotide binding and protein phosphorylation have been implicated in the regulation of metabolic events in diverse mammalian cell–virus interactions (*10–14*). Although the role of phosphorylation in viroid replication or pathogenesis is not clear, several possibilities exist. One potential function, in both normal and infected cells, is in the regulation of protein synthesis. A dsRNA-dependent protein kinase is found in reticulocyte lysates and regulates the initiation of translation by interfering with the recycling of the eIF-2/GTP/Met-tRNA ternary complex (*16*). The only known physiological substrate for this enzyme and the P_1 dsRNA-dependent kinase from interferon-treated cells is the α subunit of eIF-2 (*7, 10*). Recently, Kitajewski *et al.* (*15*) have shown that induction of P_1 kinase activity is associated with the inhibition of protein synthesis.

Data presented by Morrow *et al.* (*12*) suggest that the host factor necessary for poliovirus replication is an M_r 67,000 phosphoprotein associated with dsRNA-dependent protein kinase activity. The α subunit of eIF-2 is a substrate for this enzyme (*12*). We have also shown that an M_r 68,000 host-encoded protein from tobacco is phosphorylated in crude tobacco mosaic virus replicase preparations, suggesting a possible role in replication in that system (*17*).

Evidence has been provided to support a model for viroid replication that involves direct RNA to RNA copying via a rolling circle mechanism; replication would be mediated by a host polymerase (*18*). RNA species in the replication complex have been analyzed but associated proteins have not yet been identified (*19*). As viroids encode no polypeptides they must manipulate host cells in a way to obtain components necessary for their replication. Analysis of interactions between viroid RNA (and intermediates found in the replication cycle) and host factors can suggest possible mechanisms of regulation. The requirement of dsRNA in vivo may be met by highly intramolecularly base-paired single-stranded RNA or by dsRNA replicative intermediates. Protein synthesis in reticulocyte lysates is inhibited by dsRNA with as few as 50 matching base pairs, and there is no specificity relative to base composition or base sequence (*20*). The hairpin-like structure of poly(A)–poly(U) is apparently sufficient structure for recognition. Samuel (*10*) found that reovirus single-stranded RNA stimulates the phosphorylation of P_1 and eIF-2. In this regard, viroid RNA as covalently closed circles may be adequate for activation of the p68 kinase. At this time we cannot exclude the possibility that the effect of PSTV infection on p68 may be a secondary event, not directly relevant to replication or viroid pathogenesis. These possibilities will be resolved, in part, by purification of the protein and evaluation of the effect of viroid RNA species on enzyme activity.

In conclusion, we have identified a viroid-induced phosphorylation of a host-encoded protein. This protein has kinase activity or is closely associated with such activity. The role of this protein in viroid pathogenesis is presently unclear, but possibilities include the regulation of protein synthesis and replication.

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21. We thank A. Branch, Rockefeller University, New York, for providing PSTV-infected and mock-inoculated tomato tissues, C. Samuel, University of California, Santa Barbara, CA, for providing anti-P_1 sera, and B. Haley, Markey Cancer Center, University of Kentucky, Lexington, KY, for providing azido nucleotide photoaffinity analogs. This work was supported by NSF grants RII-8610680 and DMB-8611424.

22 January 1988; accepted 18 May 1988

Fish Oils Inhibit Endothelial Cell Production of Platelet-Derived Growth Factor–Like Protein

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Diets rich in fish and fish oils are associated with a reduced risk of cardiovascular disease and atherosclerosis. The interaction of a commercial fish oil extract (MaxEPA) with vascular endothelial cells (ECs) was studied as a possible mechanism for this protective effect. MaxEPA almost completely inhibited EC production of platelet-derived growth factor–like protein (PDGFc) while other lipids had a lesser effect or no effect. Overall protein synthesis was not reduced, nor was the inhibition due to defective secretion or increased degradation of the growth factor. Antioxidants suppressed the inhibitory activity of MaxEPA indicating that free radical oxidative processes were required for the inhibition. These results suggest that fish oils may suppress intimal smooth muscle cell proliferation by decreasing the production of EC-derived paracrine growth factors. This inhibitory process represents a possible molecular mechanism for the antiatherosclerotic action of marine lipids.

FISH OILS ARE HETEROGENEOUS MIXtures of lipids, primarily triglycerides, containing an abundance of two unusual polyunsaturated fatty acids, eicosapen-

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taenoic acid (EPA; C20:5, ω-3) and docosahexaenoic acid (DHA; C22:6, ω-3), that are generally present at low concentrations in nonmarine animals. Bang and Dyerberg (*1*) have proposed that ingestion of ω-3 fatty acids may be responsible for the low incidence of cardiovascular disease, especially atherosclerosis, in a well-studied community of Greenland Eskimos. Beneficial effects of a fish diet have also been reported in fishing villages in Japan (*2*) and in Holland (*3*). The physiological mechanisms connecting increased fish intake with decreased vascular disease are not known, but at least two distinct hypotheses are currently being investigated. The first is that fish oils exert their beneficial effects by reducing plasma cholesterol and triglyceride levels (*4*). A second possible mechanism involves a reduction in the thrombogenicity of platelet prostaglandins derived from ω-3 fatty acids, compared to those derived from ω-6 fatty acids (*5*).

As the barrier between blood and the arterial wall, the ECs can regulate the exchange of materials and information between these tissues. Consistent with this regulatory function, cultured ECs are known to respond to a wide variety of stimuli. One specific response is the production of growth factors, including PDGFc, that stimulate the migration and proliferation of cultured vascular smooth muscle cells (SMCs) (*6*). The secretion of such cell-derived growth factors may contribute, along with platelet-derived factors, to the development of the intimal lesion that is characteristic of advanced atherosclerosis (*7*). The experiments of Barrett *et al.* (*8*) suggest that, under normal conditions in vivo, EC expression of the gene encoding one of the two chains (the B chain) of platelet-derived growth factor (PDGF) may be low compared to that under perturbed conditions, for example, in vitro or possibly over an atherosclerotic lesion. Several agonists stimulate the production of PDGFc by ECs in vitro, including tumor necrosis factor, transforming growth factor–β, thrombin, Factor Xa, and agents injurious to ECs such as bacterial endotoxin and phorbol esters (*9, 10*). We reported previously that the production of PDGFc by cultured ECs is depressed by certain modified low density lipoproteins in a process that is dependent on the presence of oxidized lipids in the lipoprotein particle (*11, 12*). These experiments suggest that in vivo production of growth factors by EC may be tightly regulated. We examined the possibility that the cardiovascular benefits of fish oils are mediated by their action on vascular ECs.

MaxEPA (Solgar), a marine lipid extract containing 30% ω-3 fatty acids (18% EPA, 12% DHA), was emulsified with egg phosphatidylcholine (PC) and incubated with bovine aortic ECs. After 3 days, the amount of PDGFc in the conditioned medium was determined by a specific radioreceptor assay (*10*). MaxEPA inhibited PDGFc production in a dose-dependent manner, with half-maximal inhibition at approximately 10 μg of lipid per milliliter and maximal inhibition (85%) at about 85 μg of lipid per milliliter (Fig. 1A). Emulsions of safflower oil and peanut oil were also incubated with ECs to test for the specific requirement of marine lipids. Safflower oil, chosen for its high content of ω-6 polyunsaturated fatty acids [approximately 74% linoleic acid (*13*); C18:2, ω-6], also inhibited PDGFc production, but was 1/10 to 1/20 as potent as MaxEPA. Peanut oil, which contains primarily saturated and monounsaturated fatty acids (*13*), did not significantly affect growth factor production. The decrease in growth factor production caused by MaxEPA was not due to a decrease in cell viability as evidenced by normal cell morphology and constant cell number. Neither the MaxEPA nor any of the other lipid emulsions significantly inhibited cell-associated or secreted protein synthesis by the ECs (Fig. 1B). These results demonstrate the viability of the ECs and further indicate that the inhibition of PDGFc production was specific, and not simply the result of a general inhibition of synthetic pathways. To determine the extent of MaxEPA uptake by the ECs, an emulsion of MaxEPA was labeled with a tracer amount of [^{3}H]cholesteryl oleyl ether, a hydrophobic, nonhydrolyzable marker lipid (*14*). After 72 hours, the cells were harvested with trypsin and centrifuged to remove bound lipid. The radioac-

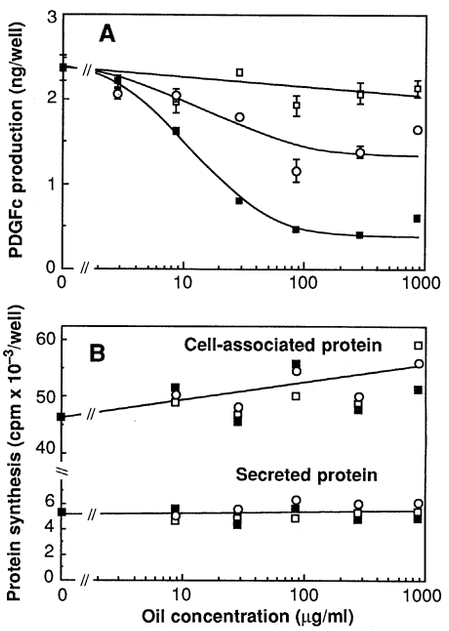

Fig. 1. The inhibition of production of PDGFc by MaxEPA. MaxEPA (■), safflower oil (○), and peanut oil (□) were dissolved in chloroform with egg PC (5% by weight) and BHT (0.03% of lipid weight). The solvent was evaporated with N_2, a few drops of ethyl ether were added, and the solvent was again removed under N_2. The lipids (15 mg of total lipid per milliliter) were emulsified in phosphate-buffered saline by sonication under N_2 for 10 min in a bath sonifier. The emulsions were incubated for 72 hours with confluent cultures of bovine aortic ECs in 12-well culture dishes containing 0.5 ml of medium (Dulbecco-Vogt modified Eagle's medium and Ham's F12, 1:1) and 2 mg of bovine serum albumin per milliliter). PDGFc in the EC-conditioned medium was measured in (**A**) by radioreceptor assay and expressed as the mean ± SEM (n = 3 independent cultures). After 72 hours, [^{3}H]leucine (1 μCi per well) was added to the medium, and the cells were incubated for an additional 8 hours. Protein synthesis was measured in (**B**) as incorporation of radioactivity into TCA-precipitable material; the synthesis of cell-associated proteins and secreted proteins was independently measured (*10*). The results shown are the average for two samples, which differed by less than 10%.

Table 1. The inhibition of PDGFc production by natural and purified oils. The natural and purified lipids were emulsified with egg PC as described in Fig. 1 and incubated with bovine aortic ECs for 72 hours. PDGFc in the EC-conditioned medium was determined by radioreceptor assay (mean ± SEM, n = 3).

Treatment	Concentration of oil (triglyceride μg/ml)	PDGFc production (ng/well)	Inhibition (%)
	Experiment 1		
EC alone		3.58 ± 0.23	
Egg PC alone		3.97 ± 0.15	<0.0
Peanut oil	57.0	3.63 ± 0.23	<0.0
Safflower oil	57.0	2.14 ± 0.08	40.2
Trilinolein	57.0	2.39 ± 0.09	33.2
Cod-liver oil	57.0	0.83 ± 0.07	76.8
MaxEPA	57.0	0.61 ± 0.08	83.0
	Experiment 2		
EC alone		2.35 ± 0.09	
Trimyristin	50.0	2.28 ± 0.15	3.0
Trilaurin	50.0	2.36 ± 0.11	0.0
Triolein	50.0	2.23 ± 0.05	5.1

tivity of the pellet was determined. The ECs took up between 16 and 20% of the emulsion (n = 5 cultures).

To show that MaxEPA and other lipids did not interfere with the radioreceptor assay for PDGFc, we incubated ^{125}I-labeled human PDGF (^{125}I-PDGF, 0.2 ng per well, 26,000 cpm/ng) with human fibroblasts in the presence of emulsions of MaxEPA, safflower oil, and peanut oil (at the highest concentration used in the experiment shown in Fig. 1). The cellular binding of ^{125}I-PDGF was 299 ± 6 cpm per well in the absence of lipid, and 301 ± 2, 305 ± 5, and 315 ± 6 in the presence of peanut oil, safflower oil, and MaxEPA, respectively (mean ± SEM, n = 3). Thus the lipids did not interfere with the binding of PDGF to its cell surface receptor, validating the radioreceptor assay under these conditions.

The inhibitory activity of several other triglycerides was also examined. The saturated triglycerides trilaurin and trimyristin, and a monounsaturated triglyceride, triolein, did not significantly inhibit growth factor production. Trilinolein (consisting of C18:2, ω-6 fatty acids), like safflower oil, partially inhibited PDGFc production (Table 1). A second marine lipid, cod-liver oil (Squibb), containing approximately 20% ω-3 fatty acids, had nearly the same inhibitory activity as MaxEPA. These results suggest that all polyunsaturated triglycerides may have some inhibitory activity, but that the ω-3 fatty acid content may be a critical factor determining the potency of a lipid. The results are also consistent with the inhibitory activity being a function of the degree of unsaturation of the constituent fatty acids, independent of the position of the terminal double bond, since the ω-3 fatty acids in fish oils generally contain five or six double bonds, whereas trilinolein and safflower oil contain primarily fatty acids with two double bonds. Although the inhibitor lipid in MaxEPA may be a minor constituent of the oil, it is not likely that the inhibitor is cholesterol [which constitutes approximately 0.6% of the oil by weight (*15*)], since we have previously reported that doubling EC cholesterol content using cholesterol/albumin complexes did not inhibit PDGFc production (*12*).

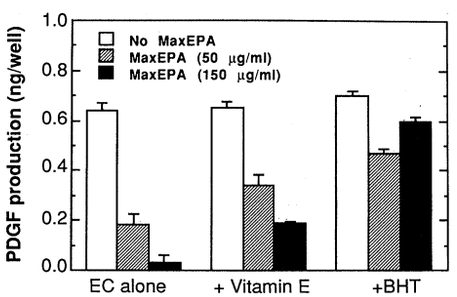

Fig. 2. The effect of antioxidants on the inhibition of PDGFc production by MaxEPA. Confluent bovine aortic ECs were preincubated with vitamin E (25 μ*M*) or BHT (20 μ*M*) delivered in 1 μl of 95% ethanol. After 24 hours, the medium was discarded, and the cells were incubated with an emulsion of MaxEPA in the presence or absence of the same concentration of the antioxidants, which were added at 24-hour intervals. Ethanol was added to the control cells that did not receive antioxidant. After 72 hours, the conditioned medium was collected and PDGFc was measured by the radioreceptor assay (expressed as the mean ± SEM, n = 3).

One possible mechanism responsible for the observed decrease in PDGFc production is the accumulation of growth factor within the cell, due perhaps to a defect in secretion induced by MaxEPA. This hypothesis was tested by lysing MaxEPA-treated ECs and measuring the amount of PDGFc released. ECs were incubated with emulsions of MaxEPA (200 μg of lipid per milliliter) for 96 hours as described in the legend to Fig. 1. The cells were lysed by six freeze-thaw cycles in phosphate-buffered saline containing 2 mg of bovine serum albumin per milliliter. Cell debris was precipitated by centrifugation, and PDGFc in the supernatant was measured by radioreceptor assay. MaxEPA reduced the amount of PDGFc in the conditioned medium from 1.17 ± 0.07 to 0.19 ± 0.05 ng. Lysis of the MaxEPA-treated ECs released only 0.04 ± 0.02 ng of PDGFc, showing that receptor-bindable growth factor was not accumulating in the cell. To show that the cell lysis procedure did not reduce the amount of measurable PDGFc, a known amount of purified human PDGF was added to control cells, and the cells were lysed. The lysis protocol did not alter the recovery of PDGF.

The high polyunsaturation level of the fatty acids in marine oils makes these lipids readily susceptible to free radical oxidative processes. Although the level of oxidized triglycerides in MaxEPA was too low to be detected by thin-layer chromatography and staining with iodine and care was taken during the handling of the lipids to prevent autooxidation, it is possible that an oxidized species present at a low concentration may be responsible for the inhibitory activity of MaxEPA. In addition, since ECs are known to promote lipoprotein lipid peroxidation by free radical reactions (*16*), an oxidized inhibitory species may be generated during the incubation itself. To test if such processes are required, we incubated ECs with MaxEPA in the presence of the free radical scavengers vitamin E and butylated hydroxytoluene (BHT). Both vitamin E and BHT reduced the inhibitory activity of MaxEPA, the latter nearly completely suppressing the activity (Fig. 2). These results clearly indicate the importance of oxidative processing in the regulation of growth factor production. Reactive free radical species, including products of lipid peroxidation, have been shown to inactivate specific proteins by cross-linking and by degradation. To test if the inhibition of PDGFc production by MaxEPA was due to degradation of secreted protein, we added exogenous purified human PDGF to ECs before incubating them for 3 days with the lipid. The amount of PDGFc produced by the cells during this interval was subtracted from the total measured amount, giving the recovery of exogenous human PDGF. The presence of MaxEPA did not affect the recovery of PDGF, suggesting that the inhibition of production of PDGFc by MaxEPA was due to decreased production rather than enhanced degradation.

Fish oil feeding causes dramatic changes in plasma fatty acid content. Harris *et al.* (*4*) reported that humans fed a diet supplemented with salmon oil exhibited a decrease in plasma triglyceride from 77 to 48 mg/dl, while the ω-3 fatty acid content increased from about 1 to 30% of the total fatty acids. This result is equivalent to an increase in plasma ω-3 fatty acid concentration from less than 10 μg/ml to approximately 150 μg/ml. Since ω-3 fatty acids account for approximately 30% of the fatty acids in MaxEPA, the concentration required to inhibit PDGFc production maximally in our studies is approximately 30 μg of ω-3 fatty acids per milliliter (Fig. 1). Therefore, dietary intake of fish oils may induce sufficient plasma lipid modification to inhibit EC production of PDGFc. Given the SMC chemotactic and growth stimulatory activities of PDGF, potential consequences in vivo of the inhibition of growth factor production by marine lipids include a decreased rate of migration of medial SMCs into the intima and decreased intimal proliferation of SMCs. Extrapolating our in vitro studies to the situation in vivo, we propose that the specific inhibition of production of EC-derived mitogens by marine lipids may be involved in the observed reduction of vascular disease. These alterations at the cellular level are consistent with several recent studies with animal models of atherosclerosis. Weiner *et al.* (*17*) reported that cod-liver oil feeding prevented intimal thickening in the coronary arteries of hyperlipidemic swine. Similarly, Landymore *et al.* (*18*) and Cahill *et al.* (*19*) reported that fish oil feeding prevented thickening of autologous vein grafts in normal dogs. These changes were not related to plasma lipoprotein levels (*17, 19*), and although serum thromboxane concentration was lowered (*17, 19*), fish oil feeding did not alter platelet thrombotic activity (*18,*

19). Our results raise the possibility that the observed decrease in intimal thickening may be a specific consequence of suppressed growth factor production by ECs, and concomitant reduction of SMC proliferation.

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20. These studies were supported by NIH grants HL29582 (P.E.D.) and HL40352 (P.L.F.), and a grant-in-aid from the American Heart Association, Northeast Ohio Affiliate (P.L.F.). P.E.D. is the recipient of a Research Career Development Award (HL1561) from NIH. We thank S. Mansell and J. Shin for technical assistance, E. Ritly for photography, and T. A. Painter for discussion.

7 March 1988; accepted 1 June 1988

Site-Specific Oligonucleotide Binding Represses Transcription of the Human c-*myc* Gene in Vitro

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A 27-base-long DNA oligonucleotide was designed that binds to duplex DNA at a single site within the 5′ end of the human c-*myc* gene, 115 base pairs upstream from the transcription origin P1. On the basis of the physical properties of its bound complex, it was concluded that the oligonucleotide forms a colinear triplex with the duplex binding site. By means of an in vitro assay system, it was possible to show a correlation between triplex formation at −115 base pairs and repression of c-*myc* transcription. The possibility is discussed that triplex formation (site-specific RNA binding to a DNA duplex) could serve as the basis for an alternative program of gene control in vivo.

SEQUENCE-SPECIFIC RNA BINDING is important for the regulation of splicing (*1*), for the control of translation (*2*), and for protein export (*3*). Even though the possibility has been discussed for some time (*4*), there has been no evidence to suggest that RNA binding could also serve as a mechanism for the regulation of transcription initiation. Boles and Hogan have shown that at a position 115 bp upstream of the transcription start site P1 (270 bp upstream from P2) the 5′ end of the human c-*myc* gene assumes a DNA secondary structure that is in equilibrium between at least two alternative helix conformations in vitro (*5*). The conformational equilibrium at 115 bp appears to be coupled allosterically to the binding of small RNA molecules, which led to the proposal (*5*) that the bound complex may be similar to a colinear triplex of the sort deduced from fiber diffraction analysis of simple synthetic polynucleotide helices (*6*).

Our experimental design was based on the following observation: DNA oligonucleotides are easier to synthesize than RNA and data suggest that a third strand of DNA should bind to a duplex as stably as an RNA molecule with the same sequence (*7*). For these reasons we prepared a series of single-strand oligonucleotides that were designed to bind to the −115-bp site on *myc*, by means of colinear triplex formation. One of this series was found to bind tightly to the *myc* sequence of interest.

The *myc* helix element, which we have shown to engage in conformational equilibrium, is a G-rich polypurine sequence (Fig. 1). If a colinear triplex were to form on that duplex site, with structural features similar to that of the poly(dT)–poly(dA)–poly(dT) triplex that has been studied by fiber diffraction, then the third strand would be positioned within the major helix groove and would be bound to the underlying duplex by hydrogen bonding (*6*).

Fresco and colleagues have shown that a poly(dA)–poly(dA)–poly(dT) forms at neutral *p*H, with properties that are as expected for a colinear triplex (*8*). The pattern of base pairing that stabilizes this complex may be similar to the A46-A22-U13 triplet that occurs in yeast $tRNA^{Arg}$ (*9*). As Cantor and Schimmel noted, the A-A-U triplet is isomorphous with the G46-G22-C13 triplet that has been detected in the yeast tRNA Phe crystal structure (*9*). These triplets are based on A-A and G-G hydrogen bonding to the underlying Watson-Crick duplex, which is identical to the pattern of self

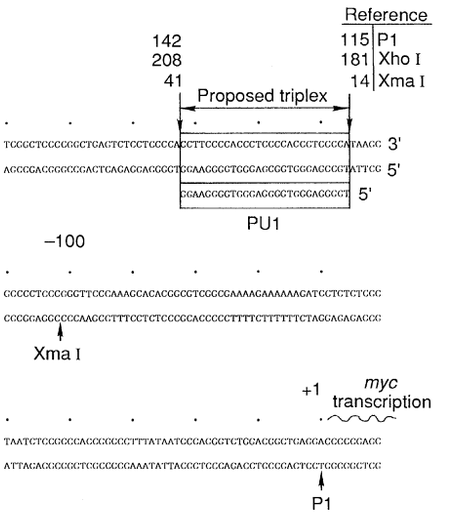

Fig. 1. A region of the human c-*myc* gene, −170 to +10 bp relative to the transcription start site P1 (*19*). The boxed segment is in conformational equilibrium (*5*) and is equivalent to the short duplex fragment described in Fig. 2. The sequence of the 27-base oligonucleotide PU1 has been presented in 3′ to 5′ alignment to emphasize its orientation relative to the duplex binding site in a colinear triplex model. To facilitate analysis of the footprinting assay (Fig. 3), the end points of the proposed triplex binding site have been numbered relative to several downstream reference points in the human c-*myc* gene (the transcription start site P1 and nearby Xma I and Xho I cleavage sites).

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