Dietary Supplementation with Fish Oil Modifies the Ability of Human Monocytes to Induce an Inflammatory Response

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Abstract

Monocytes/macrophages are key orchestrators of inflammation and are involved in the pathogenesis of chronic inflammatory disorders, including atherosclerosis. (n-3) Fatty acids, found in fish oil, have been shown to have protective effects in such disorders. To investigate possible modes of action, we used a monocyte:endothelial cell (EC) coculture model to investigate the pro-inflammatory potential of monocytes. Monocytes were isolated from the blood of donors with peripheral arterial disease (PAD) or control donors, before and after a 12-wk supplementation of their diet with fish oil. The monocytes were cultured with human umbilical vein EC (HUVEC) for 24 h, after which the ability of the HUVEC to recruit flowing neutrophils was tested. Monocytes from either group of donors stimulated the EC to support the adhesion and migration of neutrophils. Fish oil supplementation reduced the potency of monocytes from normal subjects, but not those from patients with PAD, to induce recruitment. Concurrent medication may have acted as a complicating factor. On subgroup analysis, only those free of medication showed a significant effect of fish oil. Responses before or after supplementation were not closely linked to patterns of secretion of cytokines by cultured monocytes, tested in parallel monocultures. These results suggest that fish oil can modulate the ability of monocytes to stimulate EC and that this might contribute to their protective effects against chronic inflammatory disorders. Benefits, however, may depend on existing medical status and on other treatments being received. J. Nutr. 137: 2769–2774, 2007.

Introduction

Tissue-resident macrophages are derived from circulating monocytes and act as important regulators of inflammatory responses (1,2). Following insult to tissue they release cytokines that act on endothelial cells (EC)3 and induce protective leukocyte recruitment. However, if activation of monocytes/macrophages occurs in an uncontrolled manner, chronic, pathological inflammation can ensue. For instance, during atherogenesis, cells of the monocytic lineage are present in the earliest recognizable arterial lesions and also contribute to the cellular infiltrate of the more complex lesions that are associated with pathology (3). In this case, they appear to be able to promote an inappropriate accumulation of inflammatory cells (4,5). Studies using in vitro coculture with EC have shown that monocytes can upregulate endothelial adhesion receptors and induce the ability to recruit leukocytes of all major classes (5–8). In recent studies, we showed that this pro-inflammatory potential was greater for monocytes derived from patients with peripheral arterial disease (PAD) than age-matched controls (9). The foregoing raises the possibility that treatments that modulate the ability of monocytes to stimulate EC might be therapeutic in chronic inflammatory disorders, including atherosclerosis.

A potential method of manipulating inflammation driven by monocytes/macrophages might be through increased consumption of the long chain (n-3) PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (10). These fatty acids are found in fish and in fish oils and have been demonstrated to decrease cardiovascular events and mortality (11,12) and to modify a range of inflammatory markers (10,13,14). In vitro studies involving incubation with (n-3) PUFA have demonstrated decreased adhesion molecule expression on the surface of monocytes (15,16) and EC (15,17,18). Furthermore, feeding studies reported that fish oil decreased expression of some adhesion molecules on the surface of murine macrophages (13), rat lymphocytes (19), and human monocytes (20). The (n-3) fatty acid-induced reduction in adhesion molecule expression is accompanied by a decreased ability to bind ligand-bearing

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3 Abbreviations used: ABPI, ankle:brachial pressure index; BSA, bovine serum albumin; CPDA, citrate-phosphate-dextrose-adenine; DHA, docosahexaenoic acid; EC, endothelial cell; EPA, eicosapentaenoic acid; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell; PAD, peripheral arterial disease; PC, phosphatidylcholine.
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leukocytes (15,19). However, the true functional effects of such changes for inflammatory responses and disease are unclear.

Direct demonstration and investigation of the role of monocytes in the development of human disease is difficult. However, we developed a coculture model in which the ability of monocytes to bind and migrate through endothelial monolayers and to subsequently induce the EC to recruit flowing neutrophils was tested (5,6). Subsequently, we adapted this model for studies of inter-donor variation and found that monocytes from patients with PAD were more stimulatory for EC than those from controls (9). We have also shown that fish oil supplementation improves the stability of carotid plaques in those with atherosclerotic disease awaiting endarterectomy (21). We therefore set out to test whether the coculture model would demonstrate effects of (n-3) fatty acids on monocyte function relevant to vascular disease by testing subjects before and after dietary supplementation with fish oil. We also tested whether effects might be greater in PAD patients than healthy controls, consistent with our reported effects on clinically related endpoints.

Methods

Study subjects, dietary supplementation, and blood withdrawal.
The study received ethical approval from the Southampton and South West Hampshire Research Ethics Committee. Written informed consent was obtained from all subjects. Male patients with PAD (n = 16) were recruited from general practitioner surgeries or local hospital outpatient clinics. To eliminate subjects with rapidly changing conditions and to ensure the clear presence of disease, patients were chosen who had stable claudication of >6 mo duration with an ankle-brachial pressure index (ABPI) of <0.9 and showed no critical leg ischemia. As an indicator of variation in the patient group, ABPI ranged from 0.3 to 0.89. Patients had not undergone any intervention treatment within 3 mo of starting the study. Age-matched male control subjects (n = 29) were recruited from the general public by advertisement. All donors (PAD and control) were >45 y of age. Mean and median age for PAD subjects were 64 and 60 y for PAD and 56 and 56 y for control subjects. Control subjects all had a normal ABPI of >0.9 and no history of PAD or cardiovascular disease. No participant suffered from diabetes, had any malignant disease in the last 5 y, or had any chronic or acute inflammatory conditions. Three additional subjects recruited to the study (1 PAD and 2 controls) did not yield data after fish oil supplementation; 1 was lost to follow-up and samples from 2 were the subjects of technical failure in the laboratory.

At the time of blood sampling, all subjects had been free from obvious colds or infections for at least 14 d. All PAD patients were receiving prescribed medication(s) (see Table 1). At the time of the study, all were normotensive (systolic < 140 mm Hg and diastolic < 90 mm Hg), although 11 patients were receiving antihypertensive medication. Of the 29 control subjects used for comparison, 19 were not receiving medication and 10 were receiving medication (see Table 1). Subjects did not alter their medication during the study except for cases of antibiotics for infection. Blood samples were taken at least 2 wk after any antibiotic course was finished.

All subjects received 6 × 1 g/d capsules of fish oil (MaxEPA, Seven Seas) for 12 wk. This gave a total daily intake of 1.02 g EPA and 0.69 g DHA. The dose and duration of supplementation were based on our previous work, where we studied the effects of fish oil on modulation of immune cell functions (14,22,23). Before and after supplementation, venous blood was collected into citrate-phosphate-dextrose-adenine [CPDA; concentrate diluted (1:10) in blood to yield final concentrations of 20 mmol/L Na-glucose, 11 mmol/L citric acid\(\cdot\)\(\text{H}_2\text{O}\), and 0.2 mmol/L adenosine; pH 7.4] and into a plastic universal tube without anticoagulant. The latter was allowed to coagulate for 60 min and serum was retrieved after centrifugation at 800 \(\times\) g; 10 min.

Coculture and adhesion assay protocol.
The development of the model for culture of monocytes with human umbilical vein EC (HUVEC) and the subsequent neutrophil adhesion assay used for these treatment- and patient-based studies has been described in detail recently (9). The key stages here were to thaw a frozen aliquot of HUVEC and establish cultures in microslides (glass capillary tubes 50 mm long with a rectangular cross section of 3 × 0.3 mm; Camlab). On the same day, we drew blood from study subjects and prepared serum. Serum and CPDA blood were held on wet ice overnight, and monocytes were isolated after EDTA had been added to the blood for 1 h. The monocytes were seeded on HUVEC (1 h), nonadherent cells were washed out, and the numbers adherent were counted before coculture for a further 23 h. Monocytes were also seeded in 24-well dishes. The next day, neutrophils were isolated from freshly drawn blood (see below) and adhesion to monocyte-HUVEC cocultures tested. Supernatants from monocyte cultures were collected and stored at \(-80^\circ\) C until assay for cytokines. The same neutrophil donor was used for testing cocultures before and after fish oil supplementation. Aliquots from the same batch of frozen HUVEC were used before and after fish oil supplementation. The protocol was developed over a 9-mo period, as described (9), and the same person (N.-T.L.) carried out all the coculture and adhesion assay procedures to standardize methodology and avoid any operator-based changes in results.

Isolation of monocytes or neutrophils.
EDTA (1.5 g/L) was added to the CPDA blood that had been stored for 24 h on wet ice and 1 mL dextran (MW 500 kD, 6% w:v in PBS; Sigma Aldrich) was added. Adding EDTA improved the yield of monocytes from the stored blood (9). The tube was placed at a 45° angle from the vertical and red cells were allowed to sediment for 1 h. The leukocyte-rich supernatant was retrieved and placed on top of 3 mL Nycoprep 1068 medium (Amersham Biosciences) and centrifuged at 400 \(\times\) g; 15 min. The middle layer, rich in monocytes, was decanted. The monocytes were washed twice in Ca2\+ and Mg2\+-free PBS/horse serum albumin (BSA, 0.15% culture tested; Sigma), resuspended in Medium 199 (M199; Invitrogen) and centrifuged at 400 \(\times\) g; 15 min. The middle layer, rich in monocytes, was decanted. The monocytes were washed twice in Ca2\+ and Mg2\+-free PBS/horse serum albumin (BSA, 0.15% culture tested; Sigma), resuspended in Medium 199 (M199; Invitrogen) containing 20% autologous serum, and counted using a Coulter Multisizer II (Coulter Electronics).

Neutrophils were isolated from EDTA-treated blood within 1 h of withdrawal using 2-step density gradients of Histopaque 1119 and 1077 (Sigma) as described (24). Cells were washed twice in PBS containing 1 mmol/L Ca2\+ and Mg2\+, 0.5 mmol/L Mg2\+-free PBS/horse serum albumin (BSA, 0.15% culture tested; Sigma) and 5 mmol/L glucose (PBS/BSA) and adjusted to 107/L in the same medium.

Culture of EC in microslides.
HUVEC were isolated from umbilical veins as described (25). Primary HUVEC were cultured in M199 containing 20% fetal calf serum (FCS), 28 mg/L gentamicin, 1 mg/L hydrocortisone, and 10 \(\mu\)g/L epidermal growth factor (all from Sigma) until confluent (4–6 d). The HUVEC from individual confluent 25-cm2 flasks were detached using trypsin/EDTA (Sigma), resseeded in 2 flasks (first passage), and cultured until confluent. This process was repeated to obtain expanded 2nd passage cultures. The confluent HUVEC were detached, centrifuged, and suspended in a mixture of FCS (90%) and 10% dimethyl sulfoxide. Aliquots equivalent to 1 25-cm2 flask were slow frozen at \(-80^\circ\) C overnight and then transferred to liquid nitrogen until use. Our previous studies indicated that aliquots from these passaged, frozen cultures supported similar leukocyte adhesion to primary cultures (9).

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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<tbody>
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<td><strong>Statins</strong></td>
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<tr>
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<td><strong>Aspirin</strong></td>
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<td>1</td>
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</table>

1 10 of 29 control donors and 16 of 16 patients with PAD receiving medication.
For assays, a frozen aliquot of HUVEC was rapidly defrosted in a water bath at 37°C and cells were seeded and cultured in the microlides as previously described (25,26). Seeding was at a density yielding confluent monolayers within 24 h.

**Stimulation of HUVEC by coculture with monocytes.** Monocytes were cocultured with the HUVEC as described (6,9). Microslides containing confluent HUVEC were injected with 50 μL of isolated monocytes adjusted to 3 × 10^5/L, yielding a ratio of monocytes to EC of 1:1 (based on a typical EC surface density of 1000/mm²). After incubation for 1 h at 37°C to allow monocyte adhesion, nonadherent monocytes were washed with culture medium. We placed the microslides on a phase-contrast video microscope, and connected to a flow system. We flushed the microslides with PBS/BSA and recorded a series of 8 video fields along the centerline for evaluation of the number of adherent monocytes (see below). We then reconnected the microslides to the culture system for a further 23 h, with the culture medium containing 20% serum autologous to the monocytes rather than FCS. In studies after dietary supplementation, the use of autologous serum ensured that monocytes continued to experience medium with modified lipid content.

**Adhesion assays.** The flow-based assay of neutrophil adhesion and migration was as previously described (5,9). Monocyte-treated microslides were glued to a glass microscope slide, mounted on the stage of a phase-contrast video microscope, and connected to a flow system allowing perfusion of neutrophils or cell-free PBS/BSA at a wall shear stress of 0.1 Pa (equivalent to a wall shear rate of 140 s⁻¹). We flushed microslides with PBS/BSA and recorded a series of 8 video fields along the centerline for evaluation of the number of adherent monocytes remaining. Neutrophil suspension was perfused through the microslide for 4 min followed by washout with PBS/BSA. A video record of adhesive events was made in at least 8 video microscopic fields after 1 and 5 min of washout.

Video records were analyzed off-line with the assistance of a computerized image analysis program (ImagePro; DataCell). For monocytes, the number of adherent cells was counted, averaged per field, and converted to cells/mm² using the calibrated field dimensions. We classified the monocytes as either adhered to the apical surface of the monolayer (phase-bright) or transmigrated (spread and phase-dark). Adherent neutrophils were counted and classified as either: 1) rolling slowly over the surface (phase bright and stationary); 2) activated on the surface (phase bright and stationary or migrating slowly on top); or 3) transmigrated (phase dark, spread, and migrating under the HUVEC). Because adherent monocytes were still present, their numbers were subtracted from the total adhesion to obtain the number of adherent neutrophils. The monocyte phase dark number was separately subtracted from the final phase dark number to quantify the level of neutrophil transmigration. Monocytes never rolled or detached from the surface under flow.

**Cytokine release by cultured monocytes.** Monocytes (3 × 10⁵ cells) were seeded in culture dishes in the same medium as above (with 20% autologous serum) and cultured for 20 h at 37°C without purposeful stimulation (as was the case in cocultures). The supernatant was collected and the concentrations of TNFα, IL-1β (IL-1), and IL-6 determined using a Cytometric Bead Array assay according to the manufacturer’s instructions (BD Biosciences).

**Analysis of plasma phospholipid fatty acid composition.** Total lipids were extracted from 500 μL plasma using chloroform:methanol (2:1, v/v) containing 50 mg/L butylated hydroxytoluene as antioxidant and 1 mol/L NaCl followed by centrifugation at 800 × g for 10 min. The lower lipid phase was collected and dried under nitrogen. Separation of the phosphatidylcholine (PC) fraction was performed using solid phase extraction (27). Total lipid was dissolved in dry chloroform and applied to a Bond Elut-CH₂₃ cartrige (Varian). The column was washed with dry chloroform and the PC fraction was eluted using chloroform:methanol (60:40, v/v). Samples were dried and redissolved in toluene.

Fatty acid methyl esters were prepared by adding methanol containing 2% (v/v) H₂SO₄ and heating at 50°C overnight. Samples were neutralized with 0.25 mol/L KHCO₃, 0.5 mol/L K₂CO₃. The PC-fatty acid methyl esters were extracted into toluene. They were then collected from the upper phase and dried under nitrogen. Samples were redissolved in hexane and fatty acid methyl esters resolved using a Hewlett Packard 6890 GC (Agilent) equipped with a 30-m × 0.25-μm × 0.25-mm BPX-70 fused silica capillary column. The concentration of each fatty acid methyl ester was determined by the area under the peak using ChemStation software (Agilent) and each fatty acid is expressed as a percentage of the total.

**Statistical analysis.** Comparisons between treatments were by paired t test and comparisons between groups were by Student’s t test. Correlations between measured parameters or between changes in parameters induced by fish oil were tested by linear regression. P < 0.05 was considered significant.

**Results**

**Monocyte adhesion to HUVEC and subsequent neutrophil adhesion to cocultures.** Monocytes isolated from blood that had been stored on ice in CPDA for 24 h were able to induce activation of thawed, batch-frozen HUVEC, so that the HUVEC were able to capture and induce migration of flowing neutrophils. Although there was considerable inter-donor variation between the number of monocytes initially binding to the HUVEC (e.g. CV was 31% for 29 controls), there was even greater variation in the subsequent binding of neutrophils that the monocytes induced (e.g. CV was 56% for 29 controls). These phenomena are similar to those we previously reported (9) and indicate that while different donors may have differently adhesive monocytes, there are also inter-donor variations in their potency in causing endothelial activation. Also, as before, monocytes from patients with PAD tended to adhere in greater number than those from controls (Table 2), although this difference was not significant (P = 0.16). Smaller

| TABLE 2 | Adhesion and migration of monocytes on HUVEC after 1 and 24 h, and adhesion of neutrophils to HUVEC that were cultured for 24 h with monocytes using cells from control donors or PAD patients before and after dietary fish oil supplementation |
| --- | --- | --- |
| | Adherent monocytes | Migrated monocytes | Adherent neutrophils |
| | After 1 h | After 24 h | After 1 h | After 24 h | After 24 h coculture |
| Control (n = 29) | | | | | |
| Before fish oil | 245 ± 14 | 115 ± 7 | 122 ± 8 | 79 ± 5 | 392 ± 41* |
| After fish oil | 240 ± 18 | 128 ± 11 | 137 ± 9 | 86 ± 7 | 278 ± 43* |
| PAD patients (n = 16) | | | | | |
| Before fish oil | 359 ± 77 | 137 ± 15 | 181 ± 39 | 94 ± 9 | 447 ± 42 |
| After fish oil | 269 ± 19 | 151 ± 14 | 168 ± 14 | 95 ± 10 | 391 ± 64 |

1 Data are means ± SEM. *Different from before, P = 0.013 (paired t test).
numbers of donors were studied here and we also noted a complicating effect of drug treatment received by some normal donors (see below) that was absent in the previous study (9).

Monocyte adhesion to HUVEC and subsequent neutrophil adhesion to cocultures: effect of fish oil. Fish oil did not affect the number of monocytes from healthy donors binding to the HUVEC or the proportion migrating within the first hour (Table 2). After 24 h, about one-half of the monocytes had actually detached from the HUVEC, some of which had reverse migrated from the subendothelial layer, but this behavior was not affected by fish oil supplementation. However, dietary supplementation with fish oil significantly decreased the ability of the monocytes (from healthy donors) bound to the HUVEC to induce subsequent capture of flowing neutrophils (Table 2; Fig. 1). Of the neutrophils bound, just over 20% went on to migrate through the endothelial monolayer, but fish oil did not affect this parameter (data not shown).

Fish oil supplementation did not affect the number of monocytes from patients with PAD binding to the HUVEC or the proportion migrating within the first hour (Table 2). Fish oil supplementation did not significantly decrease neutrophil recruitment by cocultures of HUVEC with monocytes from patients with PAD (Table 2; Fig. 1).

Monocyte adhesion to HUVEC and subsequent neutrophil adhesion to cocultures: effect of medication. We considered that a complicating factor influencing monocytes may be the use of medication. All donors with PAD, except 1, were receiving prescribed medication and 10 of 29 controls were also receiving medication (some similar to those used by PAD patients). When the controls were divided into 2 groups according to medication use or not, the effect of fish oil on monocyte-induced neutrophil recruitment was still marked and significant for the 19 healthy donors not using medication (Fig. 2). There was no effect in the 10 healthy donors that received medication. Nevertheless, monocyte-induced neutrophil recruitment, either before or after fish oil treatment, was significantly lower if monocytes came from subjects not receiving medication compared with those from subjects receiving medication (Fig. 2). Moreover, before fish oil supplementation, monocytes from nonmedicated control donors had a significantly lower ability to induce neutrophil adhesion to HUVEC than monocytes from patients with PAD (Fig. 2) as previously found (9). The initial number of adherent monocytes also tended to be lower for the nonmedicated controls than PAD patients, but this was not significant (P = 0.11) (Fig. 2).

When all donors were considered together, regardless of treatment or PAD, then monocyte-induced neutrophil recruitment to HUVEC was decreased by fish oil supplementation (from 412 ± 31 to 319 ± 36 neutrophils/mm²; P = 0.02).

![FIGURE 1](https://academic.oup.com/jn/article-abstract/137/12/2769/4670086) Comparison of neutrophil adhesion to HUVEC that was cultured for 24 h with monocytes from normal controls (A) or donors with PAD (B) before and after dietary supplementation with fish. Data for individual donors are shown.

![FIGURE 2](https://academic.oup.com/jn/article-abstract/137/12/2769/4670086) Monocyte adhesion to HUVEC and neutrophil adhesion to cocultures: effect of fish oil. Fish oil did not affect the number of monocytes from healthy donors binding to the HUVEC or the proportion migrating within the first hour (Table 2). After 24 h, about one-half of the monocytes had actually detached from the HUVEC, some of which had reverse migrated from the subendothelial layer, but this behavior was not affected by fish oil supplementation. However, dietary supplementation with fish oil significantly decreased the ability of the monocytes (from healthy donors) bound to the HUVEC to induce subsequent capture of flowing neutrophils (Table 2; Fig. 1). Of the neutrophils bound, just over 20% went on to migrate through the endothelial monolayer, but fish oil did not affect this parameter (data not shown).

Fish oil supplementation did not affect the number of monocytes from patients with PAD binding to the HUVEC or the proportion migrating within the first hour (Table 2). Fish oil supplementation did not significantly decrease neutrophil recruitment by cocultures of HUVEC with monocytes from patients with PAD (Table 2; Fig. 1).

### Plasma phospholipid fatty acid composition.

At study entry, patients with PAD had a higher content of arachidonic acid in their plasma PC compared with controls (Table 3). Fish oil supplementation resulted in significant changes in the fatty acid composition of plasma PC in both groups of donors (data for arachidonic acid, EPA, and DHA are shown in Table 3). Fish oil significantly decreased the proportion of arachidonic acid and significantly increased the proportions of EPA and DHA (Table 3). Changes in arachidonic acid were greater for PAD patients (P = 0.008) and a similar tendency was also observed for EPA (P = 0.085) and for DHA (P = 0.106) (see Table 3). The magnitude of the changes in fatty acids did not correlate with the magnitude of changes in monocyte-induced neutrophil recruitment on a donor-by-donor basis (tested by linear regression), nor was there any correlation between the baseline values for fatty acids and monocyte-induced neutrophil adhesion or migration.

### Cytokine secretion by monocytes.

Cytokine production by monocytes might be important in determining their proinflammatory effects, and changes in monocyte function induced by fish oil might correlate with changes in secretion. We therefore measured monocyte secretion of TNFα, IL-1β or IL-6, but found that fish oil supplementation did not affect their release by monocytes from PAD patients or controls (data not shown). Also, the release of these cytokines by monocytes from the 2 groups did not differ (data not shown). We tested whether there was any relationship between the initial values for these
cytokines and neutrophil adhesion or migration on HUVEC induced by monocyte coculture or whether changes in secretion after fish oil supplementation correlated with changes in these readouts. However, in no case (for PAD or control subjects) did linear regression show linkage between these parameters.

### Discussion

We have shown for the first time, to our knowledge, that monocytes from donors receiving dietary supplementation with long chain (n-3) PUFA undergo a functional change that is directly linked to reduced potential to induce an inflammatory response in the vessel wall. Using a culture model incorporating primary human cells, we found that monocytes potently induced EC to recruit flowing neutrophils. However, this potency was reduced when donors supplemented their diet with fish oil (6 g/d for 12 wk). The efficacy of this treatment varied quite widely between donors and effects on patients with PAD were less than those on age-matched controls without known vascular disease. This difference did not relate to the ability to incorporate the (n-3) fatty acids, which was similar between groups and, in fact, tended to be greater in the PAD patients. Variation may have been due in part at least to concurrent medication received by all PAD patients and about one-third of control subjects. The influence of fish oil on the ability of monocytes to stimulate EC was not strongly linked with any reduction in production of individual inflammatory cytokines by the monocytes, assayed in separate monocultures.

Previous studies have shown the potential of (n-3) PUFA to decrease adhesion molecule expression on monocytes (15,16,20), EC (15,17,18), macrophages (13), and lymphocytes (19) and have demonstrated that this reduces adhesive interactions with leukocytes (15,19). Here, we investigated how (n-3) fatty acids modified the ability of monocytes to drive an inflammatory response, which is one of their main functions. This would itself necessarily depend on their adhesion and migration on EC. In fact, we found little effect on the recruitment of the monocytes themselves but a reduction in the subsequent binding of flowing neutrophils to the cocultures when the monocytes were isolated after fish oil supplementation. Thus, although previous studies suggest the potential for fish oil to inhibit the contribution of monocyte/macrophages to inflammation, the model used here is the first to our knowledge to report on the amelioration of effects of these cells on other cells of the vascular system. The advantage of this relatively complex multicellular model is that it provides an integrated functional readout relevant to a range of inflammatory processes. This is likely to be more sensitive, and indeed relevant, than the assay of single cytokines released or adhesion molecules or chemokines expressed, because inflammation requires coordinated responses via several such mechanisms.

Indeed, we did not detect obvious changes in monocyte cytokine secretion here, despite the change in the functional recruitment readout. The lack of simple correlation between cytokine release and the effects on EC might occur because of complex interactions between effects of several cytokines and growth factors, or because the spontaneous secretion analyzed might not be the same as secretion in the actual cocultures, whose release products we could not assay.

Reduction in the pro-inflammatory potential of monocytes was significant when data from all donors was pooled, but there was no significant effect in patients with PAD (who were all receiving medication) or in the subset of controls who were receiving medication. The medications were of a wide variety and no linkages could be demonstrated with specific agents (such as statins or aspirin), because the study was not designed, or indeed powered, for such a purpose. It is possible that monocytes from patients with PAD and those receiving medication might have a more activated phenotype that was less responsive to (n-3) fatty acids. Indeed, there was evidence in this direction here and in our previous study (9), where monocytes from patients with PAD induced greater recruitment of neutrophils than those from controls. In addition, the PAD patients inevitably had variation in duration and severity of atherosclerotic disease (e.g. judged from ABPI), which may have added to the variability in their responses. Thus, although fish oil appeared beneficial overall, efficacy in individuals probably depends on their state of health at the start of supplementation. It was notable that PAD patients had significantly higher arachidonic acid in their plasma PC than healthy controls at study entry and tended to have lower EPA and DHA. This pattern is consistent with the idea that an excess of arachidonic acid over long-chain (n-3) fatty acids is associated with inflammatory conditions (10) and that higher risk of cardiovascular disease is associated with low intake and low status of long chain (n-3) fatty acids (28). However, with fish oil supplementation, plasma PC of PAD patients became similarly enriched in EPA and DHA as that of control subjects, indicating that the deficit in these fatty acids seen at study entry was most likely related to a lower dietary intake.

Leukocyte recruitment is an essential part of all inflammatory responses and neutrophil recruitment, in particular, is relevant to a range of pathologies. Although neutrophils are not typically major constituents of atherosclerotic plaques, the underlying process of macrophage-induced endothelial activation is important in this and other chronic disorders. In fact, coculture of monocytes with EC also induces the ability to recruit flowing monocytes and lymphocytes (5) and neutrophils were used here because they give a robust readout with which we have great experience. However, the results of this study will also be relevant to a range of disorders not usually linked with these cells.

### Table 3

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<th>Control</th>
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<tr>
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<td>After FO</td>
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<td>% of total fatty acids</td>
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<td>DHA</td>
<td>4.1 ± 0.3</td>
<td>5.9 ± 0.2**</td>
<td>1.8 ± 0.4</td>
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1 Data are means ± SEM. *Different from before, P = 0.001; **P < 0.001 vs. before fish oil (paired t test); ***P = 0.002 vs. controls before fish oil; ****P = 0.008 vs. controls (Student’s t test).
The observation of decreased monocyte-induced leukocyte recruitment to EC may explain in part the protective effect of long chain (n-3) fatty acids from fish and fish oils on atherosclerosis. This effect has been well demonstrated in animal models (29–34) and there are suggestions from human studies of decreased vessel wall inflammation associated with reduced macrophage infiltration (21) and of slowed lesion progression (35) with dietary fish oil. Future studies could usefully identify the features of monocytes that are altered by (n-3) fatty acids such that their interaction with EC is dampened.

This study suggests that their initial adhesiveness and ability to secrete classic inflammatory cytokines are unaltered, but other cytokines or chemokines might be involved. In addition, it leaves open the more basic question of how (n-3) fatty acids influence the cellular signaling or secretion pathways that lie upstream of the functional responses.

**Literature Cited**


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