Prostaglandins, Leukotrienes and Essential Fatty Acids (2000) **62**(3), 201–207

doi:10.1054/plef.2000.0142, available online at http://www.idealibrary.com on IDEAL®

Effects of n-3 fatty acids on growth and survival of J774 macrophages

D. J. Fyfe, M. Abbey

CSIRO Health Sciences and Nutrition, PO Box 10041, Adelaide BC, SA 5000, Australia

Summary To further understand potential mechanisms underlying the protective effects of eicosapentanoic acid (EPA) against atherosclerosis, J774 macrophages were used to explore cellular responses to growth in the presence of PUFA in vitro. Clonogenic assays indicated that 15μg/ml of EPA killed over 90% of J774 populations. Docosapentaenoic acid (DPA) was more cytotoxic than either EPA or docosahexaenoic acid (DHA). EPA was shown to be elongated to DPA. Cytotoxicity induced by EPA was not inhibited by the presence of alpha-tocopherol (a-toc) in the medium. Immunological screening for caspase enzymes and microscopic examination indicated that apoptosis was not the major cause of cell death. Proliferation assays demonstrated that total cell numbers of EPA-treated cells were not significantly different to control cells. Increasing does of EPA were correlated with increasing levels of intracellular malondialdehyde (MDA). These observations suggest that EPA may influence the growth parameters of macrophages whilst inducing moderately elevated levels of oxidative stress. © 2000 Harcourt Publishers Ltd

INTRODUCTION

There is strong evidence that EPA (20:5n-3) from fish oil protects against the development of atheromatous plaques in cholesterol-fed rats,[1] quails,[2] rabbits,[3] pigs[4] and monkeys.[5] EPA is also extremely susceptible to oxidative damage, and it has previously been shown in our laboratory that EPA from fish oil can facilitate the oxidation of low-density lipoprotein by J774 macrophages.[6] This raises a paradox, since elevated levels of oxidative stress and oxidation of LDL in macrophages is strongly implicated in atherogenesis.[7] How can fatty acids which have increased propensity to lipid peroxidation protect against atherosclerosis? Accordingly, the present investigation aimed to further our understanding of potential mechanisms underlying this phenomenon by exploring the effects of EPA on isolated cells. In vitro, the presence of EPA has previously been shown to influence the growth patterns of many cell types. For example, EPA inhibits growth in murine[8] and human[9] lymphocytes, bovine carotid endothelial cells[10] and vascular smooth muscle cells,[11,12] as well as inhibiting accelerated growth in a range of tumour cells. Proposed mechanisms for these phenomena include modulation of eicosanoid synthesis,[13] membrane function and lipid peroxidation[14] and the induction of cell death by apoptosis.[15] However, the effects of EPA in comparison with other PUFA on growth and survival of macrophages in vitro have not been extensively investigated. We have previously demonstrated that EPA from fish oil was appreciably cytotoxic towards J774 macrophages in vitro.[16] In the present study, we have extended our investigations to further explore the nature and specificity of the cytotoxic effects of EPA in J774 cells.

Received 29 November 1999
Accepted 12 January 2000

Correspondence to: DJ Fyfe PhD, CSIRO Health Sciences and Nutrition, PO Box 10041, Adelaide BC, SA 5000, Australia. Tel.: +61-8-8303-8800; Fax.: +61-8-8303-8899; E-mail: daren.fyfe@hsn.csiro.au

METHODS

Cell culture

Murine macrophage J774 cells were grown in a humidified incubator at 37°C, 5% CO_2 in minimal essential medium supplemented with 10% foetal calf serum, 0.01% glutamine, 0.01% penicillin/streptomycin, 0.5% sodium bicarbonate and PUFA (in free fatty acid form) or alpha-tocopherol (a-toc) dissolved in ethanol. Eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), docosapentaenoic acid (DPA, 22:5n-3), alpha-linolenic

acid (ALA, 18:3n-3), linoleic acid (LA, 18:2n-*6*) and oleic acid (OA, 18:1n-*9*) were purchased from Sigma Chemical, New South Wales, Australia. In the clonal survival experiments, 5×10^2 cells were incubated with graded doses of PUFA (0–30 μg/ml) or a-toc (0–8 μg/ml) for 10–14 days in 25 cm^3 cell culture flasks (Sarstedt Australia, Adelaide, South Australia) until colonies were visible with the naked eye. At this point, the cells were fixed and stained with Crystal violet in absolute ethanol (2% m/v), and scored for colonies of >50 cells. The proliferation assays were carried out by initially plating out approximately 10^4 cells into cell culture dishes (3 cm diameter, Sarstedt Australia, Adelaide, South Australia) in medium supplemented with fixed doses of PUFA (15 μg/ml) for 8 days. Cells were counted (using a haemocytometer) and media were changed daily.

Detection of cell death

The nature of cell death induced by EPA was investigated using an ELISA-based kit for the detection of apopain/Caspase-3–induced apoptosis (Bio-Rad Laboratories, California, USA). Briefy, J774 cells were grown in media supplemented with or without 15 μg/ml EPA and 16 μg/ml a-toc for *96* h to give approximately 10^6 cells per flask. Apopain enzymes were then labelled in each whole cell sample with the fluorometric peptide substrate, carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Z-DEVD-AFC). The accumulation of fluorescent, free AFC was measured following cleavage of Z-DEVD-AFC molecules by apopain enzymes. Samples were compared to the activity of an apopain standard as a positive control.

HPLC measurement of MDA

Approximately 10^5 cells were seeded into 75 ml flasks in MEM supplemented with EPA or LA or octadecanoic acid (18:0) (Sigma Chemical) for comparison. The methods of Fukunaga et al.[17] were used to measure levels of MDA produced by the cells. Briefly, identical numbers of cells (1×10^6) were centrifuged at 1000 × g for 5 min. Samples (50 μl) of the cells in suspension in conditioned medium were treated with a thiobarbituric acid (TBA) solution. TBA formed a fluorescent complex with MDA (TBA-MDA) and levels of this complex were measured by comparison to standards (prepared with 1,1,2,2-tetraethoxypropane) using high performance liquid chromatography (HPLC). Samples were passed through a 150 mm × 4.6 mm C18 column using 7:3 mixture of acetonitrile:MilliQ water as the mobile phase. Samples were analysed using WinChrom analysis software following fluorescence detection. Hardware and software for HPLC analyses were purchased from GBC Scientific Equipment Pty Ltd, Dandenong, Victoria, Australia.

Analysis of cellular fatty acids by gas chromatography

J774 cells were grown in media supplemented with or without different fatty acids for *96* h to give approximately 10^6 cells per flask. Cells were centrifuged (1000 × g; 5 min) and washed and resuspended in phosphate-buffered saline twice. Total cell fatty acids were extracted using the phenyl-chloroform method originally described by Bligh and Dyer.[18] Total fatty acid composition was determined on the fatty acid extracts after methylation with 1% sulphuric acid in dry methanol. The fatty acid methyl esters were extracted with petroleum ether (B.P. 40–60°C) and contaminants removed by affinity chromatography on florisil (Sigma Chemical). The eluate was dried under nitrogen and the samples redissolved in isooctane. Aliquots (0.2 μl) were injected onto a silica gas chromotography column (30 m × 0.53 mm) coated with cross-linked free fatty acid phase in a Hewlett-Packard 5711A gas chromatograph (Hewlett-Packard, Avondale, PA). Fatty acids were identified by comparison with authentic standards supplied by Nu-Chek-Prep (Elysian, MN). Data were captured and analysed using WinChrom software (Hewlett-Packard).

Statistics

The significance of differences between means was estimated by one-way analysis of variance followed by Student's *t*-test using GraphPad Instant statistics program. $P<0.05$ was taken as the criterion of significance.

RESULTS

Growth parameters

Data from the proliferation assays (Fig. 1) indicate that neither EPA nor the ethanol control had any significant effect on total cell number compared to untreated control cells over 7 days. Towards the end of the proliferation assay, however, an increasing number of floating cells was apparent in the cells grown in media supplemented with EPA. Results from the clonal survival experiments (Fig. 2) demonstrated that n-3 PUFA were cytotoxic towards J774 cells in a dose-dependant manner. Survival curve parameters D_0 and D_{37} (as previously described[19]) for the different fatty acids are summarised in Table 1. These data indicate that the most cytotoxic PUFA was DPA. EPA, DHA and ALA were appreciably more cytotoxic than LA. The data also indicate that ALA is more cytotoxic than DHA. Alpha-tocopherol was also shown to be strongly cytotoxic towards J774 cells (Fig. 3) and did not have any protective effects against the cytotoxicity of EPA, since co-supplementation of media with both EPA and a-toc (5 μl/ml, 12 μM) resulted in clonal survival of 0%. Survival curve parameters for Fig. 3 are as follows: $D_0 = 0.72 \pm 0.4$ μg/ml, $D_{37} = 1.28 \pm 0.7$ μg.ml.

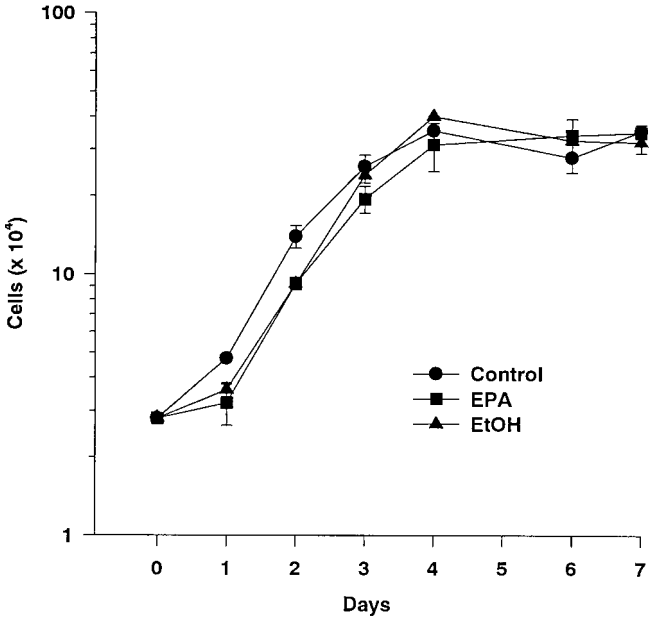

Fig. 1 Growth curves for J774 cells +/– the presence of EPA (15 μg/ml). Values are means (+/– SEM) of three experiments.

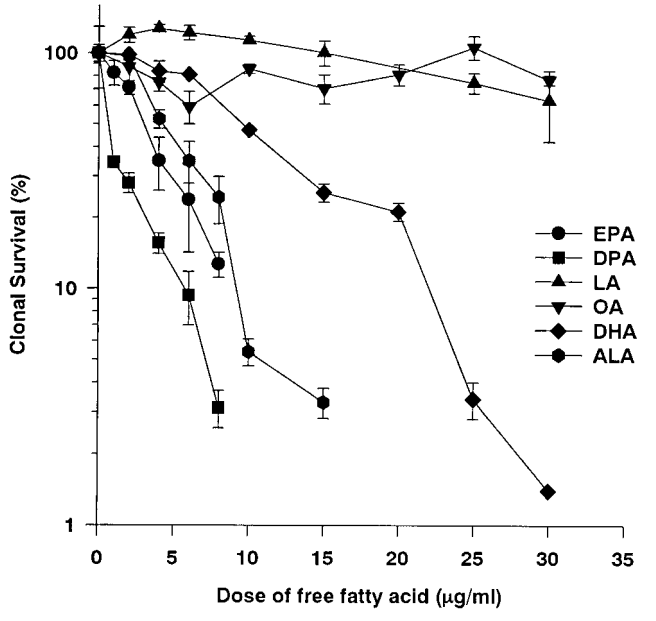

Fig. 2 Survival curves for J774 cells grown in graded concentrations of EPA, DPA, ALA, LA and OA for 12d. Values are means (+/– SEM) of three experiments.

Table 1 Survival curve parameters (derived from data in Fig. 2 for J774 macrophage grown in graded doses of long chain unsaturated fatty acids. The curves are described in terms of a linear decrease in cell survival, the slope of which is termed D_0, and is defined as the concentration required to lower survival from any point on the slope to 37% of that value. D_0 values therefore reflect the sensitivity of the cells to a given PUFA and are not to be confused with those for D_{37}, which state the full dose required to reduce survival to 37%

	D_{37} (μg/ml)[1]	D_0 (μg/ml)[1]
Eicosapentaenoic acid	3.9 ± 0.4[a]	1.7 ± 0.3[a]
Docosapentaenoic acid	1.0 ± 0.2[b]	1.2 ± 0.2[a]
Alpha-linolenic acid	6.0 ± 0.5[c]	2.9 ± 0.4[b]
Docosahexaenoic acid	12.5 ± 0.9[d]	3.8 ± 0.4[b]
Linoleic acid	>30.0	>30.0
Oleic acid	>30.0	>30.0

[1]Mean ± SEM from 3 experiments.
[2]Values differed significantly ($P \leq 0.05$) in columns with different superscript letters.

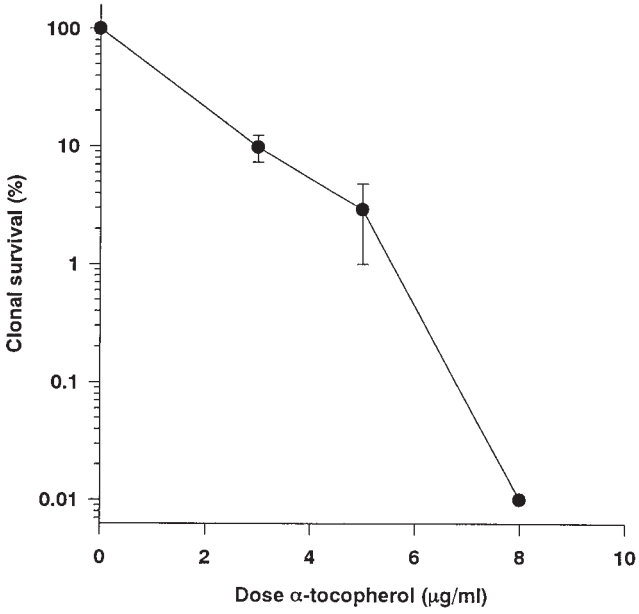

Fig. 3 Survival curve for J774 cells grown in graded concentrations of alpha-tocoperol. Values are means (+/– SEM) of three experiments.

Cell death

No significant change in fluorescence was detected in either the untreated control cell samples or cell samples that had been grown in the presence of EPA +/- a-toc. In contrast, there was a significant ($P<0.05$) time-dependant increase over 20 h in the fluorescence of the positive control (active caspase) from zero to 40.7 ± 1.5 arbitrary fluorescence units. Analyses of the same cell samples using light microscopy prior to the caspase assay did not reveal the presence of regions of chromatin condensed into 'apoptotic bodies', previously described as features characteristic of apoptotic cells.[20]

HPLC measurement of MDA

The change in MDA concentration produced by the cells in the presence of graded doses of EPA for *96* h is illustrated in Fig. 4. The graph demonstrates a linear increase in MDA formation (up to approximately 100%) with

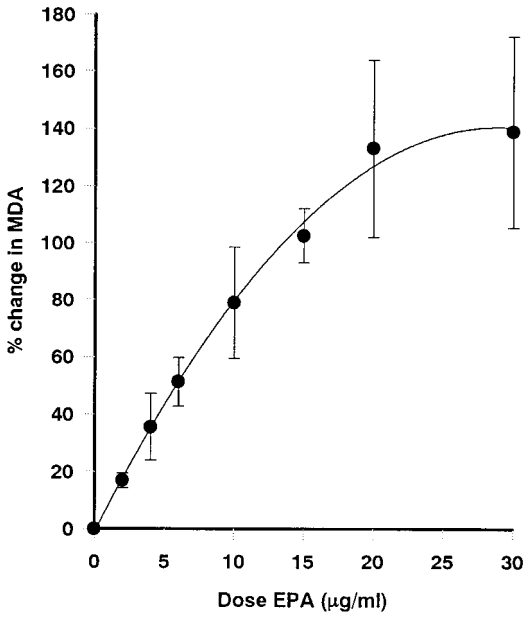

Fig. 4 Effect of growth in graded doses of EPA for 96 h on MDA produced by J774 cells. Values are means (+/– SEM) of three experiments and are expressed as '% increase' with respect to untreated control cells.

increasing doses of EPA from 0 to 15 μg/ml at which point the results tend to plateau. The plateau signifies no further increase in MDA formation, with increasing doses of EPA, probably because at these higher doses EPA was substantially cytotoxic (Fig. 2). The percentage increases in MDA concentration produced by cells grown in 30 μg/ml octadecanoic acid (18:0) and LA (18:2n-*6*) for *96* h were –2.3 ± 3.6% and 25.2 ± 1.1% respectively.

Fatty acid profiles

Fatty acid analyses of total cell lipids by gas chromatography are given in Table 2. Supplementing the media with PUFA (EPA, DPA, DHA and ALA) resulted in a significant rise ($P<0.05$) in the percentage of each added PUFA respectively. The main consequence of this rise was a decrease in the proportions of fatty acids 18:1 and 20:4. Supplementation with either DHA, DPA or ALA did not result in dramatic increases in the proportions of any other fatty acids. Therefore, there was no evidence of significant elongation or desaturation of ALA. However, supplementation with EPA resulted in a striking rise in the proportion of DPA (to 21%) within cells, indicating elongation of EPA. Data also indicate that DPA was not significantly metabolised further by desaturation to DHA, as shown by the lower values for percentages of DHA in the EPA, DPA and ALA samples compared to the controls.

DISCUSSION

The putative causative effects of elevated levels of oxidative stress[7] and the protective effects of dietary fish oil[1–5] in atherogenesis are well documented. However, the effects of PUFA from fish oil on the growth parameters and survival of macrophages have not been extensively explored in the past. There is no doubt, however, that PUFA from fish oil are extremely vulnerable to lipid peroxidation. How PUFAs, which have increased propensity to undergo lipid peroxidation, protect against atherosclerosis is not understood. Evidence surrounding the putative causative role of oxidative stress in atherosclerosis is often conflicting, retrospective and inferential, even though there is ample evidence that oxidative stress is present and associated with the pathogenesis of atherosclerosis (particularly during platelet aggregation).[21] Unfortunately, it is not known whether these elevated levels of oxidative stress are causal or consequential in atherogenesis, and to extrapolate the experimental evidence available to the full complexity of atherogenesis is considered unwise.[22] Most importantly, there is still no unequivocal evidence that oxidative stress actually impairs vascular function and augments atherogenesis. To further our understanding of potential mechanisms underlying atherosclerosis, the present study describes the effects of PUFA on growth parameters and MDA generation in macrophages in vitro.

Table 2 Fatty acid profile of cells grown for 96 h in unsupplemented medium (Control) or medium supplemented with 15 mg/ml EPA, ALA, DPA and DHA. Values are expressed as percentages of total cell fatty acids

Fatty acid	Control[1]	EPA[1]	ALA[1]	DPA[1]	DHA[1]
C16:0	32.41±5.82	32.28±6.48	34.26±6.51	38.07±0.85	45.24±2.53
C16:1	0.96±0.48	0.75±0.40	0.69±0.34	0.88±0.10	1.03±0.10
C18:0	14.63±3.54	14.6±1.99	19.41±7.77	14.16±0.76	13.84±1.51
C18:1	26.44±3.14	18.02±2.09*	18.91±1.39*	16.65±0.33*	16.24±2.58*
C18:2	6.61±0.54	2.72±0.06*	ND	ND	ND
C18:3	ND	ND	2.53±1.00*	ND	ND
C20:4	10.91±1.58	ND	9.30±0.75	ND	ND
C20:5	0.50±0.25	7.83±0.29*	2.88±1.44*	5.91±0.33*	3.18±0. 57*
C22:5	4.61±1.00	21.86±3.22*	7.54±0.71	22.84±0.55*	6.82±4.1 7
C22:6	5.88±1.11	1.95±0.17*	4.48±0.18	1.49±0.10*	13.65±6.27*

[1]mean % ± SEM of 3 experiments. ND signifies none detected; *, significantly different from control ($P<0.05$).

Results demonstrated that EPA presented as a free fatty acid killed a large proportion (>90%) of cell populations in vitro whilst elevating levels of MDA by approximately 40% (as compared to untreated control cells) at a dose of approximately 5 μg/ml (Figs 2, 4). The levels of EPA are physiologically feasible, since other workers have demonstrated that similar levels of EPA (7.6 ± 3.6 μg/ml) are found in free fatty acid form in normal human plasma.[23] The nature of cell death induced by EPA was shown not to be apoptosis, since ELISA assays did not identify the presence of Caspase-3 with or without the presence of EPA in J774 cells. Caspase-3 is derived from the proenzyme CPP32 and plays a primary role at the onset of programmed cell death.[24] Furthermore, microscopic examination of cells grown in the presence of EPA did not reveal the presence of 'apoptotic bodies' previously described as features characteristic of apoptotic cells.[20] These observations imply that a necrotic pathway of cell death was induced by EPA in the J774 cells.

Analyses of fatty acid profiles provides evidence that EPA was elongated and desaturated to DPA in the surviving cells. This is not surprising, since it has been shown that EPA and DHA are actively interconverted, leading to the accumulation of DPA, the intermediate metabolite, in cell membrane phospholipids in macrophages.[25] It is interesting to note that Kanayasu-Toyoda et al.[26] suggested that the anti-atherogenic effects of EPA may act via elongation to DPA, and proposed that 'the potency of DPA stimulation of endothelial cell migration is ten-fold greater than that of EPA'. Interestingly, DPA was found to be considerably more cytotoxic than EPA in the present study (Table 1). These data raise the possibility that it may be the elongation of EPA to DPA that influence the growth rates of macrophage in this study.

The physiological significance of these observations is difficult to clarify. Certainly there is evidence from other cell models demonstrating that elevated levels of oxidative stress and MDA are potently cytotoxic.[27] This, however, does not explain how a sub-population of cells can continue to survive (Fig. 1) in the presence of elevated levels of MDA. It has been demonstrated that increased lipid peroxidation (estimated by measuring concentrations of MDA) arises in cells during growth in the presence of EPA in vitro, and after dietary supplementation with fish in humans.[28] EPA has also been reported to have increased potential to raise levels of oxidative stress compared to OA and LA as shown by measurement of conjugated diene formation in endothelial cells.[29] Comparison of the increase in MDA concentrations produced by the cells following growth in the presence of EPA (>100%) with the increase measured in cells grown in 18:0 (effectively 0%) and LA (25%) confirmed that increases in MDA were specific to each fatty acid rather than a non-specific symptom of the cell culture and incubation procedures. There is also evidence that EPA, but not DHA, may increase fatty acid oxidation in rats, possibly explaining why EPA is more efficient than DHA in lowering triglyceride levels in vivo.[30] Thus, differential levels of lipid peroxidation may help to explain the cytotoxicity of EPA towards a proportion of J774 cells in the present study compared to the other PUFA tested. Accordingly, the possibility that cytotoxic effects of EPA could be inhibited by the presence of antioxidant enzymes was explored by examining the clonal survival of cells grown in media supplemented with both EPA and 5 μg/ml (12 μM) a- toc. Levels of a-toc as high as 33.8 ± 11μM are common in human plasma.[31] In contrast to the results of other workers using tumour cells,[14] no protection against EPA-induced cell death was given by a-toc, and a-toc itself was shown to be cytototoxic (Fig. 3). It is difficult to explain these observations in the context of a-toc in its role as an antioxidant. However, there is evidence that under certain circumstances a-toc can adopt pro-oxidative properties and facilitate the oxidation of LDL in humans,[32] presumably following its oxidation to alpha-tocopherolquinones.[33] Furthermore, it has been shown that 5–30 μM a-toc can cause a dramatic increase in cell death which is accompanied by a large increase in products of lipid peroxidation in postischemic cells from the cerebellum of rats.[34] Therefore, the possibility arises that a-toc may have amplified the elevation of levels of MDA caused by EPA to such an extent that clonal survival was reduced to 0%. It would be of interest to investigate this possibility.

The observation that ALA was not further desaturated or elongated and yet was more cytotoxic than DHA, but not as cytotoxic as EPA or DPA, is not understood. However, these results imply that it may be factors other than chain length and degree of unsaturation that govern the cytotoxicity of PUFA, certainly in this model. There is evidence that dihomogammalinolenic acid (20:3;n-6) and arachidonic acid (20:4;n-6) are both appreciably cytotoxic in other cell systems in vitro.[35] Therefore, the possibility exists that the cytotoxic effects of PUFAs may not necessarily be specific to the n-3 class. In addition, macrophage foam cell death, either by necrosis or apoptosis in atheromatous plaques, is thought to be either causal or consequential or both in atherogenesis.[36] How fish oil, which contains fatty acids that increase levels of oxidative stress whilst inducing necrotic cell death, can prevent atherosclerosis remains to be clarified. It is tempting to speculate that the elevated levels of MDA are perhaps a symptom specific only to the model system used in the present study in vitro. However, there is evidence to confirm that dietary supplementation with fish oil and PUFA can raise levels of lipid peroxidation in vivo as well as in vitro.[23,37] Several possibilities exist to explain this apparent paradox. These include: (1) that other benefits of PUFA

outweigh the harmful effects of the increase in oxidative stress, (2) the increase in oxidative stress may not be sufficient to have a physiological effect, and (3) moderately elevated levels of oxidative stress may be desirable in the prevention of atherosclerosis, perhaps by generating resistance to oxidative attack. Parthasarathy et al.[38] lend strength to this possibility by proposing that the beneficial effects of PUFA 'might be derived from their intrinsic vulnerability to oxidative stress'. The results of the present study suggest that this might occur in two ways; one involving cell death and the other the surviving cells, since the cytotoxic effect itself induced by EPA was not absolute, leaving a sub-population of cells which continued to proliferate at enhanced rates. There is evidence that depleted levels of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) revert to normal levels in the plasma of patients with systemic lupus erythematosus following dietary supplementation with fish oil.[39] Furthermore, Fogt and Nanji[40] reported that the expression of mRNA for GPX and CAT was significantly enhanced in liver cells following increased lipid peroxidation induced by dietary supplementation with fish oil. This lends strength to the possibility that moderately elevated levels of oxidative stress induced by dietary supplementation with fish oil may be desirable in the prevention of atherosclerosis via a similar mechanism: by up-regulating the biosynthesis of antioxidant enzymes to generate resistance to oxidative attack. This may explain the resistance to elevated cytotoxic levels of MDA seen in the surviving cells following growth in the presence of EPA. This hypothesis warrants further investigation.

These observations present an interesting finding with respect to current views regarding the unequivocally detrimental effects of moderately elevated levels of oxidative stress in cell growth and survival,[27] one of the main components in the aetiology of disease. Moreover, in some studies, fish oil supplements have been given together with antioxidant vitamins. If part of the beneficial effect of fish oil PUFA is dependent upon inducing a moderate increase in oxidative stress, this may be compromising their preventative effects. Dormandy[41] states that 'inadequate peroxidation and free radical activity may need boosting. Normal peroxidation and free radical activity should be left alone'. We believe these considerations are worthy of further investigation in animal and human studies, since the results of this study suggest that EPA may influence the growth parameters of macrophages whilst inducing moderately elevated levels of oxidative stress.

ACKNOWLEDGMENTS

The authors wish to thank Mark Mano and Caroline Bignell for their technical support.

REFERENCES

1. Adan Y., Shibata K., Sato M. et al. Effects of docosahexaenoic acid and eicosapentaenoic acid on lipid metabolism, eicosanoid production, platelet aggregation and atherosclerosis in hypercholestrolemic rats. *Biosci Biotechnol Biochem* 1999; **63(1)**: 111–119.
2. Chamberlain J. G., Belton C. Effects of long term consumption of fish oil (MaxEPA) on serum lipids and arterial ultrastructure in Japanese quail. *Atherosclerosis* 1987; **68**: 95–103.
3. Zhu B. Q., Smith D. L., Sievers R. E., Isenberg W. M., Parmley W. W. Inhibition of atherosclerosis by fish oil in cholesterol-fed rabbits. *J Am Coll Cardiol* 1988; **12**: 1073–1078.
4. Kim D. N., Ho L. T., Lawrence D. A. et al. Modification of lipoprotein patterns and retardation of atherogenesis by a fish oil supplement to a hyperlipidemic diet for swine. *Atherosclerosis* 1989; **76**: 35–54.
5. Davis H. R., Bridenstine R. T., Vesselinovitch D., Wissler R. W. Fish oil inhibits development of atherosclerosis in rhesus monkeys. *Arteriosclerosis* 1987; **7**: 441–449.
6. Suzukuwa M., Abbey M., Clifton P., Nestel P. J. Enhanced capacity of n-3 fatty acid-enriched macrophages to oxidise low density lipoprotein mechanisms and effects of antioxidant vitamins. *Atherosclerosis* 1996; **124**: 157–169.
7. Witzum J. L. The oxidation hypothesis of atherosclerosis. *Lancet* 1994; **344**: 973–975.
8. Shapiro A. C., Wu D., Meydani S. N. Eicosanoids derived from arachidonic and eicosapentaenoic acids inhibit T cell proliferative response. *Prostaglandins* 1993; **45(3)**: 229–240.
9. Khalfoun B., Thibault G., Lacord M. et al. Docosahexaenoic and eicosapentaenoic acids inhibit human lymphoproliferative responses in vitro but not the expression of T cell surface activation markers. *Scand J Immunol* 1996; **43(3)**: 248–256.
10. Yang S. P., Morita I., Murota S. I. Eicosapentaenoic acid attenuates vascular endothelial growth factor-induced proliferation via inhibiting FIK-1 receptor expression in bovine carotid artery endothelial cells. *J Cell Phsiol* 1998; **176(2)**: 342–349.
11. Shiina T., Terano T., Saito J. et al. Eicosapentaenoic acid and docosahexaenoic acid suppress the proliferation of vascular smooth muscle cells. *Atherosclerosis* 1993; **104(1–2)**: 95–103.
12. Terano T., Tanaka T., Tamura Y. et al. Eicosapentaenoic acid and docosahexaenoic acid inhibit vascular smooth muscle cell proliferation by inhibiting phosphorylation of Cdk2-cyclinE complex. *Biochem Biophys Res Commun* 1999; **254(2)**: 502–506.
13. Saito J., Terano T., Hirai A. et al. Mechanisms of enhanced production of PGI2 in cultured rat vascular smooth muscle cells enriched with eicosapentaenoic acid. *Atherosclerosis* 1997; **131(2)**: 219–228.
14. Cantrill R. C., Ellis G. W., DeMarco A. C., Horrobin D. F. Mechanisms of the selective cytotoxic action of certain essential fatty acids. *Adv Exp Med Biol* 1997; **400A**: 539–544.
15. Hawkins R. A., Sangster K., Arends M. J. Apoptotic death of pancreatic cancer cells induced by polyunsaturated fatty acids varies with double bond number and involves an oxidative mechanism. *J Pathol*; **185(1)**: 61–70.
16. Fyfe D. J., Abbey M. Long chain fatty acids and alpha-tocopherol are cytotoxic towards J774 macrophage in vitro. *Proc Nut Soc Australia* 1998; **21**: 198.
17. Fukunaga K., Takama K., Suzuki T. High performance lipid chromatographic determination of plasma malondialdehyde level without a solvent extraction procedure. *Analytical Biochem* 1995; **230**: 20–23.
18. Bligh E. G., Dyer W. J. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; **37**: 911–917.

19. Musk S. R. R., Stephenson P., Smith T. K. et al. Selective toxicity of compounds naturally present in food toward the transformed phenotype of human colorectal cell line HT29. *Nutr Cancer* 1995; **24**: 289–298.
20. Wyllie A. H., Kerr J. F. R., Currie A. R. Cell death: the significance of apoptosis. *Int Rev Cytology* 1980; **68**: 251–306.
21. Durand P., Blache D. Enhanced platelet thromboxane synthesis and reduced macrophage-dependent fibrinolytic activity related to oxidative stress in oral contraceptive-treated female rats. *Atherosclerosis* 1996; **121(2)**: 205–216.
22. Hoeschen R. J. Oxidative stress and cardiovascular disease. *Can J Cardiol* 1997; **13(11)**: 1021–5.
23. Shimomura Y., Sugiyama S., Takamura T. et al. Quantitative determination of the fatty acid composition of human serum lipids by high-performance liquid chromotography. *J Chromatography* 1986; **383**: 9–17.
24. Rotonda J., Nicholson D. W., Fazil K. M. et al. The three-dimensional structure of apopain/CCP32, a key mediator of apoptosis. *Nat Struct Biol* 1996; **3**: 619–625.
25. Chapkin R. S., Miller C. C. Chain elongation of eicosapentaenoic acid in the macrophage. *Biochim Biophys Acta* 1990; **1042**: 265–267.
26. Kanayasu-Toyoda T., Morita I., Murota S. Docosapentaenoic acid (22:5, n-3), an elongation metabolite of eicosapentaenoic acid (20:5, n-3), is a potent stimulator of endothelial cell migration on pre-treatment in vitro. *Prostaglandins* 1996; **54(5)**: 319–325.
27. McCord J. M. Iron, free radicals and oxidative injury. *Semin Hematol* 1998; **35(1)**: 5–12.
28. Nelson G.J., Morris V. C., Schmidt P. C., Levander O. The urinary excretion of thiobarbituric acid reactive substances and malondialdehyde by normal adult males after consuming a diet containing salmon. *Lipids* 1993; **28(8)**: 757–761.
29. Crosby A. J., Wahle K. W. J., Duthie G. G. Modulation of glutathione peroxidase activity in human vascular endothelial cells by fatty acids and the cytokine interleukin-1-beta. *Biochim Biophys Acta* 1996; **1303**: 187–192.
30. Willumsen N., Vaagenes H., Lie O. et al. Eicosapentaenoic acid, but not docosahexaenoic acid, increases mitochondrial fatty acid oxidation and upregulates 2,4-dienoyl-CoA reductase gene expression in rats. *Lipids* 1996; **31(6)**: 579–592.
31. Stephens N. G., Parsons A., Schofield P. M. et al. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996; **347**; 781–786.
32. Croft K. D., Williams P., Dimmitt S. et al. Oxidation of low-density lipoproteins: effect of antioxidant content, fatty acid composition and intrinsic phospholipase activity on susceptibility to metal-ion-induced oxidation. *Biochim Biophys Acta* 1995; **1254(3)**: 250–256.
33. Lieber D. C., Burr J. A., Philips L., Ham A. J. Gas chomatography-mass spectrometry analysis of vitamin E and its oxidation products. *Anal Biochem* 1996; **236(1)**: 27–34.
34. Dyatlov V. A., Makovetskaia V. V., Leonhardt R. et al. Vitamin E enhances Ca(2+)-mediated vulnerability of immature cerebellar cells to ischemia. *Free Rad Biol Med* 1998; **25(7)**: 793–802.
35. Sangeetha S. S., Das U. N. Cytotoxic action of cis-unsaturated fatty acids on human cervical carcinoma (HeLa) cells in vitro. *Prostaglandins Leukotrienes Essential Fatty Acids* 1995; **53**: 287–299.
36. Ball R. Y., Stowers E. C., Burton J. H. et al. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis* 1995; **114**: 45–54.
37. Allard J. P., Kurian R., Aghdassi E. et al. Lipid peroxidation during n-3 fatty acid and vitamin E supplementation in humans. *Lipids* 1997; **32(5)**: 535–541.
38. Parthasarathy S., Santanaman N., Auge N. Oxidised low-density lipoprotein: a two-faced janus in coronary artery disease? *Biochem Pharmacol* 1998; **56**: 279–284.
39. Mohan I. K., Das U. N. Oxidant stress, anti-oxidants and essential fatty acids in systemic lupus erythematosus. *Prostaglandins* 1997; **56(3)**: 193–198.
40. Fogt F., Nanji A. A. Alterations in nuclear ploidy and cell phase distribution of rat liver cells in experimental alcoholic liver disease: relationship to antioxidant enzyme gene expression. *Toxicol Appl Pharmacol* 1996; **136(1)**: 87–93.
41. Dormandy T. L. In praise of peroxidation. *Lancet* 1988; **8620**: 1126–1128.

