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Effect of eicosapentaenoic acid, an omega-3 polyunsaturated fatty acid, on UVR-related cancer risk in humans. An assessment of early genotoxic markers

Lesley E.Rhodes[1,2,6], Hassan Shahbakhti[1,2], Richard M.Azurdia[2], Ralf M.W.Moison[3], Marie-Jose S.T.Steenwinkel[4], Marie I.Homburg[5], Michael P.Dean[1,2], F.McArdle[2], Gerard M.J.Beijersbergen van Henegouwen[3], Bernd Epe[5] and Arie A.Vink[4]

[1]Photobiology Unit, Dermatology Centre, University of Manchester, Manchester, UK, [2]Department of Dermatology/Medicine, Royal Liverpool University Hospital and University of Liverpool, Liverpool, UK, [3]Department of Medicinal Photochemistry, Leiden/Amsterdam Centre for Drug Research, Leiden University, The Netherlands, [4]TNO Nutrition and Food Research, Zeist, The Netherlands and [5]University of Mainz, Mainz, Germany

[6]To whom correspondence should be addressed
Email: lesley.e.rhodes@man.ac.uk

Dietary omega-3 polyunsaturated fatty acids (ω-3 PUFAs) protect against photocarcinogenesis in animals, but prospective human studies are scarce. The mechanism(s) underlying the photoprotection are uncertain, although ω-3 PUFAs may influence oxidative stress. We examined the effect of supplementation on a range of indicators of ultraviolet radiation (UVR)-induced DNA damage in humans, and assessed effect on basal and post-UVR oxidative status. In a double-blind randomized study, 42 healthy subjects took 4 g daily of purified ω-3 PUFA, eicosapentaenoic acid (EPA), or monounsaturated, oleic acid (OA), for 3 months. EPA was bioavailable; the skin content at 3 months showing an 8-fold rise from baseline, $P < 0.01$. No consistent pattern of alteration in basal and UVR-exposed skin content of the antioxidants glutathione, vitamins E and C or lipid peroxidation, was seen on supplementation. Sunburn sensitivity was reduced on EPA, the UVR-induced erythemal threshold rising from a mean of 36 (SD 10) mJ/cm^2 at baseline to 49 (16) mJ/cm^2 after supplementation, $P < 0.01$. Moreover, UVR-induced skin p53 expression, assessed immunohistochemically at 24 h post-UVR exposure, fell from a mean of 16 (SD 5) positive cells/100 epidermal cells at baseline to 8 (4) after EPA supplementation, $P < 0.01$. Peripheral blood lymphocytes (PBL) sampled on 3 successive days both pre- and post-supplementation, showed no change with respect to basal DNA single-strand breaks or oxidative base modification (8-oxo-dG). However, when susceptibility of PBL to *ex vivo* UVR was examined using the comet assay, this revealed a reduction in tail moment from 84.4 (SD 3.4) at baseline to 69.4 (3.1) after EPA, $P = 0.03$. No significant changes were seen in any of the above parameters following OA supplementation. Reduction in this range of early markers, i.e. sunburn, UVR-induced p53 in skin and strand breaks in PBL, indicate protection by dietary EPA against acute UVR-induced genotoxicity; longer-term supplementation might reduce skin cancer in humans.

Abbreviations: CPD, cyclobutane pyrimidine dimers; EPA, eicosapentaenoic acid; MDA, malondialdehyde; MED, minimal erythemal dose; MM, malignant melanoma; ω-3 PUFAs, omega-3 polyunsaturated fatty acids; ROS, reactive oxygen species; SSB, single-strand breaks; TM, tail moment; UVR, ultraviolet radiation.

Introduction

Skin provides a protective barrier against environmental insults and is the primary target for ultraviolet radiation (UVR) effects. Skin cancer is now the commonest form of cancer in white Caucasian populations, and the incidence continues to rise due to the trend for greater recreational exposure to ambient UVR (1). Basal cell carcinoma (BCC), arising from the basal epidermal layer, is the commonest skin cancer, followed by squamous cell carcinoma (SCC), derived from supra-basal keratinocytes, whereas malignant melanoma (MM), derived from melanocytes, is less common but carries a high mortality rate. UVR is implicated as the main aetiological factor in all three types.

The mechanisms of UVR-induced carcinogenesis have been extensively reviewed (2,3). UVR is a complete carcinogen, capable of the initiation and promotion of cancer, inducing both DNA damage and immunosuppression. DNA may be damaged directly by UVR, or indirectly via UVR induction of free radicals and reactive oxygen species (ROS). While the shorter wavelength ultraviolet-B (UVB, 290–320 nm) typically causes direct damage and ultraviolet-A (UVA, 320–400 nm) causes indirect damage, there is considerable overlap of effects. The commonest DNA lesions caused by direct damage are cyclobutane pyrimidine dimers (CPD), while a range of types of oxidative DNA damage have been observed including single-strand breaks (SSB) and base modifications. When UVR-induced DNA damage is not removed from the genome, this may lead to mutations and cancer development. In normal skin, the transcription factor p53 is a key element in the response to UVR-induced DNA damage, facilitating either repair by regulation of the cell cycle, or destruction of the pre-cancerous cells by apoptosis (4,5). Mutations in the p53 tumour suppressor gene, causally linked to UVR exposure, are a very early event in skin cancer induction (6). The data suggest an important role for p53 mutations in SCC and BCC, as they are present in the majority of these lesions and also in the pre-malignant actinic keratoses, and a smaller association with MM (2,3).

Strategies to protect against UVR-induced skin damage include topical sunscreens (7), but studies that have examined sunscreen application methods by consumers have consistently found these lacking, with insufficient amounts applied and uneven spread (1). A systemic means of protection, particularly a safe dietary method, would therefore have much appeal (7). While dietary agents seem unlikely to be capable of intervening to reduce direct DNA damage by UVR, they could potentially influence DNA damage due to free radicals/ROS. In addition, they might intervene at the promotion stage of photocarcinogenesis by modulating immunosuppression.

Dietary omega-3 polyunsaturated fatty acids (ω-3 PUFAs), principally eicosapentaenoic acid (EPA) and docosahexaenoic acid, extracted from oily fish, show promise as photoprotective agents. Members of the ω-3 and ω-6 PUFA families profoundly influence biological responses (8), mediating these effects both via their membrane-bound forms and as free fatty acids. In many situations, ω-3 PUFAs act as competitive antagonists for ω-6 PUFAs. A large body of evidence now demonstrates the influence of dietary PUFAs on UVR-induced carcinogenesis in animals. A linear relationship exists between ω-6 PUFA intake and photocarcinogenesis in hairless mice (9,10), whereas in contrast, diets rich in ω-3 PUFAs markedly inhibit photocarcinogenesis, with respect to both tumour latency and multiplicity (9,11). In humans, an epidemiological study of $>50\,000$ individuals with cutaneous MM indicated that PUFA intake was associated with a significantly increased risk of MM in women (12), whereas a population-based case-control study suggested that high intake of fish oils and ω-3 PUFAs reduced the risk of MM (13). Prospective human studies with mixed ω-3 PUFAs designed to examine the effect on the clinical sunburn response, have shown promising results. Significant rises were found in the skin's threshold to sunburn in a short-term study in healthy humans (14), and in uncontrolled studies in patients with the common photosensitivity disorder, polymorphic light eruption (15,16). As the UVR action spectrum for the sunburn response is very similar to that for photocarcinogenesis, this might indicate the potential for ω-3 PUFAs to protect against UVR-induced cancers in humans (17).

Whereas some studies in animal models report that ω-3 PUFAs exert protection against skin cancer at the promotion stage (18,19), there is also evidence that they can protect against carcinogenesis during initiation (9,20), although the mechanism is unknown. ω-3 PUFAs reportedly modulate oxidative stress, although the effects appear variable. The highly unsaturated ω-3 PUFAs may become targets for free-radical attack, resulting in the production of oxidation products. Seemingly paradoxically, they protect against oxidative injury in some models (21). It has been speculated that the susceptibility of these unstable fatty acids to damage may confer protection of other cellular structures from free-radical attack (15,22).

The aim of the current study was to examine the potential for dietary photoprotection by a purified ω-3 PUFA, EPA, in humans in a double-blind randomized trial. Bioavailability in skin was confirmed, the influence of supplementation on the skin's basal and UVR-modulated oxidative status was assessed, and the effects on a range of markers of UVR-induced carcinogenesis were examined. Parameters studied comprised the sunburn response, UVR-induced p53 expression in skin, and oxidative DNA modifications and UVR-induced SSB in peripheral blood lymphocytes (PBL), all of which might be modulated by an alteration in oxidative status. In addition, CPD expression, an indicator of direct DNA damage, was assessed in skin following UVR exposure.

Materials and methods

Subjects and study design

The study was performed according to the Declaration of Helsinki (Edinburgh, UK, 2000). Approval was granted by the Local Research Ethics Committee, and written informed consent was obtained from each volunteer. The subjects were 42 healthy white Caucasians, median age 44 years (range 21–65 years), 22 female, non-smokers and sun-reactive skin type II or III. Exclusion criteria were systemic medication or supplementation, photosensitivity and sunbathing or sunbed exposure in the previous 6 months. The study design was double-blind, randomized and controlled. Randomization to purified EPA or purified oleic acid (OA) was performed within the pharmacy department, with subjects randomized at the outset into six subgroups of $n = 7$ for examination of a range of different parameters, due to ethical limitations for tissue sampling in humans. Subjects continued with their usual diets throughout the study. Volunteers underwent phototesting and skin and blood sampling at the beginning and end of the 3-month supplementation period.

Dietary supplements

The ω-3 PUFA supplement was purified EPA ethyl ester (95% EPA, C20:5, other ω-3s 4%, ω-6s <1%) taken as 8× 0.5 g capsules daily; the control was ethyl esters of monounsaturated fatty acids (95% OA, <1% ω-3s), taken in 8× 0.5 g capsules of identical appearance. Both supplements contained 0.0015% (wt/wt) butylhydroxyanisole as an antioxidant. Supplements were provided by Croda Oleochemicals (Goole, UK) and encapsulated by R.P.Scherer (Swindon, UK).

Phototesting

The UVR source used was a Philips TL12 fluorescent broadband UVR lamp (range 270–400 nm, peak 311 nm). The irradiance at the skin surface was 30 mW/cm^2 (IL1400 radiometer, International Light, Boston, MA). Doses given were erythemally weighted UVR. The erythemal (sunburn) sensitivity of the volunteers' skin was assessed at the beginning and end of supplementation by the same investigator. Geometric series of UVR doses (1 cm diameter) were applied in a horizontal row to the skin of the upper buttock. At 24 h, sites were assessed visually to determine the minimal erythemal dose (MED), i.e. the lowest dose of UVR that produced a perceptible erythema. The individual's MED at baseline was used for calculation of the UVR doses (all 1 cm diameter) to be administered throughout the study.

Skin sampling

Samples were taken from buttock skin. This comprised both unexposed skin and skin at 1 and 24 h following 2× MED of UVR (Philips TL12 lamp), i.e. a measured dose sufficient to induce mild-moderate sunburn. Skin samples were taken under local anaesthesia (2% lignocaine without adrenaline) as 5 mm 'punch' biopsies. Samples for immunostaining were fixed in buffered formaldehyde and subsequently transferred to a 70% ethanol solution and embedded in paraffin. Before immunostaining (p53, CPD) sections (5 μm) were deparaffinized; slides were boiled for 10 min in 10 mM citrate buffer (pH 6.0) and rinsed with PBS (×2). Skin samples for analysis of antioxidant parameters were snap-frozen at −70°C.

Blood sampling

Blood (20 ml) samples were taken from the antecubital fossa at the same time each morning on 3 consecutive days, after a 30 min resting period. Subjects were all non-smokers, due to the potent effect of smoking on the comet assay (23), and samples were taken during the winter months (November to February) to avoid possible seasonal influences (24). They were instructed not to drink alcohol and to undergo minimal exertion on the preceding evening and morning of the visit.

Bioavailability of EPA

Whole skin (epidermis + dermis) was analysed from samples taken from unexposed buttock skin and skin at 1 h following 2× MED of UVR. Fatty acids (including EPA and OA) were hydrolysed and derivatized to methyl esters and measured by gas chromatography (25).

Antioxidant status and lipid peroxidation

Whole skin (epidermis + dermis) was analysed for content of vitamin E, vitamin C and glutathione in samples from unexposed skin and skin at 1 h following UVR (2× MED). For measurement of vitamin E, weighed aliquots of skin sample were extracted for 20 h using acetonitrile with 0.5 g/l butylated hydroxytoluene as an antioxidant; vitamin E was then analysed using HPLC and fluorometric detection (26). For assessment of vitamin C, 150 μl of 5% (w/v) meta-phosphoric acid containing 1 mM desferrioxamine was added to an aliquot of skin sample. Analysis of total and oxidized vitamin C was performed using HPLC and fluorometric detection (27,28). For measurement of total (reduced + oxidized) glutathione, 175 μl of 3% (v/v) perchloric acid was added to an aliquot of skin sample, whereas for the measurement of oxidized glutathione, 3% perchloric acid containing 10 mM *N*-ethylmaleimide was used. Spectrophotometric analysis was based on a glutathione reductase-mediated recycling assay (29). Lipid peroxidation was assessed by measuring malondialdehyde (MDA) by HPLC, using fluorometric detection (30).

P53 expression in skin

Paraffin sections (see skin sampling section) were assessed for p53 immunostaining. The endogenous peroxidase activity was removed by treatment with

0.3% hydrogen peroxide in 70% methanol for 10 min. Sections were blocked with 10% normal rabbit serum for 20 min at room temperature before treatment with the primary antibody (mouse anti-p53 IgG, BP53-12-1, BioGenex, San Ramon, CA) overnight at 4°C. Finally, sections were incubated with biotin–streptavidin HRP–DAB complex (rabbit anti-goat, ITK, Amsterdam, The Netherlands) and counterstained with haematoxylin. Sections were assessed by two blinded observers; for every sample at least two sections were assessed, and the number of p53-positive cells/100 epidermal cells recorded.

Quantification of CPDs in skin

A mouse monoclonal anti-CPD antibody (H3; IgG1-lambda subclass) and goat anti-mouse IgG fluorescein-labelled secondary antibodies were used for CPD immunostaining of paraffin skin sections (see skin sampling section). The antibody was developed against cyclobutane thymine dimers in single-stranded DNA (31) and has high affinity for 5′-T-containing dimers (32). Nuclei of skin cells were counterstained with propidium iodide. Nuclear green fluorescence in the epidermal cells proportional to the level of CPDs was assessed with a scanning laser microscope (Zeiss LSM-41) by using image processing and image analysis (33).

Analysis of basal oxidative DNA damage in peripheral blood lymphocytes

Catalase was added to whole blood in order to prevent artefactual oxidation, to obtain a final concentration of 315 U/ml. Lymphocytes were isolated by centrifugation with Histopaque-1077 separation medium. They were re-suspended in freezing medium [FCS 90% and DMSO 10% (v/v)] to obtain a final concentration of 2.0–2.5 $\times 10^6$ cells/ml, and stored at -70°C. These unirradiated PBL were analysed for DNA single-strand breaks and oxidative purine modification sensitive to the repair glycosylate Fpg protein using a modified elution assay, as described previously (34).

Comet assay (single cell gel electrophoresis) in peripheral blood lymphocytes

The comet assay was conducted in duplicate, on three samples taken from each volunteer pre- and post-supplementation. Procedures were performed on ice and, where possible, in protective containers to avoid ambient UVR. Lymphocytes were isolated from whole blood by centrifugation with Histopaque-1077 separation medium (Sigma, Poole, UK), and suspended in Ham's medium without phenol red (Gibco BRL, Paisley, UK). The alkaline comet assay was performed on unirradiated cells and cells irradiated *in vitro* with 15 and 30 mJ/cm^2 UVR (Philips TL12 source). Cells were assessed for viability by trypan blue exclusion. Following UVR-irradiation or mock irradiation, cells were incubated for 80 min at 37°C, before returning to ice. This incubation period was chosen as it has been demonstrated to be optimal for augmentation of measurable DNA damage (35). The comet assay was performed using a modified method of Singh (36). In brief, cells were embedded in soft agar on frosted microscope slides, which were then placed in a high salt lysis mixture. Following lysis, cells were transferred to alkaline buffer (pH 12), and the nuclei electrophoresed (20 V/m for 24 min). Slides were stained with ethidium bromide, and DNA damage was assessed as the tail moment (TM), i.e. a product of the percentage of DNA in the tail and the tail length. One hundred cells in six fields were scored using image analysis (Optomax V image analyser, Synoptics, Cambridge, UK).

Statistical analysis

Comparison of results before and after supplementation and before and after UVR exposure was performed using the Mann–Whitney *U* test; comparison of results from the two supplementation groups was performed using the Wilcoxon signed ranks test. *P* values <0.05 were regarded as significant.

Results

Tolerance of supplements

Supplements were well tolerated by all subjects. No side-effects were experienced other than increased flatulence.

Bioavailability of EPA

Skin content of EPA and OA, and ratio of total ω-3/ω-6 fatty acids, was determined before and after 3 months dietary EPA or OA supplementation, both in skin exposed to 2× MED of UVR and unexposed skin (Table I, Figure 1). Pre-supplementation values did not differ between subjects subsequently supplemented with EPA or OA, and therefore these values are presented as originating from one group of subjects. Skin fatty acid analysis ($n = 14$) revealed an 8-fold rise in actual EPA content in the EPA-supplemented group after

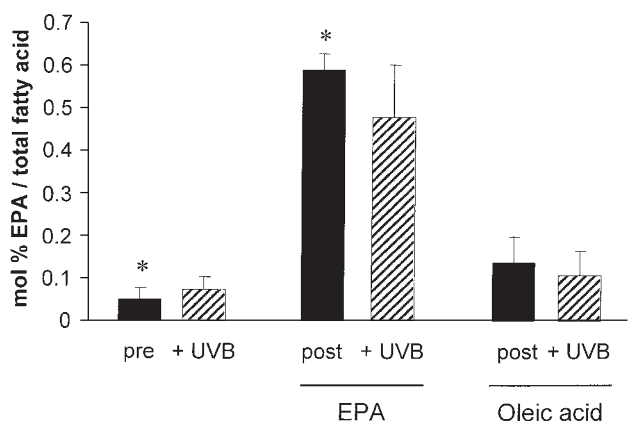

Fig. 1. The EPA content of skin, in mol% total fatty acids, increases after 3 months supplementation with EPA, but not OA, $^*P < 0.01$. Solid columns are unexposed skin, hatched columns are skin at 1 h following exposure to UVR (2× MED). Data shown are mean ± SE; $n = 7$ in each supplementation group.

Table I. Levels (mean ± SE) of EPA, OA, ratio of total ω-3/ω-6 fatty acids, antioxidants and lipid peroxidation in skin before and after 3 months EPA or OA supplementation, in unexposed skin and skin at 1 h post-UVR

	Pre-supplementation ($n = 14$)		Post-EPA supplementation ($n = 7$)		Post-OA supplementation ($n = 7$)	
	Unexposed	UVR	Unexposed	UVR	Unexposed	UVR
EPA (mol%)	0.05 ± 0.02	0.07 ± 0.03	0.59 ± 0.04**‡	0.48 ± 0.12	0.14 ± 0.06	0.13 ± 0.06
OA (mol%)	35.2 ± 1.8	34.3 ± 2.3	31.7 ± 2.6	31.0 ± 2.6	31.6 ± 2.9	27.0 ± 3.4
Total ω-3/ω-6	0.10 ± 0.01	0.10 ± 0.01	0.19 ± 0.01**‡	0.19 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
Vitamin E[a]	35.3 ± 3.8	46.6 ± 4.3	25.6 ± 4.2	26.2 ± 3.6*	30.0 ± 4.7	31.0 ± 8.4
Red. vitamin C[a]	132 ± 21	118 ± 18	81 ± 28	106 ± 25	170 ± 46	122 ± 42
Oxid. vitamin C (%)	21 ± 3	30 ± 8	56 ± 14*†	40 ± 14	19 ± 5	40 ± 14
Red. glutathione[a]	514 ± 50	538 ± 88	517 ± 146	418 ± 102	520 ± 56	447 ± 65
Oxid. glutathione (%)	7.9 ± 1.6	15.1 ± 4.9	6.0 ± 2.3	6.7 ± 3.8	5.9 ± 2.9	2.8 ± 0.8
Lipid peroxidation[b]	1.6 ± 0.4	2.2 ± 0.9	1.9 ± 0.5	2.6 ± 0.5	2.5 ± 1.1	2.9 ± 1.1

[a]Expressed as pmol/mg.
[b]Expressed as pmol malondialdehyde/mg.
$^*P < 0.05$, $^{**}P < 0.01$ compared to pre supplementation.
$^†P < 0.05$, $^‡P < 0.01$ compared with OA supplementation after same treatment.

3 months, $P < 0.01$, accompanied by a doubling of the ratio of total ω-3/ω-6 fatty acids, $P < 0.01$. In contrast, OA supplementation did not influence EPA, or OA, skin levels. UVR-exposure did not induce significant changes in either group.

Antioxidant status and lipid peroxidation in skin

Pre-supplementation values did not differ between subjects later supplemented with EPA or OA ($n = 14$), and therefore these are presented as originating from one group of subjects (Table I). The mild-moderate dose of UVR (2× MED) did not induce significant changes in antioxidant status or lipid peroxidation (indicated by MDA levels) in comparison with unexposed skin, although the increase in % oxidized vitamin C (+43%), % oxidized glutathione (+91%) and MDA (+38%), suggested a tendency for increase in oxidative stress.

No significant change occurred in MDA or glutathione levels after EPA or OA supplementation. MDA showed a tendency to increase following supplementation with both OA (+79% in unexposed skin, +16% in UVR-exposed skin) and EPA (+12% in unexposed skin, +37% in UVR-exposed skin). In the EPA-supplemented group, the % oxidized vitamin C in unexposed skin increased significantly, from 21 (SE3) at baseline to 56 (14)% after 3 months, $P < 0.05$ versus baseline and OA-supplementation. Additionally in the EPA-supplemented group, vitamin E content in UVR-exposed skin fell from a mean of 46.6 (SE4.3) mol/mg at baseline to 26.2 (3.6) mol/mg at 3 months, $P < 0.05$ versus baseline, but there was no significant difference between the EPA and OA groups. Overall, the changes were slight, and no consistent difference in pattern was observed between the active and control supplementation groups.

UVR-induced erythemal response

UVR skin testing ($n = 28$) showed that threshold to sunburn increased in those supplemented with EPA, whereas there was no change in the OA-supplemented subjects (Figure 2). The MED increased from a mean of 36 (SD 10) mJ/cm^2 at baseline to 49 (16) mJ/cm^2 after EPA ($n = 14$), $P < 0.01$, whereas the MED was 35 (11) and 39 (18) mJ/cm^2, respectively, pre and post 3 months supplementation with OA ($n = 14$).

p53 expression in skin

p53 expression was assessed immunohistochemically ($n = 14$). In unexposed skin and 1 h following UVR exposure, p53 was below the level of detection, but at 24 h following UVR-exposure p53 was induced in all subjects. After 3 months, there was a pronounced reduction in UVR-induced p53 expression in the EPA supplement group, from a mean of 16 (SD 5) at baseline to 8 (4) positive cells/100 epidermal cells post-supplementation, $P < 0.01$ (Figure 3). No change was seen in the OA group, with 20 (3) and 22 (6) positive cells/100 epidermal cells pre- and post-supplement, respectively.

CPD induction and repair in skin

Analysis of CPDs was performed in samples of unexposed skin, and skin at 1 and 24 h following UVR ($n = 14$). Fluorescence was expressed in arbitrary units and considered CPD-specific after subtraction of fluorescence in non-exposed skin. In all subjects, exposure to 2× MED UVR resulted in a significant increase in fluorescence compared with the non-irradiated control. CPD induction at 1 h post-UVR was a mean (SD) of 43 (17.9) arbitrary units pre-EPA and 61.9 (SD 22.5) post-EPA ($n = 7$), whereas values of 32.1 (SD 13.5) and 42 (28.8) were seen pre- and post-OA ($n = 7$), respectively, no significant difference. CPD repair was signified by % CPD remaining at 24 h post-UVR. This was highly variable between individuals, ranging from 60% to no repair, with no significant difference on supplementation.

Basal oxidative DNA modifications in PBL

Blood samples were taken in triplicate both pre- and post-supplementation and PBL extracted ($n = 14$). No significant difference was seen in basal (non-UVR exposed) PBL pre- and post-supplementation in either the EPA or OA group, with respect to the background levels of SSB and oxidative DNA base modification sensitive to the repair glycosylate Fpg protein, including 8-hydroxy-deoxyguanosine (8-oxodG). Explicitly, the mean level of Fpg-sensitive modifications in the EPA group was 0.219 (SD 0.045) per 10^6 bp before and 0.206 (0.046) per 10^6 bp after supplementation ($n = 7$), whereas in the OA group the pre- and post-supplementation values were 0.270 (0.044) per 10^6 bp and 0.246 (0.065) per 10^6 bp, respectively ($n = 7$). Mean levels of SSB pre- and post-EPA supplementation were 0.088 (SD 0.023) per 10^6 and 0.075 (SD 0.013) per 10^6 bp, respectively ($n = 7$), whereas pre- and post-OA values were 0.081 (SD 0.018) per 10^6 bp and 0.091 (SD 0.026) per 10^6 bp, respectively ($n = 7$).

Comet assay for DNA damage in peripheral blood lymphocytes

Blood samples were taken in triplicate both pre- and post-supplementation and PBL extracted ($n = 14$). A reproducible

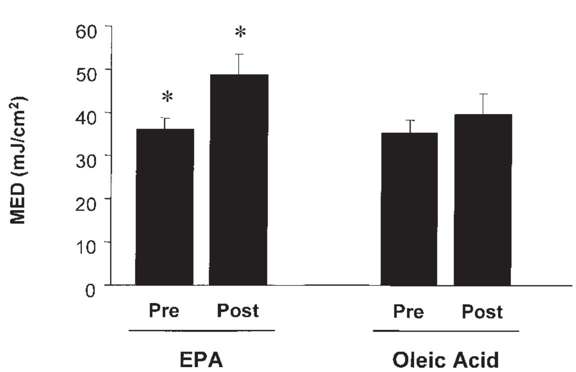

Fig. 2. The erythemal threshold to UVR (minimal erythema dose, MED, in mJ/cm^2) rises after 3 months dietary supplementation with EPA, but not OA, $^*P < 0.01$. Data shown are mean ± SE; $n = 14$ in each supplementation group.

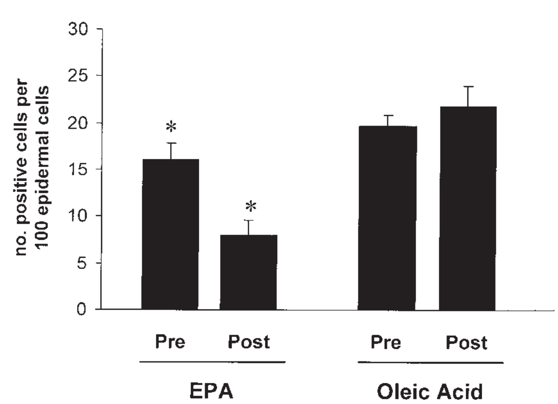

Fig. 3. UVR-induced p53 expression in skin is reduced after 3 months supplementation with EPA but not OA, $^*P < 0.01$. Data shown are mean ± SD; $n = 7$ in each supplementation group.

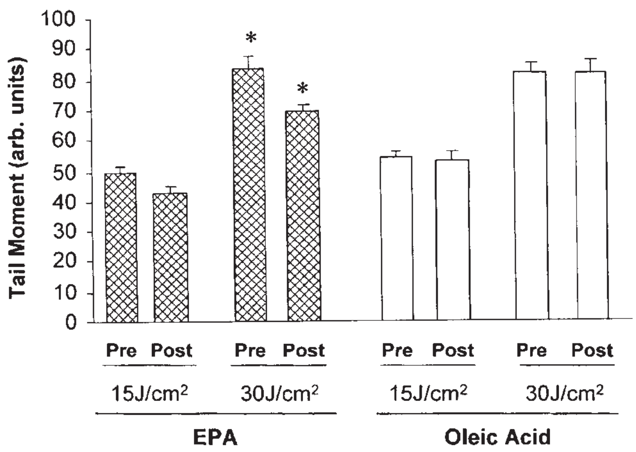

Fig. 4. The comet assay in UVR-exposed PBL before and after dietary EPA and OA supplementation. Tail moment due to *ex vivo* UVR is reduced after supplementation with EPA (shaded columns), but not OA (open columns), $^{*}P < 0.05$ for the 30 mJ/cm^2 UVR dose. Data are mean ± SD; $n = 7$ in each supplementation group. Results are the mean of three samples taken on consecutive days in each subject.

dose–response in the TM of comets was observed with increasing dose of *ex vivo* UVR. No significant change was seen in the TM of non-UVR exposed lymphocytes after supplementation with EPA or OA, or in UVR-exposed lymphocytes after OA. However, the TM of UVR-irradiated cells was reduced following EPA supplementation; at 15 mJ/cm^2 the UVR-induced TM was reduced from a mean of 49.9 (SD 2.2) arbitrary units pre-supplement to 42.9 (1.9) after EPA, $P = 0.07$, whereas at 30 mJ/cm^2 the TM was reduced from 84.4 (3.4) to 69.4 (3.1), $P = 0.03$ (Figure 4).

Discussion

In this double-blind randomized study, it has been demonstrated that the ω-3 PUFA, EPA, conveys significant protection against UVR-induced erythema in humans. EPA was incorporated into the skin, where it protected not only against the clinical sunburn response but also against UVR-induced p53 expression, frequently interpreted as a biomarker of DNA damage. The protection against p53 induction was greater than the protection against erythema, supporting p53 as the more sensitive indicator of skin damage. No change was seen in these parameters in the OA-supplemented subjects. Protection was also observed against *ex vivo* UVR-induced strand breaks (or alkali-labile sites) in extracted PBL in the single cell gel electrophoresis (comet) assay. Significant reduction in tail moment was seen following EPA supplementation *in vivo*, compared with no effect in the control group. As anticipated, there was no evidence of protection by EPA against direct DNA damage in skin, i.e. CPDs. Hence, EPA supplements caused a series of events resulting in protection against UVR-induced erythema and p53 induction in skin, and reduced UVR-induced strand breaks in PBL. All three effects might indicate protection against free-radical mediated mutagenesis by the ω-3 fatty acid.

The tumour suppressor gene, p53, is induced in response to DNA damage and facilitates repair (37). The observed reduction in UVR-induced p53 expression in skin following dietary EPA is therefore anticipated to reflect the presence of less oxidative DNA damage. We were unable to assess immunohistochemically for direct evidence of reduced oxidative DNA damage in skin. As many tumours are associated with loss of normal p53 function, it is important to consider that a negative consequence of reduction in p53 might be reduced removal of DNA damage and consequently reduced skin protection. However, the reduced UVR-induced p53 expression was consistent with other evidence of skin photoprotection, i.e. a significant reduction of the sunburn response.

We also examined the effects of EPA on PBL, both as a surrogate for UVR-induced DNA damage in epidermal cells, and to assess for evidence of systemic protection by EPA. PBL are known to be particularly sensitive to UVR, which is attributed to their unusually low nucleoside pools, limiting the rejoining step and resulting in a higher frequency of measurable repair-associated DNA strand breaks (38). In our studies, blood was repeatedly sampled pre- and post-supplementation and PBL extracted. Basal levels of oxidative DNA base modification damage including 8-oxodG were measured by a modified alkaline elution assay (34), whereas UVR-induced DNA damage (SSB/alkali-labile sites) was assessed using single-cell alkaline gel electrophoresis, following *ex vivo* irradiation. While no effect of supplementation was observed on basal oxidative DNA damage, there was significant protection against UVR-induced SSB. The latter were measured after an incubation of 80 min following UVR, which was chosen since this is the optimal time-point for the augmentation of measurable damage (35). Hence, a broad indicator of DNA damage was assessed, and the measured strand breaks could reflect primary single-strand DNA, which has not undergone ligation due to the low nucleotide pool, or reduced incision or increased activity of polymerase or ligase at the repair stage. Having demonstrated a significant effect of dietary EPA, further studies are now indicated, including detailed examination of other time points following UVR and use of restriction enzymes, to fully assess the influence of EPA on UVR-induced DNA damage.

The changes we observed in oxidative parameters in skin following dietary supplementation were in the main small and inconsistent. After EPA, a fall in vitamin E level occurred in UVR-exposed skin alone and a fall in % oxidized vitamin C only in unexposed tissue, whereas there were no significant changes in glutathione or level of lipid peroxidation in either unexposed or UVR-exposed skin. The reported effects of ω-3 PUFA supplements on oxidative parameters in a range of animal and human tissues have been quite variable, possibly due to differences in vitamin E content of supplement, concentration of ω-3 PUFAs, and lipid peroxidation markers examined (21). In our previous uncontrolled *in vivo* study in UVR-exposed human skin, we found a significant rise in another indicator of lipid peroxidation, thiobarbituric acid reactive substances, after supplemental mixed ω-3 PUFAs containing a low amount of vitamin E to prevent *in vitro* oxidation (15). Differences in protocol that may have influenced the measured effects on lipid peroxidation are that the skin samples used for assessment of this parameter in the earlier study were predominantly epidermal, as opposed to whole epidermis and dermis, and that a high dose of UVR was employed previously, whereas in the current study a dose was selected that would produce a mild-moderate sunburn response. Whilst there is no consistent evidence of increased oxidative stress in skin following purified EPA supplements in this study, it is also uncertain whether the ω-3 PUFAs can be capable of acting as a free-radical buffer (15,22). There are several reports of ω-3 PUFA enhancement of tissue protection against oxidative injury, despite their susceptibility to peroxidation (21,39,40). It has therefore been proposed that the highly unstable fatty acids are preferentially damaged by free radicals, sparing more vital structures from attack (15,22). Another potential mechanism is a reduction in ROS generated during arachidonic acid metabolism (21). UVR triggers arachidonic acid release from cell membranes, and as the arachidonic acid cascade itself generates ROS, its modulation by ω-3 PUFAs, both by competition for release from cell membranes and by competition of the free fatty acids, could reduce the excessive UVR-induced ROS production.

ω-3 fatty acids are increasingly recognized to have a wide range of anti-inflammatory and immunomodulatory functions, related both to their membrane-bound and free forms. They are well described to compete with arachidonic acid, an ω-6 PUFA, for metabolism by cycloxygenase and lipoxygenase (8). This results in the formation of the less active eicosanoids, including prostanoids and leukotrienes, of the three series. In particular, it has been reported previously that dietary ω-3 fatty acids reduce UVR-generated prostaglandin E_2 (PGE_2) in human skin (16), as well as reducing PGE_2 in animal tissue including skin (41). As this pivotal mediator plays a major role in the sunburn response (42) and is an immunomodulator involved in cancer promotion and progression (43), PGE_2 appears likely to contribute to aspects of the photoprotection conveyed by ω-3 PUFAs. Moreover, alteration in PUFA content influences membrane fluidity (44) and could be accountable for modification of a range of signalling events that might impact on UVR-induced responses (45).

Interestingly, it has been proposed that PBL, in view of their exquisite UVR-sensitivity, might be directly damaged by ambient UVR exposure during their passage through the skin microvasculature, with resultant suppression of T lymphocytes (35). This is supported by the observation of a significant association of strand breaks and DNA repair in peripheral mononuclear cells with ambient UVR levels (24), the summer months (23) and sunny holidays abroad (46). This raises the intriguing possibility that improved immunosurveillance on EPA could contribute to the observed protection by ω-3 PUFAs against promotion of skin cancer in animal models (19). This is in addition to their observed, though presently poorly understood, protection against cancer initiation (9,20). Clearly, ω-3 PUFAs are complex agents with many activities potentially conveying photoprotection; these require further exploration.

Dietary photoprotection by ω-3 PUFAs is a promising and novel area for research. This original, prospective study supports animal and epidemiological data associating ingestion of ω-3 PUFAs with protection against harmful UVR effects, principally skin cancers. In healthy humans, there is evidence of protection by dietary EPA against a range of early genotoxic markers, i.e. sunburn, p53 induction in skin and strand breaks in PBL. Even a modest reduction in these acute UVR-effects could translate to a major impact on skin cancer induction in the human population, given long-term dietary modification (47).

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