# Cell proliferation, apoptosis and accumulation of lipid droplets in U937-1 cells incubated with eicosapentaenoic acid

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The monocytic cell line U937-1 was cultured in the presence of eicosapentaenoic acid (20:5, $n-3$) (EPA) or oleic acid (18:1, $n-9$) (OA). EPA caused a dose-dependent inhibition of cell proliferation, whereas OA had no effect. At the highest EPA concentrations, 120 and 240 $\mu$M, inhibition of cell proliferation was accompanied by initiation of apoptosis. A concentration of 60 $\mu$M EPA caused a 35% reduction in cell proliferation without inducing apoptosis, and was therefore used for further studies. Addition of antioxidants or inhibitors of eicosanoid synthesis had no influence on the reduced cell proliferation after EPA treatment. The inhibition required continuous presence of EPA in the incubation medium as the cells resumed a normal proliferation rate when they were placed in EPA-free medium. The inhibition of proliferation was not accompanied by differentiation into macrophage-like cells, as expression of serglycin and the ability to perform respiratory burst was unaffected by EPA. Expression of CD23 mRNA increased when the cells were incubated with EPA, but to a smaller extent than after retinoic acid (RA) or PMA treatment. Furthermore, expression of the monocytic differentiation markers CD36 and CD68 was lower in cells treated with EPA or OA when compared with untreated cells. The cell cycle distribution of U937-1 cells was similar in cells incubated with EPA or PMA, whereas RA-treated cells accumulated in the $G_1$ phase. Side scatter increased in cells incubated with EPA and OA, which was ascribed to an accumulation of lipid droplets after examination of the cells by electron microscopy. The number of droplets per cell was higher in cells exposed to EPA than OA. The cellular triacylglycerol (TAG) increased 5.5- and 15.5-fold after incubation with OA and EPA respectively. No difference in the cellular content of cholesterol compared with untreated cells was observed. The TAG fraction in EPA-treated cells contained high amounts of EPA and docosapentaenoic acid and minor amounts of docosahexaenoic acid, whereas OA-treated cells had high levels of OA in the TAG. In cells incubated with a sulphur-substituted EPA, only minor effects on cell proliferation and no accumulation of cellular TAG were observed. These findings may indicate the existence of other mechanisms for regulation of cell behaviour by very-long-chain polyunsaturated $n-3$ fatty acids than the well established lipid peroxide and eicosanoid pathways.

## INTRODUCTION

Most studies on the effects of very-long-chain polyunsaturated $\omega-3$ ($n-3$) fatty acids (PUFA) on humans have to a large extent focused on cardiovascular diseases. Diets rich in $n-3$ fatty acids may reduce the incidence of ischaemic heart disease in populations such as Greenland Eskimos and Japanese living in coastal areas [1,2]. Over the past years, atherosclerosis has been recognized as a chronic inflammatory disease [3], and it is possible that some of the beneficial effects of diets rich in $n-3$ fatty acids on this disease may be ascribed to modulatory effects on cells in the immune system. The effects of $n-3$ fatty acids on chronic inflammatory diseases are still somewhat contradictory. No improvement was observed in patients with psoriasis taking $n-3$ rich capsules [4] but some improvement occurred in patients with atopic dermatitis [5] and rheumatoid arthritis [6].

To gain further insight into the effects of $n-3$ fatty acids on cells from the immune system, several studies have been focused on human leucocytes [7]. The proliferation of freshly isolated T-lymphocytes decreased when the cells were cultured in the presence of PUFA. This inhibition was dependent on the presence of antigen-presenting cells, either monocytes or Langerhans cells. When cells in the monocyte–macrophage lineage were exposed to PUFA, they mediated inhibition of proliferation of mitogen-stimulated T-cells [7]. A reduced proliferation of smooth muscle cells has been demonstrated in co-cultures with macrophages exposed to PUFA, compared with cells cultured with untreated macrophages [8]. It is therefore reasonable to assume that functional properties of cells in the monocyte–macrophage lineage as well as the T-lineage, may be altered by exposure to PUFA. This may have implications for several processes where monocytes and macrophages play important roles. First, the conversion of monocytes to macrophages and foam cells is an important step in the formation of atherosclerotic plaques [9]. Secondly, monocytes and macrophages are cells of prime importance both in inflammatory conditions and autoimmune diseases. Thirdly, circulating monocytes are derived from stem cells through complex differentiation processes in the bone marrow. A further understanding of the effects of nutrients on cells in this lineage, at the cellular and molecular level, would accordingly increase our understanding of potential dietary effects on the immune system and haematopoietic cell differentiation.

In a recent study, it was found that arachidonic acid (20:4, $n-6$) (AA), eicosapentaenoic acid (20:5, $n-3$) (EPA) and docosahexaenoic acid (22:6, $n-3$) (DHA) reduced the multiplication of U937-1 cells [10]. The aim of the present study was to use this monocytic cell line as a model for differentiation in the

Abbreviations used: EPA, eicosapentaenoic acid; OA, oleic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; AA, arachidonic acid; RA, retinoic acid; TAG, triacylglycerol; PUFA, very-long-chain polyunsaturated fatty acids; PI, propidium iodide; HO342, Hoechst 33342.

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monocyte–macrophage lineage and investigate the effects of EPA on these important immune cells in further detail. A concentration of 60 $\mu$M EPA caused lower cell proliferation without affecting cell viability or promoting cell differentiation. At higher concentrations of EPA, apoptosis was observed in U937-1 cells. The presence of antioxidants or eicosanoid synthesis inhibitors did not counteract the effects of EPA. Furthermore, cells exposed to EPA accumulated triacylglycerol (TAG) in a reversible manner, whereas a sulphur analogue of EPA did not affect TAG accumulation, inhibition of cell proliferation or induction of apoptosis.

## MATERIALS AND METHODS

Oleic acid (18:1, $n-9$) (OA), EPA, PMA, BSA (essentially fatty acid free), 2,7-dichlorofluorescein, butylated hydroxytoluene, propidium iodide (PI), Hoechst 33342 (HO342), indomethacin, nordihydroguaretic acid, vitamin C and potassium ferrocyanide were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). All-*trans* retinoic acid (RA), 2,2-dimethoxypropane, vitamin E and Epon were obtained from Fluka (Buchs, Switzerland). $OsO_4$ was from Chemi-Teknik (Oslo, Norway) and uranylacetate from Kebo-Lab (Oslo, Norway). [$^3$H]thymidine and [$\alpha^{32}$P]dCTP were purchased from DuPont–NEN Research Products (Stockholm, Sweden). Flow cytometric analyses of TUNEL-labelled cells, cell cycle distributions, light scatter characteristics and CD36 and CD68 expression were done using a FACScan flow cytometer (Beckton Dickinson, San José, CA, U.S.A.). Permeabilization Solution (10×) and IgG–FITC antibody were from the same supplier. FITC-labelled mouse anti-human CD68 antibody (clone KP1) was from DAKO (Glostrup, Denmark) and the CD36 antibody (clone FA6-152) was from Immunotech (Marseilles, France). The 0.24–9.5 kb RNA molecular mass markers were from Gibco-BRL (Paisley, Scotland, U.K.) and oligo(dT)$_{25}$ Dynabeads from Dynal A/S (Oslo, Norway). The open reading frame of serglycin was obtained via PCR from a U937 library [11]. The probe for the human $\beta$-actin gene was from Clontech (Palo Alto, CA, U.S.A.). A *Pst*I–*Bam*HI fragment of Fc($\epsilon$)RII cDNA was used as probe for CD23 [12]. Probes were labelled with Megaprime DNA labelling system 1606 (Amersham, Little Chalfont, Bucks., U.K.). The *In Situ* Cell Detection kit and fluorescein was purchased from Boehringer Mannheim (Mannheim, Germany). Cellular TAG and cholesterol content was determined enzymically using reagents supplied by BioMérieux (Lyon, France). Triheptadecanoin was from Larodan (Malmö, Sweden). Cellular protein content was determined by the bicinchoninic assay (Pierce, Rockford, IL, U.S.A.). Other chemicals used were of analytical grade and were from commercial suppliers.

### Cells

U937-clone 1 was derived *in vitro* from the less mature U937-GTB cell line, a lymphoma cell line with monoblastic characteristics [13]. The cells were cultured in RPMI medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, L-glutamine (2 mM) and gentamycin (0.1 mg/ml), all purchased from BioWhittaker (Verviers, Belgium). Cells were incubated with fatty acids complexed to fatty-acid-free BSA at a molar ratio of 2.5:1. RA, PMA, vitamin E, nordihydroguaretic acid and indomethacin were dissolved in ethanol to give a final concentration of 0.1% (v/v) ethanol in the cell cultures. Control experiments showed that this concentration of ethanol did not affect cell number or cell viability. The cells were maintained in a logarithmic growth phase at densities of $0.2\times10^6$ to $1.2\times10^6$ cells/ml. For experimental purposes, the cells were normally seeded at a density of $0.3\times10^6$ cells/ml. Cell counting experiments were performed in duplicate (Coulter Z1, Coulter Electronics Ltd., Luton, Beds., U.K.) and each experiment was repeated three times. Cells incubated in fatty-acid-free medium were used as controls in the cell counting experiments and the numbers of control cells were taken as 100%, relative to cell proliferation in experimental cells.

### Microscopy

For microscopic analyses of cell viability [14], 1 ml of the respective cell cultures was first incubated with 10 $\mu$l of PI (0.5 mg/ml) in the dark for 30 min. Thereafter, 10 $\mu$l of HO342 (1 mg/ml) was added to each culture and the cells were incubated for a further 30 min in the dark. The cells were then centrifuged at 514 ***g*** for 5 min in a swing-out rotor. The cell pellet was resuspended in PBS, recentrifuged and the cells were then suspended in 10 $\mu$l of fetal-bovine serum. A drop of each cell suspension was smeared on to a microscope slide and dried. The smears were analysed in a Leitz Ortholux II fluorescence microscope (Leica, Wetzlar, Germany) and at least 200 cells were counted on each slide. PI and HO342 associates with DNA and emits red and blue light respectively after UV light treatment. The fluorescence from both PI and HO342 after UV light exposure was detected using an A filter in the microscope. Cells were photographed with Fujichrome Provia 400 professional colour reversal film with a MPS 48/52 camera (Leica). The film was exposed as 800 ASA but developed as 400 ASA. Cells subjected to electron microscopy [15] were prefixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer at 4 °C overnight and postfixed in 2% (w/v) $OsO_4$ in 1.5% (w/v) potassium ferrocyanide before incubation in 1.5% (w/v) uranylacetate for 30 min in the dark. The specimens were dehydrated in graded ethanols, embedded in Epon, sectioned and stained with lead citrate. The sections were examined in a JEM 1200 EX transmission microscope (LEOL, Tokyo, Japan).

### [$^3$H]Thymidine incorporation

Cells were incubated for different time periods in the absence or presence of fatty acids in 96-well microtitre plates (Costar, Cambridge, MA, U.S.A.). [$^3$H]Thymidine (0.4 $\mu$Ci) was added to each 200 $\mu$l culture. After incubation for 2 h, cells were harvested, lysed and the DNA was transferred to filters by a cell harvesting system (Flow Laboratories–Skatron A/S, Lier, Norway) and counted as described previously [16].

### Flow cytometry

DNA fragmentation was determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL), using the *In Situ* Cell Death Detection kit [17]. DNA content was determined by simultaneous staining with PI (10 $\mu$g/ml). From each culture, 5000 cells were analysed. Cell cycle analyses were performed as described previously [18]. Briefly, cells were permeabilized with Nonidet P-40 and DNA was stained with PI. The DNA content of 10000 cells was measured and the percentage of cells in the different phases of the cell cycle were estimated from the PI histograms. Changes in forward and side scatter were determined by defining the average forward scatter and side scatter in control cultures as 100%, and presenting the changes in forward and side scatter after different treatments as a percentage of the control. Before CD68 analysis, the cells were permeabilized in 500 ul of 1× Permeabilization Solution. IgG–FITC antibodies were used as isotype controls.

Labelling of the cells with CD36 or CD68 was according to manufacturer's instructions. The markers were set by including 1% of the isotype-labelled cells and 10000 cells were analysed from each sample. The data obtained by flow cytometry were analysed with the Lysis II or Cell Quest software (Beckton Dickinson).

### mRNA analyses

Poly(A)$^+$ RNA was isolated by the use of oligo(dT)$_{25}$ Dynabeads as described by the manufacturer. The poly(A)$^+$ RNA from each sample was eluted from the Dynabeads in sample buffer 10% (v/v) 10 × Mops (0.5 M Mops, pH 7.0/0.01 M EDTA) containing 5.6% (v/v) formaldehyde and 40% (v/v) formamide and loading buffer [2 mM sodium phosphate buffer, 1% (v/v) Ficoll-400 and 0.025% (w/v) Bromophenol Blue]. The samples were denatured for 5 min at 68 °C and incubated 5 min on ice before transfer to a 1% (w/v) agarose gel made in 1 × Mops and 6.7% (v/v) formaldehyde. Gel electrophoreses and blotting were performed as described previously [16]. The RNA mass markers were cut from the membrane and stained with 0.03% (w/v) Methylene Blue in 0.3 M sodium acetate, pH 5.2. cDNAs were labelled by random priming and hybridized to the membrane overnight according to the method of Church and Gilbert [19]. Before autoradiography (Medical X-ray film; Fuji, Tokyo, Japan), excess radioactivity was removed from the membrane by washing in 40 mM sodium phosphate and 1% (w/v) SDS at 65 °C. The signals were analysed (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, U.S.A.). Before further hybridizations, the membranes were stripped in 0.1% (w/v) SDS in 0.1 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate) at 95 °C. Each membrane was hybridized up to four times. The signals for each sample were calibrated to those of the housekeeping gene, $\beta$-actin.

### Respiratory burst

U937-1 cells were subjected to various pretreatments and the respiratory burst was initiated by exposing the cells to PMA. The change in absorbance of cytochrome *c* was measured (Shimadzu UV-2100 spectrophotometer; Kyoto, Japan) [16].

### Analyses of cellular TAG and cholesterol content

From each sample, $12 \times 10^6$ cells were lysed and sonicated for 5 s using VirSonic 50 (Virties Company Inc., Gardiner, NY) in 600 $\mu$l of water. Lipids were extracted from 500 $\mu$l of cell homogenate into silanized glass tubes [20]. The lipid extracts were dissolved in 50 $\mu$l of propan-2-ol and 10 $\mu$l aliquots were used for enzymic determination of cellular cholesterol and TAG content. The cellular lipid content was related to the protein concentration in each sample. For analysis of fatty acids incorporated into cellular TAG, 4 $\mu$l of triheptadecanoin (1.1 mg/ml) was used as internal standard and the lipids were extracted as described above in the presence of butylated hydroxytoluene (0.1 mg/ml). The samples were then solubilized in 200 $\mu$l methanol/chloroform (2:1, v/v) and separated by TLC in the presence of argon, using hexane/diethyl ether/acetic acid (80:20:1, by vol.). The TAG bands were detected in UV light after the plates were sprayed with 0.2% (w/v) 2,7-dichlorofluorescein and were scraped into glass tubes. Hydrolysis and transmethylation of the samples were achieved by incubation in a mixture of 1 ml benzene, 2 ml methanol/HCl (3 M) and 200 $\mu$l 2,2-dimethoxypropane in the dark at room temperature overnight. The samples were then neutralized with 4 ml $NaHCO_3$ (0.7 M), extracted twice with 2 ml hexane, dried and taken up into 50 $\mu$l of hexane. The fatty acids were separated by GLC [GC-14A (Shimadzu) equipped with a 100-m polar SP 2560 capillary column, 0.25-mm diameter (Supelco, Bellafonte, PA, U.S.A.)] using helium as carrier gas. The oven temperature was ramped at 135–180 °C at 5 °C/min, 180 °C for 30 min, 180–193 °C at 6 °C/min and 193 °C for 70 min. Retention times and peak areas were computed using a Shimadzu C-R4AX Chromatopac. Identification of individual methyl esters was by comparison with authentic standards analysed under the same conditions.

## RESULTS

### Effect of EPA on cell viability and cell number

To investigate a possible relationship between effects on cell number and viability, U937-1 cells were incubated with EPA at concentrations ranging from 30–240 $\mu$M for three days and the cells were then counted and the extent of membrane leakage and DNA fragmentation was established. There was a dose-dependent decrease in cell number when compared with proliferating untreated cells (Figure 1A). The inhibition of cell multiplication was also seen when incorporation of [$^3$H]thymidine

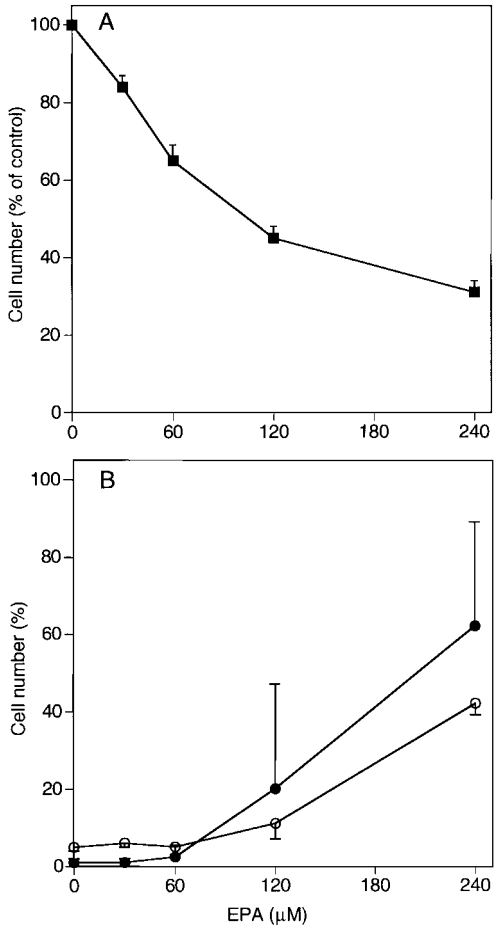


**Figure 1 Effect of EPA on cell number and viability**

U937-1 cells were cultured in the presence of various concentrations of EPA and after three days the cells were counted. The numbers of cells are expressed as a percentage of those of untreated cells (**A**). Cell membrane leakage (○) and DNA fragmentation (●), which are indicative of cell death, were determined by PI–HO342 staining and the TUNEL method (**B**). Each point represents the mean ± S.D. of duplicates in three separate experiments ($n = 6$).

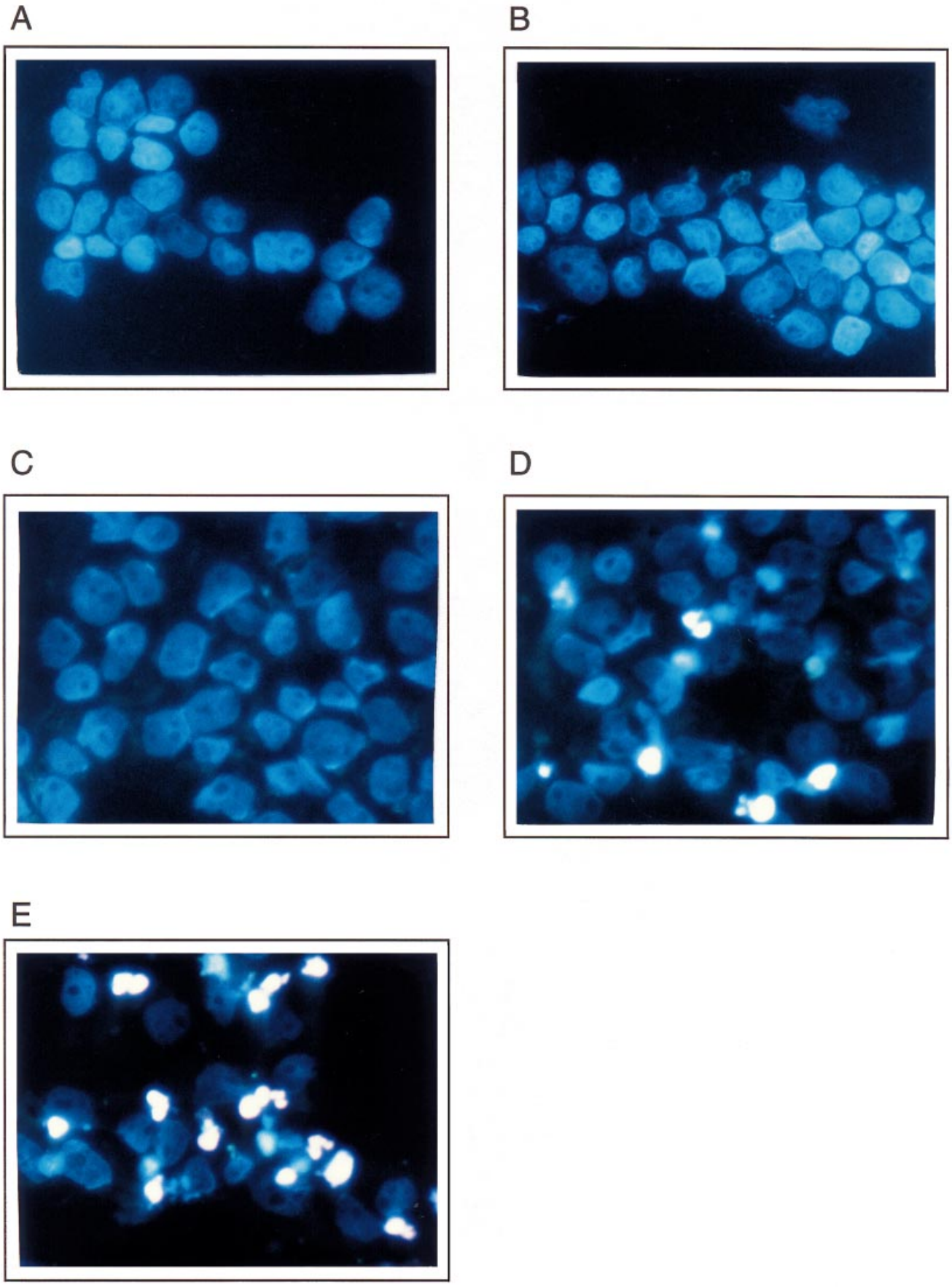


**Figure 2** For legend see opposite page.

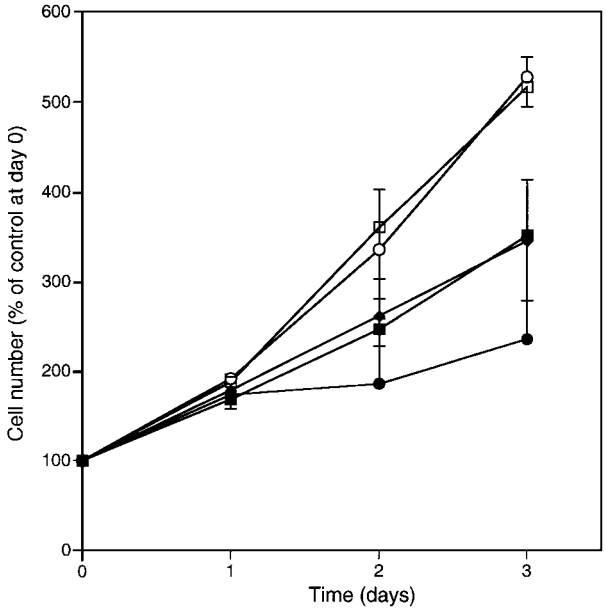


**Figure 3 Effects of OA, EPA, RA or PMA on cell number**

U937-1 cells were cultured without any supplement (○) or in the presence of 60 $\mu$M of OA (□), 60 $\mu$M EPA (■), 1 $\mu$M RA (◆) or 160 nM PMA (●) for three days. Cell numbers were determined and are presented as a percentage of the cell numbers in untreated cells at the start of the experiment. Each point represents the means ± S.D. from duplicates in three separate experiments ($n = 6$).

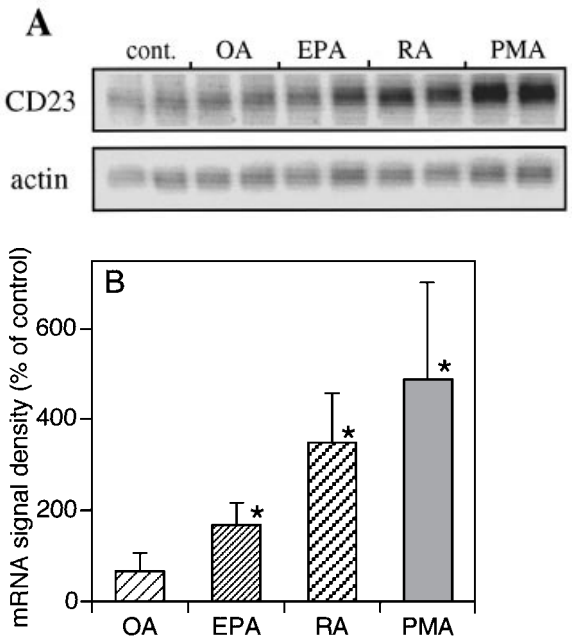


**Figure 4 CD23 mRNA levels**

U937-1 cells were untreated or cultured with 60 $\mu$M OA, 60 $\mu$M EPA, 1 $\mu$M RA or 160 nM PMA for three days. (**A**) mRNA was isolated from the respective cell cultures and Northern-blot analyses performed with a CD23 probe. The expression levels were related to $\beta$-actin in each sample. (**B**) The signals obtained in untreated cultures was defined as 100% and the intensity of the signals observed after the respective treatments are presented as a percentage of control values. The values are the means ± S.D. of three separate experiments, each performed in duplicate ($n = 6$). *, $P \leqslant 0.05$ compared with OA-treated cells.

was used as a measure of cell proliferation. After three days of treatment with EPA, RA or PMA, [$^3$H]thymidine incorporation after a 2 h pulse was 75, 88 and 49 % of the control respectively. The number of cells with leaky membranes and fragmented DNA (Figure 1B) was negligible at 30 and 60 $\mu$M EPA. However, at 120 and 240 $\mu$M EPA increasing membrane leakage and DNA fragmentation was observed. Micrographs of these cultures stained with PI–HO342 showed features typical of apoptosis (Figures 2D and 2E). At 120 $\mu$M EPA, light blue to white, intense nuclear HO342 staining was seen in many cells, indicating chromatin condensation and at 240 $\mu$M EPA several fragmented nuclei were also observed. In contrast, U937-1 cells treated with 30 and 60 $\mu$M EPA (Figures 2B and 2C) were similar to control cells (Figure 2A). A concentration of 60 $\mu$M EPA was chosen for further studies, as this concentration reduced cell proliferation but not viability of U937-1 cells.

To investigate further the decrease in cell number with 60 $\mu$M EPA, effects on cell proliferation and cell cycle distribution were compared with that of 1 $\mu$M RA and 160 nM PMA, which are both known to inhibit proliferation of U937-1 cells and induce differentiation into macrophage-like cells [21]. Cells incubated with 60 $\mu$M OA were used as negative controls in these experiments. After three days of incubation, the cell numbers in cultures treated with PMA were 44 % of those in untreated cells (Figure 3), and 28 % of the cells were PI positive (results not shown). Furthermore, the cell number in cells exposed to RA or EPA was 65 % of untreated cells (Figure 3), and 90–95 % of the cells were viable after three days (results not shown). When the distribution of cells in the different phases of the cell cycle was determined after three days, untreated cells and cells incubated with EPA, OA or PMA had closely similar distributions in the $G_1$, S and $G_2$/M phases. In these cultures 63–67 % of the cells were in the $G_1$ phase, 29–35 % in the S phase and 2–4 % in the $G_2$/M phase. In RA-treated cells, however, a $G_1$ phase arrest was observed after three days, as 93 % of the cells were in this phase and only 5 % were in the S phase and 2 % in the $G_2$/M phase of the cell cycle (results not shown).

### Effect of EPA on cell differentiation

Reduced proliferation of U937-1 cells with RA or PMA is accompanied by differentiation of U937-1 cells into two different types of macrophages [21]. The reduction in cell number observed after incubation with EPA might also be linked to differentiation of U937-1 cells into more mature macrophage-like cells. To test this possibility the cells were incubated with 60 $\mu$M OA or EPA, 1 $\mu$M RA or 160 nM PMA for three days and the mRNA levels for the differentiation markers serglycin [11], CD23 [22] and CD44 [23], and the protein levels of CD36 [24] and CD68 [25]

**Figure 2 Cell micrographs**

U937-1 cells were cultured with various concentrations of EPA for three days, stained with PI–HO342 and photographed. PI and HO342 associates with cellular DNA and emits red and blue light respectively when exposed to UV light. Staining with PI is indicative of leaky cell membranes as this dye cannot cross intact cell membranes. The HO342 stain crosses intact cell membranes and stains the nuclei in all cells. When this stain associates with condensed chromatin, such as is found in apoptotic cells, the blue colour becomes more intense. Control cells (**A**), 30 $\mu$M EPA (**B**), 60 $\mu$M EPA (**C**), 120 $\mu$M EPA (**D**) and 240 $\mu$M EPA (**E**). Magnification: 390 ×. One representative set of photographs from three separate experiments is shown.

were measured. The mRNA levels for CD44, serglycin and CD23 in OA-treated cells were similar to those observed in untreated cultures. The levels of serglycin and CD44 mRNA were not affected by EPA, but were increased in RA-incubated cells and reduced in cells incubated with PMA when compared with the levels in cells exposed to OA (results not shown). However, the mRNA level for CD23 was 1.7 times higher in EPA-treated cells than in cells cultured with OA, and 3.5 and 4.9 times higher after incubation with RA and PMA respectively (Figures 4A and 4B). U937-1 cells differentiated with vitamin $D_3$ but not with RA or PMA, express mRNA for the cell surface antigen CD14 [26]. However, incubation of U937-1 cells with EPA did not induce expression of CD14 mRNA.

CD36 was expressed in 96% of untreated cells, and a similar level of expression was observed in cultures treated with 60 $\mu$M OA or 160 nM PMA, whereas only 81% of the cells expressed this cell surface marker in cultures exposed to 60 $\mu$M EPA. After treatment with RA, 100% of the cells expressed CD36 and the mean intensity of the FITC peak was shifted to the right of the peak of control cultures, indicating increased expression of CD36 (results not shown). Furthermore, the number of CD68-positive cells increased to 49% after incubation with RA compared with 19% in the control culture. In contrast, only 1–5% of the cells were CD68 positive after treatment with OA, EPA or PMA.

Two typical features of activated macrophages are the ability to adhere to different surfaces and to perform respiratory burst [27]. U937-1 cells treated with PMA adhered to the plastic culture dish, whereas cells treated with RA or EPA did not. Furthermore, U937-1 cells treated with RA gained the capacity to perform respiratory burst, whereas cells treated with EPA did not (results not shown).

### Antioxidants, eicosanoid synthesis inhibitors and the EPA effect

To investigate if the reduction in cell number and the increased degree of apoptosis observed with EPA was caused by lipid peroxidation products, the cells were incubated with various concentrations of the antioxidants vitamin E and vitamin C for 10 min before the addition of EPA. Neither vitamin E nor vitamin C (50–100 $\mu$M) counteracted the reduction in cell numbers observed after incubation with EPA (results not shown). Furthermore, the effect of EPA on cell proliferation could, conceivably, be caused by EPA-oxidation products such as eicosanoids. The generation of these products can be inhibited by the cyclo- and lipo-oxygenase inhibitors indomethacin and nordihydroguaretic acid respectively. The presence of these inhibitors did not reverse the reduction in cell numbers observed with EPA after three days of incubation (results not shown).

### Effect of EPA on cellular morphology

Flow-cytometric analyses, measuring forward scatter and side scatter, can be used to obtain information about cell size and morphology, and to define sub-populations of leukaemic cells for diagnostic purposes [28]. Incubating U937-1 cells with 60 $\mu$M OA, 60 $\mu$M EPA, 1 $\mu$M RA or 160 nM PMA for three days did not have any major influence on cellular forward scatter. This indicates that the size of the cells was unchanged after the respective treatments. However, cells incubated with PMA, OA or EPA showed a higher side scatter than control cells (Figure 5). This particular feature was most prominent for EPA-treated cells, where the side scatter was 2.6 times higher than in untreated cells. Staining with PI revealed that cells that had an increased side scatter after incubation with PMA were dead. However, no effect on cell viability was observed in EPA- or OA-treated cells with increased side scatter (results not shown).

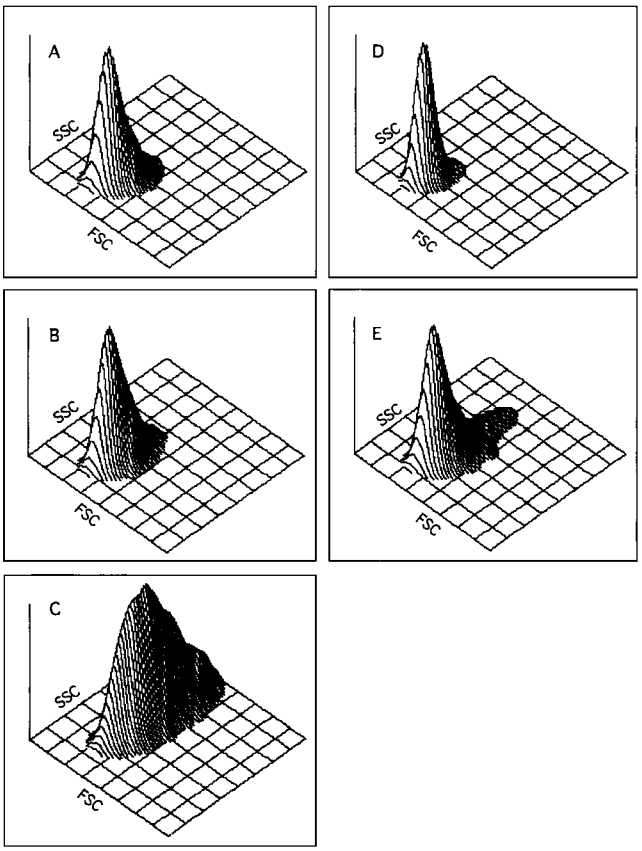


**Figure 5 Forward scatter and side scatter analyses**

U937-1 cells were cultured for three days, either untreated (**A**), with 60 $\mu$M OA (**B**), 60 $\mu$M EPA (**C**), 1 $\mu$M RA (**D**) or 160 nM PMA (**E**) and forward scatter (FSC) and side scatter (SSC) characteristics were analysed by flow cytometry. The results of one representative experiment of three are presented.

Increased side scatter has been linked to increased cell granularity and/or structural changes on the cell surface [29]. The cells were, therefore, examined for possible changes in morphology after exposure to 60 $\mu$M OA or 60 $\mu$M EPA for three days. May–Grünewald–Giemsa staining revealed that cells incubated with EPA contained 35–60 white bodies/cell, whereas in OA-treated cells 12–25 white bodies/cell were observed (results not shown). Electron microscopy of the cells revealed that these bodies were lipid droplets and no other major changes in morphology were observed.

### Reversibility of the EPA effect

To investigate if the inhibition of proliferation, accumulation of lipid droplets and increased side scatter were reversible upon removal of EPA, cells were incubated for three days with 60 $\mu$M EPA. The cells were then washed, diluted to a density of 300000 cells/ml and reincubated in EPA-free medium for three more days. Cells treated with 1 $\mu$M RA were subject to the same procedure. After preincubation for three days, the number of cells was reduced in cultures exposed to EPA and RA (shown in Figure 3). After reincubating the cells in EPA-free medium, proliferation was similar to that of control cells during the

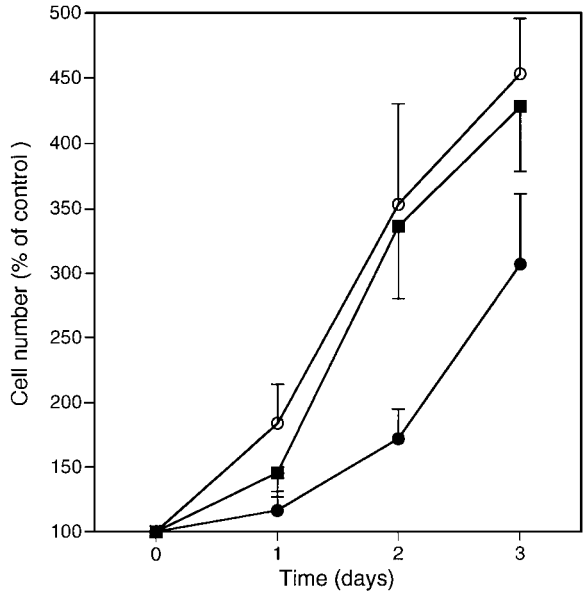


**Figure 6 Preincubation with EPA and RA and cell proliferation**

U937-1 were cultured for three days either as control (○) or with 60 $\mu$M EPA (■) or 1 $\mu$M RA (●). The cells were washed, diluted to a density of $0.3 \times 10^6$ cells/ml and incubated for three days more without EPA or RA respectively. The cell numbers are expressed as a percentage of the control cell number at the start of the experiment. Each point represents the mean ± S.D. of three separate experiments performed in triplicate ($n = 9$).

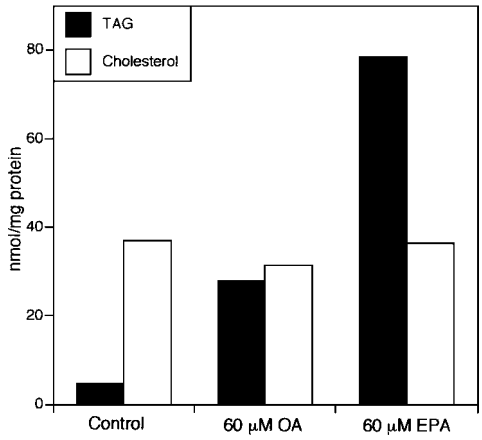


**Figure 7 TAG and cholesterol content in cells treated with EPA and OA**

The cells were cultured for three days in the presence of 60 $\mu$M of OA or EPA and the cellular content of TAG and cholesterol was estimated and compared with that of control cells. The values are the means ± S.D. from three separate experiments performed in duplicate ($n = 6$).

following three days of incubation (Figure 6). Furthermore, at this time point, side scatter was also similar to that of control cells and no lipid droplets were observed with May–Grünwald–Giemsa stain (results not shown). The proliferation of cells preincubated with RA was inhibited for two days after the removal of RA but then the rate of proliferation was similar to that of control cells. Accordingly, the inhibition of proliferation observed with both EPA and RA were reversible and required the presence of the reagents; reversal of the RA effect was slower than for EPA.

### Cellular content of TAG and cholesterol

Lipid droplets may contain TAG or cholesteryl esters [30]. Since the lower cell proliferation observed with EPA was accompanied by accumulation of lipid droplets, the cellular content of TAG and cholesterol was determined in control cultures and cells treated with 60 $\mu$M OA or EPA. After three days of incubation, the cells contained 28 and 78 nmol TAG/mg of protein respectively, whereas control cultures contained 5 nmol TAG/mg of protein (Figure 7). The cellular content of cholesterol, however, was similar in the three different cultures, indicating that the lipid droplets predominantly contained TAG.

### EPA and thia-substituted EPA in U937-1 cells

It has been shown that the thia-substituted EPA analogue, 3-thia-6,9,12,15,18-heneicosapentenoic acid (thia-EPA, $C_{18}H_{27}SCH_2COOH$), was incorporated to a smaller extent into cellular TAG than EPA in rat hepatocytes [31]. U937-1 cells were, therefore, incubated with 60 $\mu$M thia-EPA to investigate if the effects on cell proliferation and lipid accumulation would differ from those observed with 60 $\mu$M EPA or OA. From Table 1, it is evident that thia-EPA could not be detected in the TAG fraction of the cellular lipids in cells incubated with this EPA analogue. Furthermore, the levels of saturated fatty acids, monounsaturated fatty acids and PUFA in TAG did not differ significantly from those of control cells. In cells treated with EPA, however, 215 nmol PUFA/mg protein was incorporated into the TAG fraction. The majority of these were EPA and docosapentaenoic acid (22:5, $n-3$) (DPA) and a minor amount was DHA, indicating that U937-1 cells have the capacity to convert EPA to DPA and DHA. Furthermore, in OA-treated cells, the TAG fraction contained 93 nmol monounsaturated fatty acids/mg of protein of which the majority was OA.

To investigate if incorporation of EPA into cellular TAG was important for the effects observed on U9371 cells, the effect of thia-EPA on cell proliferation and viability was compared with that of EPA. Thia-EPA treatment of U937-1 cells did not affect cell number to any significant extent (Figure 8A), whereas incubation with EPA did (see Figures 1 and 3). Moreover, no induction of cell death was observed in cultures treated with various concentrations of thia-EPA (results not shown), whereas apoptosis was seen with 120 $\mu$M EPA (see Figures 1 and 2). Thia-EPA affected neither the level of TAG (Figure 8B) nor the level of cholesterol at any concentration (results not shown), whereas

**Table 1 Fatty acid content of TAGs after three days of incubation with OA, EPA or Thia-EPA (all at 60 $\mu$M)**

Means ± S.D. for three separate experiments are presented. SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; nd, not detected.

| | Fatty acid content (nmol fatty acid/mg protein) | | | |
|---|---|---|---|---|
| | Control | OA | EPA | Thia-EPA |
| SFA | 22.1 ± 3 | 18 ± 1 | 36.1 ± 2 | 29.5 ± 8.9 |
| MUFA | 12.1 ± 2 | 93.8 ± 29 | 30.0 ± 2 | 17.4 ± 8 |
| PUFA | 2.2 ± 1 | 8.2 ± 2 | 215.7 ± 41 | 3.4 ± 2 |
| OA | 8.9 ± 2.8 | 81.7 ± 26 | 19.8 ± 1.5 | 14.9 ± 9 |
| EPA | nd | nd | 57.3 ± 14.5 | nd |
| DPA | nd | nd | 139.6 ± 22 | nd |
| DHA | nd | nd | 9 ± 2 | nd |
| Thia-EPA | nd | nd | nd | nd |

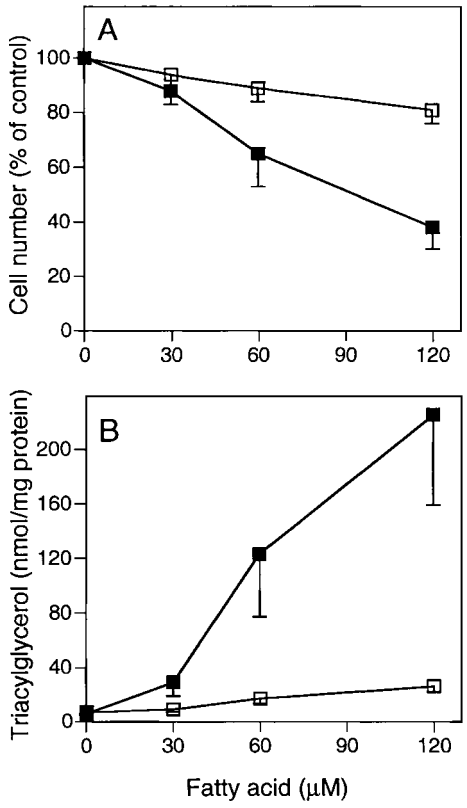


**Figure 8 Effect of thia-EPA and EPA on cell number and TAG content**

The cells were cultured for three days in the presence of 30, 60 or 120 $\mu$M of thia-EPA (□) or EPA (■) and the cell numbers (**A**) and cell content of TAG (**B**) were determined. Means ± S.D. from three separate experiments are presented.

EPA induced a dose-dependent increase in cellular TAG (Figure 8B).

## DISCUSSION

The data show a dose-dependent reduction in cell numbers of monocytic U937-1 cells after incubation with EPA. At 60 $\mu$M EPA, reduction in cell numbers was not accompanied by apoptosis, whereas apoptosis was evident at higher concentrations. No effects on these parameters were observed when U-937-1 cells were incubated with 60 $\mu$M OA. The reduction in cell number was not linked to differentiation into more macrophage-like cells, which was observed with RA- or PMA-treated cells. The mRNA level for the differentiation marker CD23 was slightly increased, whereas the protein level of CD36 and CD68 was lower in cells treated with EPA. A striking effect, however, was the accumulation of lipid droplets in cells incubated with EPA. This phenomenon was also observed in cells exposed to OA but to a lesser extent than in cells incubated with EPA. Finally, the effects of EPA were clearly reversible, as the cell proliferation rate reverted to normal and lipid accumulation disappeared when the cells were reincubated in EPA-free medium.

In several studies, the biological effects of $n-3$ fatty acids have been ascribed to the incorporation of these fatty acids into phospholipids in cellular membranes, where they may affect cellular eicosanoid synthesis and lipid peroxidation and alter membrane fluidity [32–34]. However, addition of eicosanoid synthesis inhibitors and antioxidants did not counteract the effects of EPA on the proliferation of U937-1 cells. These observations suggest alternative and yet undefined mechanisms whereby $n-3$ fatty acids may exert their biological effects, in agreement with previous reports on EPA-induced apoptosis in HL-60 and Ramos cells [10,16]. Determination of the fatty acids incorporated into TAG in EPA-treated cells revealed that 2–3 molecules of EPA or the derivatives, DPA and DHA, were incorporated per TAG molecule. In contrast, the sulphur-substituted EPA analogue (thia-EPA) was not incorporated into the TAG fraction of U937-1 cells, and had only minor effects on cell number and cell viability. It has been reported previously that thia-EPA was not incorporated into cellular phospholipids in macrophages, and to a lesser extent than EPA into phospholipid and TAG in hepatocytes [31]. The observed effects of EPA and not thia-EPA may support the hypothesis that incorporation of EPA into TAG-rich lipid droplets is the basis for as-yet-unknown mechanisms important for regulation of cell proliferation and viability.

The accumulation of lipid droplets was correlated with the reduction in cell number. Indications of a possible connection between lipid accumulation and effects on cell growth and viability has been reported. A shift of arachidonic acid (AA) from the phospholipid to the TAG fraction and reduced cell proliferation were shown in the human gastric cell line HGT [35]. In addition, choline-deficient rats developed fatty livers and accumulation of TAG was accompanied by apoptosis in liver cells grown in choline-deficient medium [36]. A possible effect of accumulation of PUFA in TAG-containing lipid droplets has also been described for human inflammatory cells. Activated neutrophils from broncheolar lavage of patients with adult respiratory distress syndrome incorporated large amounts of AA in TAG-rich lipid droplets. Furthermore, a dose-dependent accumulation of AA in TAG and a reduction in cell numbers were demonstrated in freshly isolated blood neutrophils incubated *in vitro*, concomitant with a 6-fold increase in lipid bodies [37]. Studies on cultured human alveolar macrophages showed that exogenously added AA was, to a large extent, associated with the cellular TAG fraction in the initial phase, whereafter a significant part was transferred to phospholipids. Stimulation of cells led to a release of AA from the phospholipids and a transient reacylation of AA in the TAG fraction [38]. These data suggest that phospholipid and TAG pools have different roles in the control of free AA generated during activation. In the present study, accumulation of both OA and, in particular, EPA in cellular TAG was observed. First, the storage of fatty acids in lipid droplets may function to protect the cells from exposure to high concentrations of non-esterified fatty acids. Secondly, a TAG pool, rich in PUFA, may be an important reservoir for signal molecules in immune cells [37]. The storing of EPA and EPA-derivatives in TAG, seen in U937-1 cells, may lead to changes in the general turnover of important lipid-derived signal molecules, and the effects observed on cell viability and proliferation in the present study may be secondary to TAG accumulation.

A large part of PUFA recovered from the TAG fraction of U937-1 cells incubated with EPA was DPA and a small amount was DHA. This indicates that U937-1 cells have the capacity for chain elongation and desaturation of EPA, in contrast to reports on rat hepatocytes and CaCo-2 cells [39,40]. It is possible that DHA, DPA and EPA, when liberated from an intracellular storage pool, affect intracellular signal transduction systems differently than when released from cellular phospholipids. Recent studies show that the peroxisome-proliferator-activated receptor-$\gamma$ (PPAR-$\gamma$) is up-regulated in activated macrophages. In these cells, typical markers of activation, such as expression of

the nitric oxide synthase and the scavenger receptor genes [41] and the synthesis of inflammatory cytokines, were down-regulated following activation of PPAR-$\gamma$ [42]. Since fatty acids can activate PPAR receptors [43,44], it is possible that EPA may influence U937-1 cells via PPAR-dependent signal pathways. The high intracellular concentrations of EPA obtained when cells were incubated with 60 $\mu$M of this $n-3$ fatty acid could conceivably alter the expression of genes important for regulation of proliferation and apoptosis. Possible targets in addition to PPAR-mediated pathways could be members of the protein kinase C family, the activities of which may be altered by EPA, DHA and diacylglycerol. PUFA may induce translocation of various PKC isoenzymes from the cytosol to the particulate fraction in human monocytes and macrophages [45]. Some forms of PKC may be more active in promoting apoptosis than others [46], and high intracellular levels of EPA may lead to selective activation or inhibition of some PKCs.

EPA induced only minor effects on U937-1 cell differentiation parameters when compared with those of PMA and RA. It is interesting to note that TAG accumulation was accompanied by a decrease in the expression of CD36 and CD68, both of which are receptors for oxidized low-density lipoprotein [47,48]. Whether such effects of EPA on human monocytes and macrophages are part of an inhibitory effect on atherogenic processes *in vivo* remains to be established. Some degree of differentiation has been observed in U937 cells after treatment with an AA analogue [49], and with EPA in combination with other differentiation inducers [50]. The ability of EPA to modulate phenotypic characteristics of the monoblastic U937-1 cell line was modest but still significant and suggests that dietary intake of $n-3$ fatty acids may alter monocyte and macrophage functions.

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