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Effect of Fatty Acid Supplementation on Growth and Differentiation of Human IMR-32 Neuroblastoma Cells In Vitro

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Abstract Polyunsaturated fatty acids play a critical role in the structure and function of the developing nervous system. It has been proposed that fatty acids may effect a variety of biologic processes through the activation of the peroxisome proliferator activated receptors (PPARs)–ligand activated transcription factors. In this report, we demonstrate that fatty acids can inhibit the proliferation of the human neuronal cell line IMR-32. The fatty acids linoleate, α-linoleate, arachidonate, docosahexaenoate, and oleate all inhibited [^{3}H]thymidine incorporation of IMR-32 cells after 72 h. Fatty acid supplementation also led to the morphologic differentiation of the IMR-32 cells. Linoleate and arachidonate, fatty acids of the n-6 series, induced the most extensive differentiation. Furthermore, the addition of fatty acids to IMR-32 cells led to PPAR activation, suggesting that PPAR activation may be an important event in fatty acid modulation of IMR-32 cell growth. In support of this hypothesis, clofibric acid, a specific ligand of PPARα, also inhibited IMR-32 cell proliferation and strongly induced PPAR activation. Together these results suggest that fatty acids may play an important role in the development of neuronal precursor cells and that activation of the PPARs may be one pathway by which fatty acids modulate the growth and differentiation of neuronal precursor cells. J. Cell. Biochem. 80: 266–273, 2000. © 2000 Wiley-Liss, Inc.

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Primary events in the development of the nervous system include the migration and differentiation of neuroblasts followed by the formation of interneurone connections. Failure of sympathetic neuroblasts to undergo terminal differentiation may result in the development of the childhood cancer neuroblastoma. Neuroblastoma represents the most common extracranial tumour in children, with a mean overall survival rate of about 40% at 5 years. Despite this relatively poor prognosis, and genetic abnormalities such as N-myc amplification, spontaneous differentiation of early-stage neuroblastoma into nonmalignant ganglioma in vivo is well known. Furthermore, cells isolated from human neuroblastomas readily differentiate into mature neurones in response to a variety of agonists in vitro, including retinoic acid (RA) and cAMP [Howard et al., 1993; Cohen et al., 1995]. One implication of these data is that part of the tumorigenic process in neuroblastoma could be the failure to respond to developmental cues that regulate cell division and differentiation of neuroblasts in vivo. These observations have formed the basis of clinical trials of RA as a potential differentiating agent in neuroblastoma. However, such trials have produced disappointing results. For

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example, recent evaluation of 13-*cis*-RA failed to show significant activity in advanced neuroblastoma, while toxic side effects were also observed [Finklestein et al., 1992].

Development of alternative and potentially more effective therapeutic strategies for neuroblastoma requires the understanding of the mechanisms that regulate differentiation of neuroblasts. A number of recent studies have demonstrated that a variety of fatty acids were able to alter the proliferation of several types of tumour cells. Cancer lines cells that exhibit growth inhibition in the presence of fatty acids in vitro include HL60 cells (promyelocytic leukaemia) in response to arachidonate (20:4n-6; AA) [Finstad et al., 1994], and Jurkat T-cell leukaemia [Chow et al., 1989]. In Jurkat cells, individual fatty acids induced differential inhibition of proliferation such that the effect of docosahexaenoate (22:6n-3; DHA) was greater than eicosapentaenoate (20:5n-3, EPA), which was greater than α-linolenate (18:3n-3, ALNA), linoleate (18:2n-6, LA), or oleate (18:1n-9, OA) [Chow et al., 1989]. Furthermore, DHA and EPA have been shown to significantly inhibit the growth of a human lung mucoepidermoid carcinoma in athymic mice [DeBravo et al., 1991] and the metastasis and proliferation of the murine mammary tumour MM48 [Kinoshita et al., 1996].

The mechanisms by which fatty acids modify growth and/or differentiation are not clear. Some effects on cell function may be the result of modifications to membrane receptor-mediated cell signalling via changes in the composition of phospholipid pools that are substrates for second messenger production [Hunt et al., 1991; Burdge and Postle, 1995; Heung and Postle, 1995]. There is, however, increasing evidence that fatty acids induce changes in cell function by the activation of specific receptors. Peroxisomal proliferator activated receptors (PPARs) are ligand-activated transcription factors [Schoonjans et al., 1996a] that bind a variety of hydrophobic molecules including fatty acids, eicosanoids, and fibrates such as the hypolipidaemic drug clofibric acid (CFA). Ligand binding to the PPAR leads to the formation of heterodimers with the 9-*cis*-retinoic acid receptor, which facilitates binding of the PPAR to specific DNA sequences referred to as peroxisome proliferator response elements (PPRE) [Marcus et al., 1993]. These events, in turn, result in the modulation of gene expression [Kliewer et al., 1992; Schoojaans et al., 1996b]. A role for PPAR activation in mediating the effects of fatty acids on cell proliferation is supported by the observation that treatment with ALNA induced differentiation of preadipocytes to mature adipocytes by PPAR activation [Amri et al., 1991; Chawla and Lazar, 1994]. This involved transcriptional control of genes encoding several lipid-metabolising enzymes [Schoojaans et al., 1996b].

In the present study, we have tested the hypothesis that fatty acid supplementation may alter the growth and differentiation of IMR-32 neuroblastoma cells in vitro. The results of these studies suggest that fatty acids may have a role in neurologic development distinct from incorporation into membrane phospholipids, and may be potential therapeutic agents in neuroblastoma.

MATERIALS AND METHODS

Materials

All chemicals, with noted exceptions, were purchased from Sigma (Poole, Dorset, UK). Tissue culture materials were from Gibco (Paisley, Scotland).

Preparation of Fatty Acids

Fatty acid supplements were prepared as follows. Sodium salts of ALNA, DHA, LA, AA and OA were prepared by adding of 0.1 M sodium hydroxide (300 μl) to 30 μmoles fatty acid in ethanol (2.0 ml). Fatty acid-sodium salts were dried under nitrogen at 40°C and dissolved in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS) by heating to 90°C. Ice cold 10% (w/v) fatty acid-free bovine serum in HBSS (10.0 ml) was then added to the fatty acid salts and the solution stirred at room temperature until clear. Fatty acid–albumin complexes were diluted with Dulbecco's modified Eagle's medium (DMEM) to 300 μM, filter-sterilised and stored at −20°C.

Cell Culture

IMR-32 cells were maintained in DMEM containing 10% (v/v) foetal calf serum and 100 mM Glutamax, supplemented with penicillin, streptomycin, and amphoteracin (DMEM/FCS). Cells were incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 and

were passaged by incubation with trypsin/ethylenediamine tetraacetic acid (EDTA) at three-day intervals.

Measuring Cell Growth

IMR-32 cells were seeded at 1×10^3 cells per well in DMEM/FCS (100 μl) in 96-well tissue culture plates and incubated for 18 h to allow attachment. Cells were supplemented with either fatty acids (final concentration 30 μM), all *trans*-RA (4 μM), or CFA (100 μM), and incubated for between 24 and 72 h. The concentration of RA, CFA, and fatty acid used in the experiments was determined by initial dose-response experiments. The concentration that gave maximum effect with less than 5% cell death was subsequently used (data not shown). Cell proliferation was assessed by incorporation of [^{3}H]thymidine (Amersham, Middlesex, UK) into DNA. [^{3}H]Thymidine (1 μCi/well) was added either at the same time as medium supplements or after 48 and 72 h. After 24 hours, cells were washed three times with HBSS and extracted into 62.5 mM Tris-HCL, pH7.5, containing 0.1% sodium dodecyl sulfate. Duplicate aliquots were precipitated with 750 μl of ice cold 10% trichloroacetic acid (TCA). After 30 min at 0°C, the precipitates were diluted to 5 ml with ice cold TCA and collected on G/C filters (Whatman). Filters were washed extensively with TCA, dried, and counted in optiphase Hi-safe scintillant (Fisions Chemicals). In some experiments, monoclonal anti-murine 2.5S-nerve growth factor (NGF) antibody (40 ng/ml; Promega, Wisconsin) was added to cultures at the same time as the medium supplements. Cells were harvested after 72 h.

Assessing Cell Morphology

IMR-32 cells (5×10^2) were seeded on to 13-mm diameter coverslips (Merck Ltd., Poole, Dorset, UK) in DMEM/FCS (30 μl) and allowed to attach for 6 h. DMEM/FCS (2.0 ml) was added, and the cells were incubated for a further 12 h. Cultures were supplemented with fatty acids (30 μM), RA (4 μM), or CFA (100 μM) for 72 h. Cultures were washed three times with HBSS, fixed with 1% (w/v) paraformaldehyde in HBSS for 10 min at room temperature, and rinsed with HBSS. Cell membranes were visualised using 3,3′-dioctadecyloxacarrbocyanine perchlorate (DiO; Molecular Probes Inc., Eugene, OR). Fixed cells were incubated with DiO in DMEM/FCS (80 μg/ml) for 1 h at 37°C, washed twice with HBSS, and mounted. Cell morphology was assessed by confocal microscopy using a Leica TCS 4D microscope (Heidelberg, Germany) with ×63 oil immersion objective. Excitation was at $\lambda = 488$ nm. Data were collected from eight optical slices in the X–Y plane (0.5–1.0 μm step size in the Z plane). Image analysis involved construction of extended focus images using Leica Scanware V4.2 with scaling and 2D filtering.

Transfections and Chloramphenicol acetyl transferase (CAT) Assays

IMR-32 cells (5×10^5) were plated on 9-mm^2 dishes and transfected with 2 μg of the reporter plasmid PPRE$_3$TK-CAT [Dowell et al., 1999] or with an empty vector pBL$_2$CAT [Luckow and Schutz, 1987], according to the method of Gorman [1986]. Transfected cells were then exposed to the fatty acids (30 μM), RA (4 μM), or CFA (100 μM) for 72 h, harvested, and CAT enzyme activity determined [Lillycrop et al., 1991]. Transfection efficiency was normalised using the Abken and Reinfenrath assay [1992] to ensure equal amounts of DNA were taken up into the cells. Quantitation of the CAT assay was achieved using a phosphoimager (STORM); and then an image quantifier programme was used to calculate percentage conversions of the ^{14}C chloramphenicol to its acetylated products.

Statistical Analysis

Statistical analysis was carried out using Student's *t*-test.

RESULTS

Effect of Fatty Acids on IMR-32 Cell Proliferation

The effect of fatty acid supplementation on IMR-32 cell proliferation was assessed by [^{3}H]thymidine incorporation into DNA. These results showed that cell proliferation in untreated IMR-32 cells increased significantly over a 48-h period, followed by a slight decrease between 48 and 72 h (Fig. 1). Addition of RA had little effect on cell proliferation until 72 h after addition (Fig. 1A). In contrast, fatty acid supplementation induced a marked reduction in IMR-32 cell proliferation, although each fatty acid exhibited differences both in the time

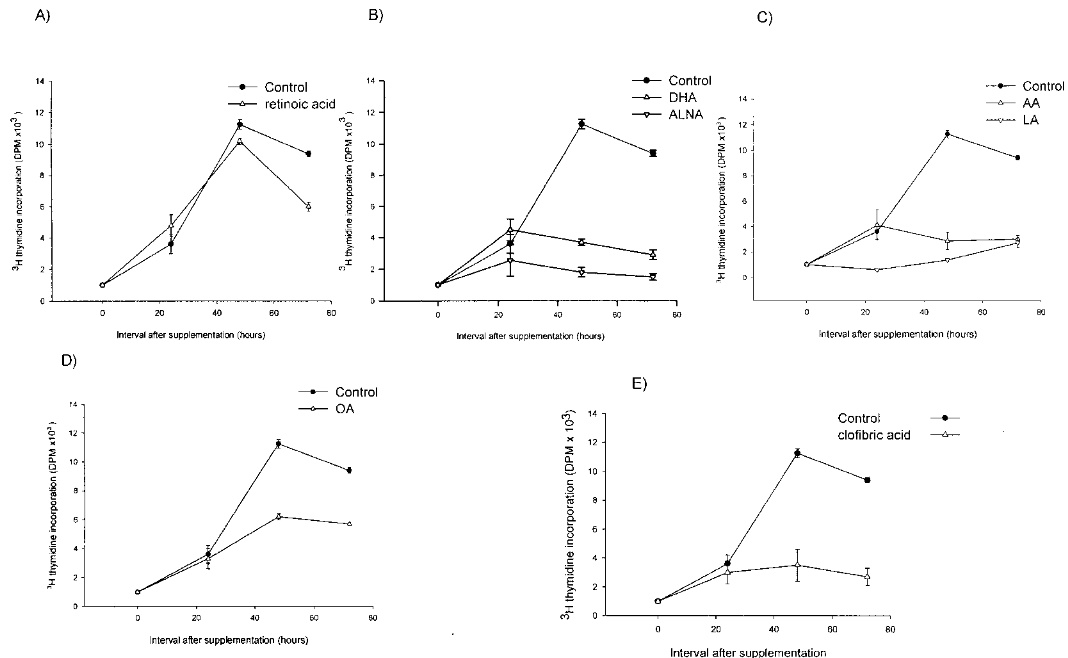

Fig. 1. Fatty acid supplementation leads to the inhibition of IMR-32 cell growth. Graphs showing the effect of retinoic acid, fatty acids, and clofibric acid on [^{3}H]thymidine incorporation in IMR-32 cells over a 72-h period. Each value represents the mean of four independent experiments ± S.E.M. DHA, docosahexaenoate; ALNA, α-linoleate; AA, arachidonate; LA, linoleate.

scale and extent of growth inhibition. [^{3}H]thymidine incorporation into DHA-, AA-, and OA- (Fig. 1B–1D) treated cells did not differ significantly at 24 h, while the growth rate of LA-supplemented cells was significantly reduced ($P < 0.05$) at this time point (Fig. 1C). Growth of ALNA-treated cells was reduced also at 24 h, although this was not significant (Fig. 1B). After 48 h, cells treated with ALNA, AA, and DHA also showed marked growth inhibition (Fig. 1B–1D). [^{3}H]thymidine incorporation was significantly reduced ($P < 0.05$) at 72 h in DHA-, ALNA-, OA-, and AA-treated cultures compared with controls.

Having shown that fatty acid supplementation inhibits IMR-32 cell growth, we also investigated whether other known ligands of the PPARs could inhibit neuroblastoma cell growth. To test this, IMR-32 cells were treated with CFA, a hypolipidaemic drug known to activate the PPAR α isoform. We found, as shown in Figure 1E, that the addition of CFA to IMR-32 cells led to a significant inhibition ($P <$ 0.05) in IMR-32 cell proliferation at 48 h, and this inhibition continued until 72 h.

Effect of Fatty Acids on IMR-32 Cell Morphology

To determine whether the addition of fatty acids to IMR-32 cells affected cell morphology as well as cell proliferation, untreated and fatty acid-supplemented cells were visualised by confocal microscopy using 3,3′-dioctadecyloxacarrbocyanine perchlorate. Representative images of IMR-32 cell cultures are shown in Fig. 2A–2G. Control IMR-32 neuroblastoma cells were typically rounded with occasional microspikes (Fig. 2A), but no significant neurite formation. Cells treated with all-*trans* RA (4 μM) for 72 h showed flattening and a triangular morphology with short neurite outgrowths (Fig. 2B). Incubation with ALNA, DHA, LA , AA, and OA (30 μM) induced varying degrees of cell flattening and neurite formation after 72 h (Fig. 2C–2G). Cells supplemented with ALNA, DHA, and OA showed flattening and the formation of a few neurites. In contrast, cells treated with LA and AA exhibited both flattened morphology and extensive neurite formation that appeared to form intercellular connections (Fig. 2 E, 2F). The addition of CFA also led to morphologic changes in

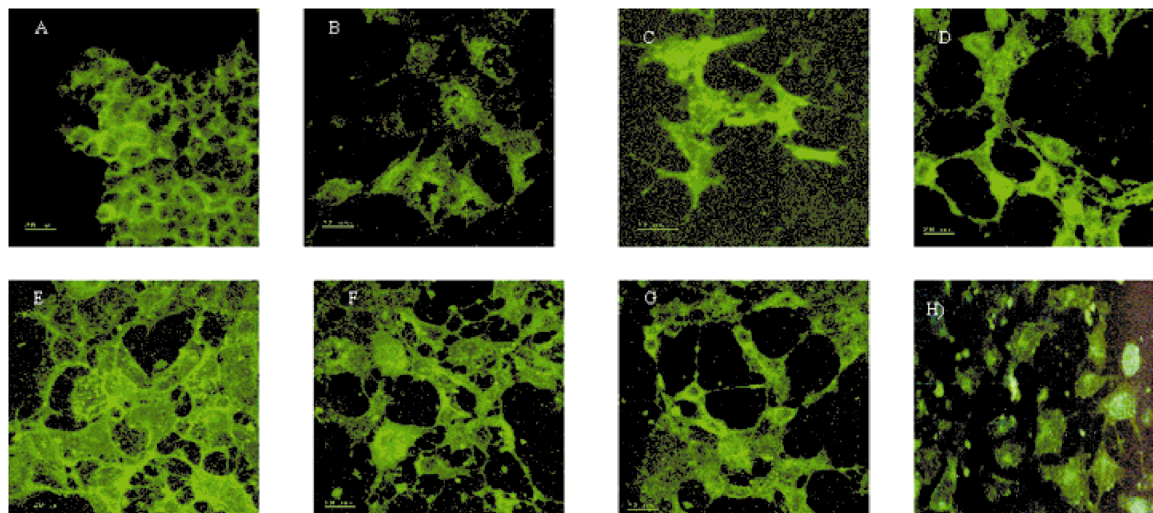

Fig. 2. Fatty acid supplementation leads to the morphologic differentiation of IMR-32 cells. Confocal microscopy images of 3,3′-dioctadecyloxacarrbocyanine perchlorate (DiO)-stained IMR-32 cells after 72 h with or without supplementation. **A:** Control. **B:** Retinoic acid (RA). **C:** α-Linoleate (ALNA). **D:** Docosahexaenoate (DHA). **E:** Linoleate (LA). **F:** Arachidonate (AA). **G:** Oleate (OA). **H:** Clofibric acid (CFA). Bar = 20 μm.

the IMR-32 cells: The cells exhibited flattening with short neurite outgrowths (Fig. 2H).

Mechanism of Fatty Acid-Induced Growth Inhibition

Fatty acids have been shown to modulate changes in cell function by binding to and activating specific transcription factors termed peroxisomal proliferator activated receptors (PPARs). To determine if fatty acid supplementation of IMR-32 cells leads to PPAR activation, IMR-32 cells were transiently transfected with a reporter plasmid ($PPRE_3$TK-CAT) in which three PPRE response elements had been cloned upstream of the thymidine kinase promoter in the vector pBL_2CAT. Transiently transfected cells were subsequently treated with either fatty acids at a final concentration of 30 μM or RA (4 μM) or CFA (100 μM) for 72 h, and CAT enzyme activity was compared in untreated and treated cells. We found that the addition of the PPAR agonist CFA led to a large increase (12-fold) in $PPRE_3$TK-CAT activity (Fig. 3). The addition of fatty acids to IMR-32 cells also led to an increase in $PPRE_3$TK-CAT activity. The fatty acids ALNA and AA were the most potent activators of $PPRE_3$TK-CAT activity, increasing $PPRE_3$TK-CAT activity by 11- and eightfold, respectively. The monounsaturated fatty acid OA was the least potent activator and increased $PPRE_3$TK-CAT activity only by 1.8-fold. Retinoic acid had little effect on $PPRE_3$TK-CAT activity compared to controls. Fatty acid and CFA activation of $PPRE_3$TK-CAT was dependent on the presence of the PPRE response elements, as the addition of fatty acids or CFA to IMR-32

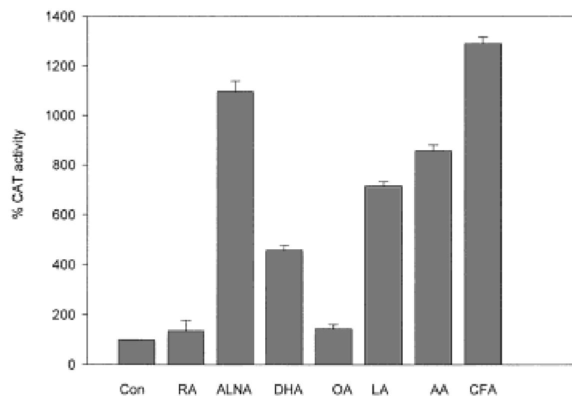

Fig. 3. Transcriptional activation of the $PPRE_3$TK-CAT construct by fatty acid supplementation in IMR-32 cells. IMR-32 cells were transiently transfected with the plasmid $PPRE_3$TK-CAT and treated for 72 h with media minus any supplementation (Con) or with retinoic acid (RA), α-linoleate (ALNA), docosahexaenoate (DHA), oleate (OA), linoleate (LA), arachidonate (AA), and clofibric acid (CFA). CAT assays were then performed. The graph shows the result of three independent experiments ± S.E.M. Results were normalised to the level of CAT activity observed in cells transfected with $PPRE_3$TK-CAT and grown in the absence of fatty acid supplementation. This value was set as 100%.

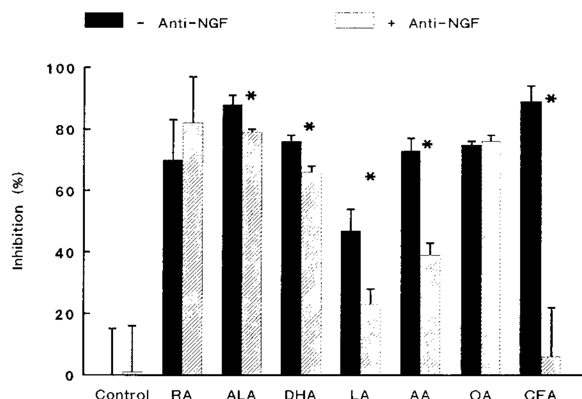

Fig. 4. [^{3}H]Thymidine incorporation in IMR-32 cells with or without addition of anti-nerve growth factor (NGF) antibody. Each value represents the mean of three independent experiments ± S.E.M. Asterisks indicate values that are significantly different ($P < 0.05$) from controls at each time point.

cells transiently transfected with the reporter vector pBL_2 CAT (which lacks the PPRE response elements) did not alter the level of CAT activity (data not shown).

Fatty Acids Inhibit Cell Growth Through an NGF-Dependent Pathway

Recent reports have shown that 1,2-dihydroxyvitamin D_3-induced neuroblastoma differentiation is mediated through an increase in NGF expression [Veenstra et al., 1997]. To gain further insights into the pathway by which fatty acids and CFA induce cell-cycle arrest, we investigated whether fatty acids and CFA induced changes in IMR-32 cells through an NGF-dependent pathway. Anti-NGF antibody was added to both untreated and fatty acid-treated cells. The extent to which cell proliferation was inhibited was determined by ^{3}H thymidine incorporation. Addition of anti-NGF antibody to the culture medium did not significantly alter the proliferation of control cells or the degree of RA-induced cell growth inhibition at 72 h (Fig. 4). However, anti-NGF antibodies impaired IMR-32 cell growth that was dependent on fatty acid species. Anti-NGF antibody reduced significantly the inhibition by ALNA (10.2%), DHA (13.2%), LA (51.0%), and AA (46.6%), but did not affect the action of OA- or RA-induced growth arrest (Fig. 4). In addition, CFA-induced inhibition of IMR-32 cell growth was also reduced significantly (93.3%) by the addition of anti-NGF antibody (Fig. 4).

DISCUSSION

The results of this study demonstrate clearly that supplementation of IMR-32 cells in culture with fatty acids of the n-3, n-6, and n-9 series resulted in significant inhibition of mitosis and the morphologic differentiation of IMR-32 cells into a mature neuronal phenotype, characterised by the production of neurite outgrowths. These findings are, therefore, consistent with previous reports that have demonstrated growth inhibition of cancer cells by fatty acid supplementation both in vitro [Chow et al., 1989; Finstad et al., 1994] or in animal models [DeBravo et al., 1991; Kinoshita et al., 1996]. In this report, we show that the fatty acids have differential actions in terms of the time scale and the extent of cell growth inhibition. Linoleate supplementation substantially reduced cell proliferation at 24 h, while no significant effect on cell growth was induced by ALNA, AA, DHA, and OA until 48 h after supplementation. This suggests a selective response of IMR-32 cells to individual fatty acids. The differences in the time taken to achieve growth inhibition may reflect differential fatty acid uptake or subsequent processing.

IMR-32 cells also showed differences in the extent of morphologic changes in response to individual fatty acids. Arachidonate and LA induced extensive neurite formation, whereas ALNA, DHA, and OA induced the formation of fewer neurite outgrowths. Both AA and LA are fatty acids of the n-6 series, whereas ALNA and DHA are n-3, and OA is an n-9 fatty acid. This further suggests selectivity in the mechanisms by which fatty acids induce differentiation in IMR-32 cells. Surprisingly, there was no correlation between the extent of neurite formation and the degree of growth inhibition induced by fatty acid supplementation. For example, treatment with AA induced both marked growth inhibition and neurite formation, whereas ALNA (although a potent inhibitor of cell proliferation) produced limited neurite outgrowth. The results indicate that differentiation and the cell-cycle inhibition may be distinct events in neuroblastoma cells. However, all of the fatty acids used in this study induced more extensive neurite outgrowths and were more potent inhitors of neuroblastoma cell growth than all-*trans* RA, which supports the suggestion that fatty acids could be used as novel therapeutic agents in

the treatment of neuroblastoma. This difference cannot simply be attributed to differences in the concentrations of fatty acids and RA used because these both represented maximal levels that did not induce greater than 5% cell death (data not shown).

The changes in cell function induced by fatty acid addition to IMR-32 cells may result from the activation of the ligand activated transcription factors—the PPARs— as fatty acid supplementation led in all cases to the activation of the reporter gene $PPRE_3TKCAT$. To date, three PPAR isoforms have been identified in mammals and these have been designated PPAR α, γ and δ. The PPAR α and δ isoforms display overlapping ligand specificities: polyunsaturated fatty acids (PUFAs) and the momounsaturated fatty acid OA have been shown to bind to and activate both isoforms. In contrast, PUFAs and OA are weak activators of PPAR γ isoform [Forman et al., 1997]. This suggests that fatty acids induce cell growth inhibition of IMR-32 cells through the activation of the PPARs, presumably the α or δ isoform. In support of this hypothesis, we found that the addition of CFA, a known potent agonist of PPAR α, also led to the inhibition of IMR-32 cell growth. These data support the role of PPARs, probably in the α isoform, in signalling events leading the growth arrest of IMR-32 cells. Interestingly, OA was the weakest activator of PPAR activity in these experiments and was also the least potent at inhibiting IMR-32 cell growth, whereas CFA, AA, LA, and ALNA were strong activators of PPAR activity and also potent inhibitors of IMR-32 cell growth. This suggests that the activation of the PPAR pathway may be involved in growth inhibition in IMR-32 cells.

Interestingly, the addition of anti-NGF antibody to the fatty acid-treated cells decreased the inhibitory effect of ALNA, DHA, LA, AA, and CFA on IMR-32 cell division. In the case of CFA, the addition of anti-NGF antibody resulted in almost complete inhibition of CFA action, suggesting that NGF expression is critical for the inhibitory effect of CFA on IMR-32 cells. The addition of anti-NGF antibody also significantly reduced the inhibitory effect of the fatty acids LA, AA, ALNA, and DHA, implying that these fatty acids may use a similar NGF-responsive pathway to inhibit IMR-32 cell growth. The addition of anti-NGF antibody to OA- and RA-treated cells had no effect. These results suggest that individual fatty acids may use different mechanisms to induce growth inhibition in these cells: n-3 and n-6 fatty acids appear to use primarily an NGF-dependent pathway, whereas n-9 fatty acids and RA use an NGF-independent pathway to suppress the growth of IMR-32 cells. Interestingly, n-3 and n-6 fatty acids were also the most potent activators of the PPARs. Such differences in the mechanism of action between LA, AA, DHA, and ALNA compared to RA and OA may explain, at least in part, the observation that n-3 and n-6 fatty acids were far more potent inhibitors of IMR-32 cell growth than RA or OA.

The ability of the fatty acids to induce morphologic changes in IMR-32 cells did not appear to correlate with the level of PPAR activation. Other mechanisms must therefore account for the induction of morphologic differentiation induced by some of the fatty acids. For example, modulating the fatty acid composition of membrane phospholipids would lead to alterations in the composition of phospholipase C-generated second messengers [Leach et al., 1991] and phospholipase D-generated second messengers [Heung and Postle, 1995]. This has been suggested to modulate protein kinase C activation [Leach et al., 1991]. In addition, cellular differentiation during foetal development [Hunt et al., 1991; Burdge and Postle, 1995] or in vitro [Murphy and Horrocks, 1993] is associated with specific changes to membranes phospholipid composition. The overall effect of fatty acid supplementation is likely to be the net product of the activation and cross-talk between different signalling pathways.

The primary implications of these data are that fatty acids are able to induce neuroblast differentiation, albeit to varying extents. This suggests the possibility that activation of PPARs, possibly by fatty acids or eicosanoids, may be important in differentiation of neuroblasts during foetal development. Such processes may explain, in part, why normal neurologic development requires specific polyunsaturated fatty acids, in particular DHA and AA, at specific time points in gestation [Innis, 1991]. This requirement is in addition to the selective incorporation of these fatty acids in membrane phospholipids. This demonstration that fatty acids are potent inhibitors of proliferation in human IMR-32 neuroblastoma cells may also

represent an novel source of potential therapeutic agents with limited anticipated toxicity.

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