N–3 and n–6 polyunsaturated fatty acids induce cytostasis in human urothelial cells independent of p53 gene function

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Abstract The role of long-chain polyunsaturated fatty acids (PUFA) in the etiopathology and treatment of cancer is poorly understood. We have studied the effects of n–3 and n–6 PUFA on the proliferation and survival of normal human uroepithelial (NHU) cells, cells with disabled p53 function after stable transfection with the human papillomavirus 16 (HPV16) E6 gene (HU-E6), and p53-disabled cells that had passed through crisis and acquired karyotypic abnormalities (HU-E6P). The n–3 and n–6 PUFA had distinct reversible antiproliferative and irreversible cytostatic effects according to concentration and exposure time. The reversible antiproliferative effect was partly due to the production of lipoxygenase metabolites. NHU and HU-E6 cells were equally sensitive to n–3 and n–6 PUFA, but HU-E6P cells were more resistant to both the antiproliferative and cytostatic effects. Cytostatic concentrations of n–3 and n–6 PUFA did not induce apoptosis, but caused permanent growth arrest ("interphase" or "reproductive" cell death) and mRNA levels for genes involved in cell cycle control (p21, p16, p27, cdk1, cdk2, and cdk4) were not altered. Neither n–3 nor n–6 PUFA promoted acquisition of karyotypic abnormalities in HU-E6 cells, suggesting that n–3 and n–6 PUFA do not cause genotoxic damage. In conclusion, our studies show that the antiproliferative and cytostatic effects of n–3 and n–6 PUFA are not dependent on p53 function and, further, that transformation results in a loss of sensitivity to n–3 and n–6 PUFA-mediated growth inhibition.— Diggle, C. P., E. Pitt, P. Roberts, L. K. Trejdosiewicz, and J. Southgate. **N–3 and n–6 polyunsaturated fatty acids induce cytostasis in human urothelial cells independent of p53 gene function. *J. Lipid Res.* 2000.** 41: **1509–1515.**

Supplementary key words fatty acids • PUFA • p53 • urothelium • bladder cancer

Long-chain polyunsaturated fatty acids (PUFA) of both n–3 and n–6 types can inhibit cell division, cause cell cycle arrest, and induce cell death in malignant epithelial cells of various tissue origins in vitro (1–4). However, little is known of the effects of long-chain PUFA on normal epithelial cells and the molecular basis of apparent differences in cancer risk associated with the dietary intake of n–3 and n–6 PUFA remains obscure. We have developed a robust, highly reproducible in vitro system in which normal human urothelial (NHU) cells can be isolated from the underlying stroma and maintained in culture for up to 12 passages before senescence (5). In monoculture, NHU cells show a proliferative, basal/intermediate cell phenotype, but retain the ability to reform a low-turnover stratified differentiated urothelium when seeded onto an appropriate stroma (6). This demonstrates the relevance of NHU cell cultures for investigating normal urothelial cell behavior.

Previously, we have shown that n–3 and n–6 PUFA are growth inhibitory to proliferating NHU cells, whereas saturated stearic acid had no antiproliferative effect (7). We suggested that these PUFA have a general antiproliferative effect on rapidly dividing cells, rather than showing any selective effect on neoplastically transformed cells. This is in accordance with findings that n–3 PUFA inhibit cell division in normal intestinal crypt cells, which are highly proliferative in situ (8, 9). The aims of this study were to define the nature of the growth inhibition. The p53 protein can negatively regulate cell proliferation by its ability to arrest cells in the mitotic cycle and to promote apoptosis in cells with genotoxic damage where DNA repair is unsuccessful (reviewed in refs. 10 and 11). To determine

Abbreviations: ALA, α-linolenic acid; BSA, bovine serum albumin; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; GTL, Giemsa–trypsin–Leishman; HU-E6 cells, human urothelial cells transduced with human papillomavirus type 16 (HPV16) E6 gene at precrisis passage; HU-E6P cells, postcrisis human urothelial cells transduced with HPV16 E6 gene; KSFM, keratinocyte serum-free medium; LA, linoleic acid; NDGA, nordihydroguaiaretic acid; NHU cells, normal human urothelial cells; OA, oleic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PUFA, polyunsaturated fatty acids; RPA, ribonuclease protection assay; SA, stearic acid; SDS, sodium dodecyl sulfate.

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whether the antiproliferative and irreversible cytostatic effects of n−3 and n−6 PUFA in NHU cells were dependent on functional p53 protein, we investigated whether ablation of p53 function, a major event in urothelial carcinogenesis (12, 13), would alter response or sensitivity to n−3 and n−6 PUFA.

MATERIALS AND METHODS

Normal human urothelial cell culture

Urothelium was isolated from a specimen of normal renal pelvis from a 6-month-old male undergoing pyeloplasty and used to initiate a normal human urothelial (NHU) cell line, as previously described (5, 14).

Urothelial cells were grown in keratinocyte serum-free medium (KSFM) containing bovine pituitary extract and epidermal growth factor, at the concentrations recommended by the manufacturer (GIBCO-BRL, Paisley, UK), and cholera toxin (30 ng/ml; Sigma, Poole, UK). Medium was replaced three times weekly. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and passaged at near confluence by incubating in 0.1% (w/v) EDTA in phosphate-buffered saline (PBS) for 5 min, followed by detachment in 0.25% (w/v) trypsin in 0.02% (w/v) EDTA and collection into medium containing trypsin inhibitor (1.5 mg/ml).

Human urothelial cells with disabled p53 function

A subline of the NHU cell line with disabled p53 function was prepared by stable integration of the E6 gene of human papilloma virus (HPV16), which targets the p53 protein for ubiquitination and consequent degradation. The E6 gene was introduced into NHU cells at passage 4 by transduction with replication-deficient amphotropic LXSN16E6 retrovirus, which contains the pLXSN plasmid with the HPV16 E6 and neomycin resistance (*neo*) genes (15). A dose−response curve for G418 over a range of 0.01−2.0 mg/ml indicated that G418 at 100 μg/ml would eliminate by apoptosis all NHU cells that did not express the neomycin resistance gene. For transduction, NHU cells were incubated for 4 h in medium containing retrovirus and polybrene (8 μg/ml), washed in PBS, and cultured for 48 h prior to addition of G418 (100 μg/ml; Sigma). Cells were maintained in the presence of G418 and analyzed for cytogenetic abnormalities every 10 passages. Two passages after retroviral infection, the supernatant from the transduced cells was transferred onto nontransduced NHU cells. No G418-resistant cell colonies arose, demonstrating the lack of persistence of infectious retrovirus.

E6-transduced urothelial cells showed an extended life span and underwent "crisis" at passage 20, eventually acquiring clonal karyotypic abnormalities and becoming immortalized (see Results). Studies comparing the differential responses of cells to the PUFA used were performed on the autologous nontransduced NHU cells between passages 4 and 14, on p53-null (HU-E6) cells prior to crisis, between passages 4 and 20, and on post-crisis immortalized (HU-E6P) cells between passages 30 and 55.

Fatty acids

Nonesterified fatty acids were prepared as complexes with fatty acid-free bovine serum albumin (BSA; Sigma) to mimic a physiologic route of presentation as previously described (7). Fatty acid complexes were prepared and dialyzed under nitrogen, stored freeze-dried, reconstituted in medium just prior to use, and filter sterilized (0.2-μm pore size membranes). In all studies, the BSA concentration was kept constant at 10 mg/ml and results were compared with BSA-only controls. The baseline fatty acid concentration of the growth medium was less than 2 μM, as determined by spectrophotometric assay for nonesterified fatty acids (Randox Laboratories, Ardmore, Ireland). For studies examining cytotoxic effects, the fatty acids were used at 100 μM, based on previous studies (7).

To determine the maximal sustainable dose of fatty acids in long-term cultures, NHU cells were maintained through serial passage in the continued presence of the specified PUFA at concentrations of 10 to 100 μM for at least three passages.

Cell proliferation and cell cycle analysis

Tritiated thymidine uptake was used to determine cell proliferation (7). Urothelial cells were plated in 96-well plates (Linbro, ICN Flow Laboratories, Horsham, UK) at an initial plating density of 1×10^4 cells/well in KSFM growth medium. After cell attachment, the fatty acid−BSA complexes were added to the wells in six replicates. Cells were cultured for a total period of 72 h in the presence of the fatty acids with [^{3}H]thymidine (0.5 μCi/well; Amersham Life Science, Amersham, UK) added for the final 18-h incubation period. Precursor incorporation was assessed with a Beta-plate liquid scintillation analyser (Wallac, Milton Keynes, UK), as described previously (7).

For cell cycle analysis, cells were trypsinized and labeled with propidium iodide, using a DNA-Prep kit according to the manufacturer instructions (Beckman Coulter UK, High Wycombe, UK). The labeled cells were analyzed by flow cytometry on a FACScan analytical instrument, running CellQuest software for acquisition and analysis (Becton Dickinson, Oxford, UK). Data were acquired by using doublet discrimination on a linear scale and presented as histograms of DNA content (fluorescence 2 intensity) against cell number. Markers were set to determine the percentage of cells in G_1, S, and G_2 phases of the cell cycle.

Inhibitor studies

Studies using inhibitors were performed as described above, with the addition of the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; Sigma), the cyclooxygenase inhibitor piroxicam (Sigma), and the antioxidant vitamin E (Sigma). Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added to the medium at a range of concentrations from 10 to 50 μM. The DMSO concentration was kept constant at 0.1% and a solvent-only control was included.

Determination of apoptosis

Phase-contrast and fluorescence microscopy was used to assess nuclear condensation and fragmentation on cells stained with acridine orange (10 μg/ml). For the quantitation of apoptosis, flow cytometric analysis was performed on cells incubated with fluoresceinated annexin V (Boehringer Mannheim, Lewes, UK) to detect the early apoptotic orientation of membrane phosphatidylserine and with propidium iodide to detect late apoptotic and dead cells (16). In addition to its use in selective medium for E6-transduced cells, G418 was used as a positive control to induce apoptosis in NHU cells.

Ribonuclease protection assays

Riboprobes were produced for ribonuclease protection assays (RPAs) for expression of genes associated with cell cycle control, using the hCC1 template set (PharMingen, Cowley, UK). RPAs were carried out with an RPA II kit (Ambion, Oxon, UK), using 5 μg of total RNA extracted from NHU cells.

Polymerase chain reactions

To detect E6, genomic DNA was extracted from transduced and nontransduced cells with a genomic DNA extraction kit from Nucleon Biosciences (Coatbridge, UK). The polymerase

chain reaction (PCR) was carried out on 100 ng of template DNA with the following primers designed to amplify a portion of the E6 gene: 5′ GCA AGC AAC AGT TAC TGC GA 3′ and 5′ CTG GGT TTC TCT ACG TGT TC 3′. PCR cycling conditions included an initial denaturation period of 5 min at 94°C followed by 30 cycles of denaturation for 30 sec at 94°C, reannealing for 15 sec at 54°C, and extension for 30 sec at 72°C, followed by a final extension for 5 min at 72°C. The PCR product was separated on a 2% agarose gel and visualized by UV light after ethidium bromide staining.

Immunoblotting for p53

Cells were harvested from the substratum, counted, and lysed by sonication in electrophoresis sample buffer containing dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and aprotinin (100 KIU/ml). A volume equivalent to 5×10^5 cells was loaded onto a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The separated proteins were transferred electrophoretically onto ECL membranes (Amersham) and probed with anti-p53 antibody DO1 (ref. 17; obtained from the Imperial Cancer Research Fund, London, UK). Antibody binding was detected by an enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer instructions.

Cytogenetic analysis

Cell cultures in exponential growth were exposed to Colcemid (0.2 μg/ml; Sigma) in growth medium for 17 h. Cells were harvested and incubated for 15 min in 0.075 M KCl before washing three times in Carnoy's fixative. Preparations were analyzed by Giemsa–trypsin–Leishman (GTL) banding and up to 10 metaphases were examined for each preparation.

RESULTS

Cytostatic effects of PUFA on NHU cells

To establish the maximal sustainable concentration of n−3 and n−6 PUFA that would permit long-term continued growth, NHU cells were maintained through serial passage in the constant presence of n−3 and n−6 PUFA at concentrations of 10 to 100 μM (**Table 1**). The maximal doses that would sustain cultures for at least three passages were 40 μM for linoleic acid (LA), 10 μM for γ-linolenic acid (GLA), and 20 μM for eicosapentaenoic acid (EPA). At the highest concentrations tested, cultures failed to reach confluence. At intermediate concentrations, cultures survived for one or two passages, but showed progressive growth arrest and were terminated on failing to reach confluence.

The reversibility of the antiproliferative effect was determined by studying the ability of NHU cells to proliferate after exposure to 100 μM fatty acids for defined periods. Exposure to 100 μM stearic acid (SA), oleic acid (OA), or α-linolenic acid (ALA) for up to 5 days did not affect the subsequent growth of cultures relative to a BSA-only control. By contrast, NHU cells exposed to 100 μM LA, GLA, EPA, or DHA for 72 h were not able to reestablish proliferation when replenished with growth medium devoid of fatty acids. The cells remained as an intact monolayer and showed no evidence of nuclear condensation or DNA fragmentation, as judged by phase-contrast microscopy or staining with acridine orange (10 μg/ml). Flow cytometric analysis with annexin V and propidium iodide confirmed that cells cultured for 24, 48, or 72 h with high dose n−3 and n−6 PUFA showed no increase in apoptosis, relative to BSA-only control cultures (**Fig. 1**). Thus, with the exception of ALA, high concentrations of n−3 and n−6 PUFA induced an irreversible cytostatic effect distinct from "classic" apoptosis.

Cell cycle analysis by flow cytometry was performed to determine the stage at which proliferation was blocked. Exposure for 48 or 72 h to 3 to 100 μM LA, GLA, or EPA caused a dose-dependent reduction of cells in S phase and an increase in the percentage of cells in G_1 phase (**Fig. 2**).

It has been postulated that arachidonic acid can affect CDK-cyclin A activity when cells are arrested in S phase (18). To establish whether the effect on cell cycle might involve changes in expression of cell cycle-regulated genes, we quantified expression of p21, p16, p27, cdk1, cdk2, and cdk4 genes by ribonuclease protection assay. No differences in gene expression were detected between cells exposed to BSA or to 50 μM GLA or EPA (data not shown).

Establishment and characterization of E6-transduced cells

A p53-disabled HU-E6 subline was generated by transduction of NHU cells with the HPV16 E6 gene, the product of which binds to and targets p53 for ubiquitination,

TABLE 1. Fatty acid effects on NHU cells

Fatty Acid	Chain Length: Double Bonds	Isomeric Form	Maximal Sustainable Dose	Irreversible Cytostatic Effect (100 μM)
			μM	
Stearic acid (SA)	18:0		—	No
Oleic acid (OA)	18:1	n−9	—	No
Linoleic acid (LA)	18:2	n−6	40	Yes
α-Linolenic acid (ALA)	18:3	n−3	ND	No
γ-Linolenic acid (GLA)	18:3	n−6	10	Yes
Eicosapentaenoic acid (EPA)	20:5	n−3	20	Yes
Docosahexaenoic acid (DHA)	22:6	n−3	ND	Yes

The maximal sustainable dose was the highest concentration the cells tolerated through at least three passages. The cells were deemed irreversibly cytostatic if they were unable to grow after a 72-h or longer exposure to 100 μM fatty acid followed by replating.

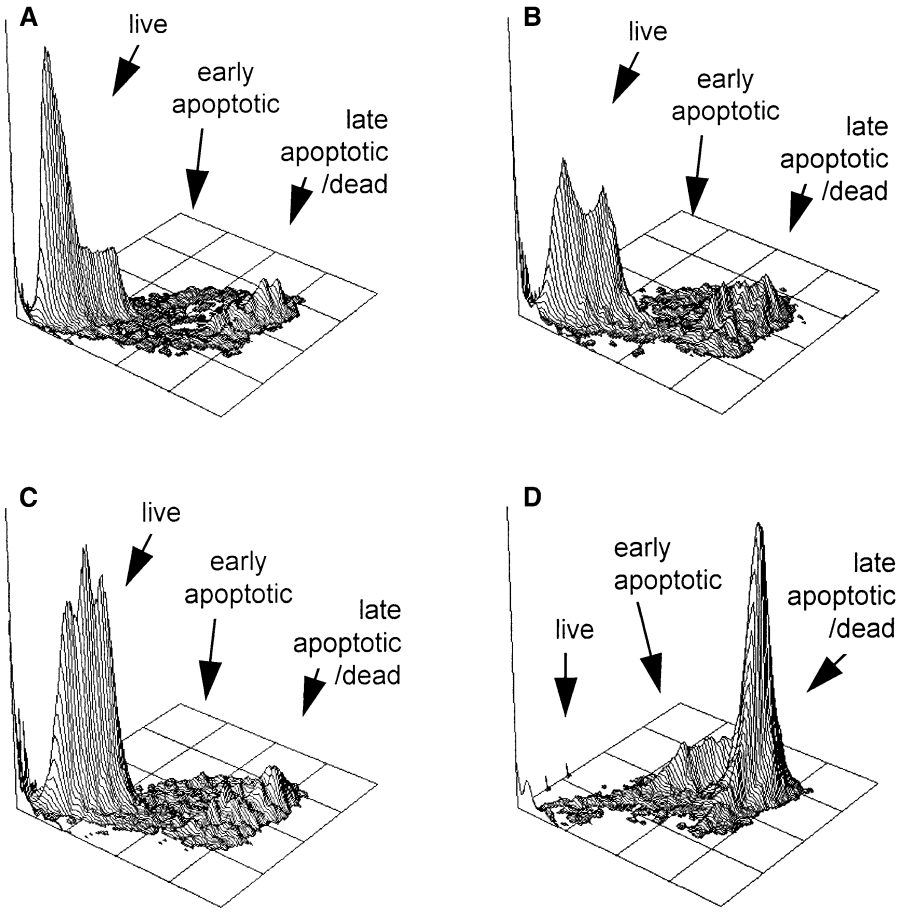

Fig. 1. Analysis of apoptosis in NHU cells cultured with n−3 and n−6 PUFA. NHU cells cultured without (BSA control) or with 100 μM PUFA were stained with propidium iodide (y axis) and fluoresceinated annexin V (x axis) to identify double-negative (live), annexin V-positive (early apoptotic), and double-positive (late apoptotic or dead) cells (16). G418 (100 μg/ml) was used as a positive control for apoptosis. Representative results shown at 72 h. (A) BSA control; (B) LA; (C) EPA; (D) G418 positive control.

effectively disabling p53 function (19). Four passages posttransduction, the nonclonally selected E6-transduced HU-E6 cells contained the E6 gene by PCR and E6 mRNA was detected by Northern blotting. Integration of the E6 gene was stable and persisted after withdrawal of G418 selection. Immunoblotting with antibody DO1 confirmed the absence of p53 protein in HU-E6 cells, the presence of p53 in NHU cells, and upregulation of p53 in control cells undergoing apoptosis in response to G418.

Absence of p53 function permits cells to accumulate genotoxic damage (10, 11). At passage 15, parental NHU and transduced HU-E6 cells were karyotypically normal. NHU cells senesced at passage 15, whereas HU-E6 cells had an extended life span (M_1 phase), entering crisis at passage 20. Crisis lasted for 10 weeks with no net gain or loss of cells; few metaphases were present and complete GTL-banded analysis was not always possible. However, random cytogenetic abnormalities were present, and there was some indication of clonal abnormalities. Eventually, subpopulations of growing cells emerged as clusters. These postcrisis (HU-E6P) cells showed a decreased cytoplasmic-to-nuclear ratio and, by passage 30, HU-E6P cells had developed clonal karyotypic changes, including loss from chromosomes 3p, 9p, and 8q, addition to chromosome 20, and a chromosome 8-to-10 translocation.

Effects of n−3 and n−6 PUFA on E6-transduced cells

In cultures maintained in the continued presence of n−3 and n−6 PUFA, the maximal sustainable concentrations of PUFA remained the same for HU-E6 cells as for nontransduced NHU cells, namely 40 μM for LA, 10 μM for GLA, and 20 μM for EPA.

Like the parental NHU cells, HU-E6 cells were also blocked in G_1 by n−3 and n−6 PUFA. Comparison of proliferation rates by NHU and HU-E6 cells exposed to n−3 and n−6 PUFA for 72 h showed an inhibition of thymidine incorporation above 10 μM for LA, EPA, and GLA. GLA was a more effective cytostatic agent than either LA or EPA. However, there was no statistical difference in sensitivity between the NHU and HU-E6 sublines (**Fig. 3**), indicating a p53-independent mechanism for the cytostatic effects of n−3 and n−6 PUFA.

Interestingly, when HU-E6 cells were maintained continually in the maximal sustainable concentration of LA, GLA, or EPA, from 72 h posttransduction, there were no differences in the time to crisis or in the frequency of colo-

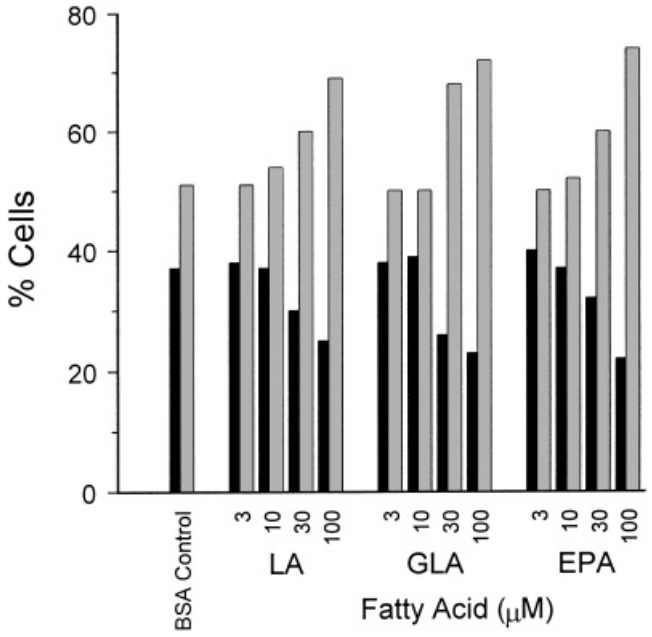

Fig. 2. Cell cycle analysis of NHU cells treated with PUFA. Cells were stained with propidium iodide and the DNA content was estimated by flow cytometry on a linear scale, using doublet discrimination. Markers were set to determine the percentage of cells in G_1, S, and G_2 phases of the cell cycle. Graph shows data for S- and G_1-phase cells derived from a representative experiment. Dark shaded columns: percentage of cells in S phase; pale shaded columns: percentage of cells in G_1 phase.

nies arising postcrisis, compared with controls. They also showed the same time scale for acquiring karyotypic abnormalities and cells at crisis and postcrisis showed no consistent specific differences in the abnormalities detected. The failure of p53-null cells to acquire more extensive karyotypic abnormalities when exposed to n−3 or n−6 PUFA suggests that PUFA do not cause gross genotoxic damage.

During exponential growth, the proliferation rates of NHU, HU-E6, and HU-E6P cells were indistinguishable, although the HU-E6P cells showed a decreased lag phase (not shown). NHU and HU-E6 cells were equally susceptible to the antiproliferative effects of n−3 and n−6 PUFA, whereas HU-E6P cells with karyotypic abnormalities were more resistant. The HU-E6P cells showed no inhibition of thymidine incorporation at 30 μM LA, EPA, or GLA (Fig. 3). At 100 μM, GLA was inhibitory, but neither LA nor EPA showed any major antiproliferative effect on HU-E6P cells.

Inhibition of PUFA antiproliferative effect

Inhibitors of the pathways for eicosanoid production from n−3 and n−6 PUFA were used to investigate potential antiproliferative mechanisms. Inhibition of cyclooxygenase or addition of antioxidant had little effect on the inhibition of thymidine uptake by n−3 and n−6 PUFA. However, inhibition of lipoxygenase resulted in a significant reversal of the antiproliferative effect with n−3 and n−6 PUFA (**Fig. 4**). In the case of LA, the growth inhibition was abolished.

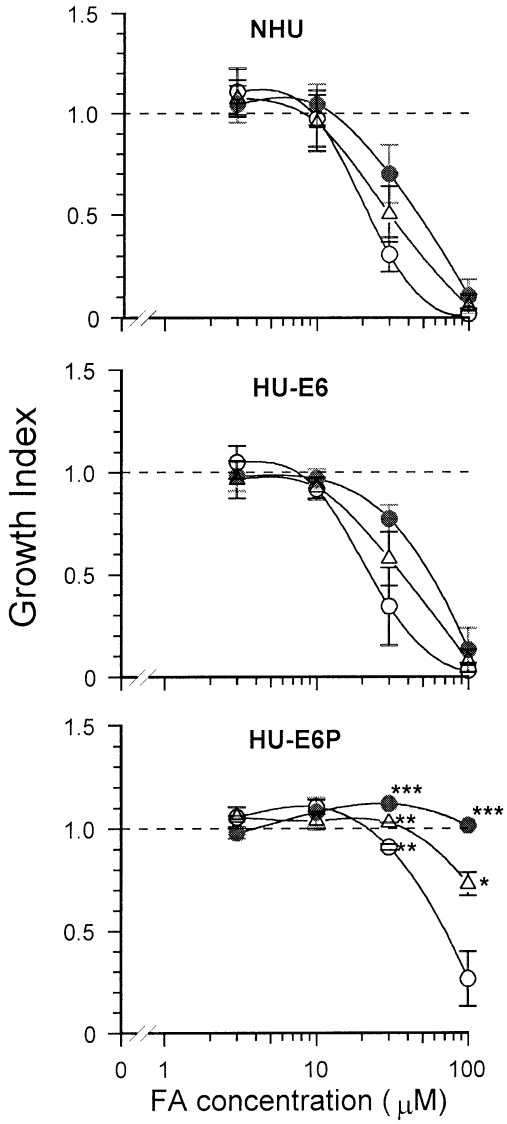

Fig. 3. Effects of PUFA on proliferation of parental NHU cells and of cells with disabled p53 function before crisis (HU-E6 cells) and postcrisis (HU-E6P cells). Cell proliferation was determined by [^{3}H]thymidine incorporation after 72 h of exposure to PUFA. Each point represents the mean (±SD) of a minimum of two separate experiments performed in replicates of six. Statistical significance (analysis of variance with Bonferroni correction) is shown versus NHU cells and is represented by asterisks: * $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$. Closed circles, LA; open circles, GLA; open triangles, EPA.

DISCUSSION

This is the first demonstration that in a well-characterized normal human epithelial cell system, n−3 and n−6 PUFA elicit concentration-dependent reversible antiproliferative and irreversible cytostatic effects, which are independent of p53 function. The small amount of previous work on PUFA and the role of p53 has been limited to tumor cell lines and has shown conflicting results (18, 20).

At present, the mechanisms of PUFA-induced antiproliferation are not fully understood. We have shown that metabolites of the lipoxygenase pathway are involved.

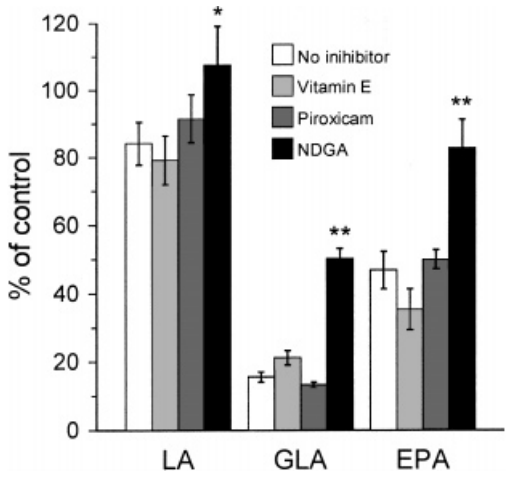

Fig. 4. Effect of metabolic inhibitors on proliferation of PUFA-treated NHU cells. Cell proliferation was determined by [^{3}H]thymidine incorporation after 72 h of exposure to 30 μM PUFA. A representative experiment is illustrated, using a 10 μM concentration of each inhibitor. Data are plotted as means of six replicates (± SD), calculated as the percentage inhibition relative to controls in the absence of fatty acid, and statistical significance (Student's *t*-test) versus no inhibitor controls is represented as follows: * $P < 0.005$; and ** $P < 0.0001$. NDGA (lipoxygenase inhibitor) had a highly significant effect in overcoming the antiproliferative effects of the fatty acids. Open columns, no inhibitor; pale shaded columns, vitamin E; dark shaded columns, piroxicam; solid columns, NDGA.

Lipoxygenase is a controlled means of peroxidation whereby unsaturated fatty acids are oxygenated, giving rise to hydroperoxy fatty acid species, which go on to form leukotrienes and hydroxy fatty acids. These fatty acid metabolites function as local hormones. In the case of GLA and EPA, inhibition of lipoxygenase only partially reversed the antiproliferation effect, suggesting that other mechanisms must also be present. In other cell systems, cytotoxicity due to PUFA has been counteracted by the use of antioxidants (21–23) or cyclooxygenase inhibitors (22). However, inhibitors of these pathways did not affect the cytotoxicity seen in NHU cells in response to LA, GLA, or EPA, implying that neither free radical production nor metabolites of the cyclooxygenase pathway are involved.

Other potential mediators of effects of PUFA include ceramides, which are products of sphingolipid hydrolysis stimulated by arachidonic acid (24, 25). We have previously demonstrated that albumin-complexed fatty acids are rapidly incorporated into normal cell membranes, and hence might be predicted to alter the functioning of proteins within cell membranes (26). Although this might influence cell membrane properties to the extent of disabling the ability of urothelial cells to respond to growth factors, it is not clear how membrane lipid perturbations could entrain an irreversible growth arrest.

The detected cytogenetic changes in HU-E6P cells were overall consistent with those described previously for E6-transduced NHU cell lines and characteristic of bladder cancer (13, 27). Our results with the HU-E6P cells contrast with frequently cited studies suggesting that PUFA have selective antiproliferative effects on neoplastically transformed cells (1, 2). Nevertheless, our observations agree with those of Griffiths et al. (28), who showed that n–6 PUFA were more growth inhibitory to normal than to adenocarcinoma-derived prostatic epithelial cells. The apparent discrepancy between studies is most likely to relate to whether the normal and neoplastic cells used for the comparison were matched, and suggests that there may be cell type-specific differences in the sensitivity of cells to the effects of PUFA.

In vivo experimental work with both chemically induced and transplantable animal tumor models has demonstrated that n–3 PUFA generally have a suppressive effect on cancer, while the n–6 PUFA LA tends to promote cancer (reviewed in refs. 29 and 30). We used the maximal sustainable doses of fatty acid, on normal and on p53-null cells, before, during, and after crisis. The conclusions from these experiments are that neither n–3 nor n–6 PUFA have any direct effect, either promotive or suppressive, on the acquisition of a postcrisis genotype. This suggests that other indirect mechanisms may be relevant in vivo.

Biochemically, LA can be converted to GLA via 6-desaturase. As the effects of LA and GLA were measurably different, this suggests that little of the LA is converted in NHU and HU-E6 cells. GLA is more antiproliferative than the other PUFA used here, and pilot clinical trials have suggested a role in cancer therapy, including a Phase II trial in breast cancer in which dietary supplementation with GLA was used in conjunction with tamoxifen (31). GLA has also been suggested as an intravesical therapy in bladder cancer (32). However, our results signal caution in the use of GLA in treatment against carcinoma cells as *a*) regenerating normal epithelial cells are sensitive to growth inhibitory effects of n–3 and n–6 PUFA and *b*) cells with genomic instability may acquire resistance.

PUFA have been shown to promote apoptosis in pancreatic cancer cells (33), and in fibroblast and lymphoblastoid cell lines (18). The relative resistance of urothelial cells to apoptosis, combined with a tendency to become irreversibly cytostatic (a phenomenon that has the hallmark "interphase" or "reproductive" cell death), may reflect a functional adaptation permitting retention of specialized urothelial barrier function in the context of cytotoxic damage by compounds in the urine.

To conclude, using a normal epithelial cell culture system, we have been able to show conclusively that n–3 and n–6 PUFA elicit distinct antiproliferative and irreversible cytostatic effects, both acting via p53-independent mechanisms. Our results also show that the antiproliferative effects of n–3 and n–6 PUFA are not tumor specific and indeed that cells with genetically unstable karyotypes can acquire resistance to n–3 and n–6 PUFA. This advocates caution in the use of PUFA as chemotherapeutic adjuncts.

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