Fish oil constituent docosahexa-enoic acid selectively inhibits growth of human papillomavirus immortalized keratinocytes

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The omega-3-fatty acids inhibit proliferation of breast cancer cells whereas omega-6-fatty acids stimulate growth. In this study, we examined effects of these fatty acids on human pre-cancerous cells. Cervical keratinocytes, immortalized with the oncogenic human papillomavirus (HPV) type 16, were treated with linoleic acid, an omega-6-fatty acid, and the omega-3-fatty acids, eicosapentaenoic and docosahexaenoic acids. Using both cell counts and bromodeoxyuridine incorporation, docosahexaenoic acid inhibited growth of these cells to a greater extent than eicosapentaenoic acid. Linoleic acid had no effect. The effect of docosahexaenoic acid was dose dependent and caused growth arrest. Docosahexaenoic acid inhibited growth of HPV16 immortalized foreskin keratinocytes and laryngeal keratinocytes grown from explants of benign tumors caused by papillomavirus, but had no effect on normal foreskin and laryngeal keratinocytes. Docosahexaenoic acid inhibited growth in the presence of estradiol, a growth stimulator for these cells. Indomethacin, a cyclooxygenase inhibitor like docosahexaenoic acid, had only minimal effect on growth. α-Tocopherol, a peroxidation inhibitor, abrogated effects of docosahexaenoic acid implying that inhibitory effects were via lipid peroxidation.

Introduction

The omega-3-fatty acids, found in fish oils from cold water fish, such as salmon and cod, inhibit proliferation and metastasis of many cancerous cells such as malignant breast cells (1). Epidemiology studies (2), animal studies (3–5) and in vitro cell culture studies (6,7) support a role for omega-3 fatty acids as adjunct therapy in prevention and treatment of breast cancer. Moreover, the omega-3-fatty acids are useful in the treatment of inflammatory diseases (8–10) including epithelial inflammatory diseases such as psoriasis (11–16).

The primary goal of this study was to determine whether the omega-3 fatty acids would inhibit growth of pre-cancerous cells prone to malignant conversion. Pre-cancerous lesions of the cervix are a recognizable type lesion associated with infection with certain types of human papillomaviruses (HPVs), e.g. types 16 and 18. Malignant conversion results in cervical cancer. In cell culture, these HPVs can immortalize genital keratinocytes, and such cells are considered pre-cancerous cells (reviewed in refs 17,18). These HPV immortalized cells provide an in vitro system to start to determine whether omega-3-fatty acids would inhibit their growth and thereby provide some protection against malignant conversion.

The secondary goal focused on the mechanism of growth inhibition caused by omega-3-fatty acids. Possibilities include lipid peroxidation, inhibition of cyclooxygenase, modulation of estrogen metabolism and estrogen receptor binding. Polyunsaturated fatty acids (PUFAs) have double bonds that are sensitive to peroxidation. The resulting peroxides are toxic to certain cells. Tumor cells lack desaturated essential fatty acids. Addition of PUFAs, such as the omega-3 fatty acids, causes cell death associated with a surge of lipid peroxidation and of superoxide formation. Normal cells are largely unaffected. In the absence of unsaturated essential fatty acids, tumor cells are resistant to lipid peroxidation (reviewed in ref. 19). Omega-3-fatty acids inhibit cyclooxygenase and thereby favorably modulate eicosanoid biosynthesis in ways that prevent tumor growth. The most notable result is that arachidonic acid and prostaglandin levels decrease in the presence of omega-3-fatty acids (20,21). Additionally, omega-3-fatty acids have the potential of suppressing estrogen enhancement of growth of certain tumors in several ways. Increased 2-hydroxylation of estradiol occurs in MCF-7 cells grown with omega-3-fatty acids, which results in more estradiol converted to inactive estrogens (22). Additionally, the omega-3-fatty acid docosahexaenoic acid (DHA) causes decreased binding and affinity of estradiol for the estrogen receptor in MCF-7 cells (23).

In this study, we show that DHA inhibits growth of cervical cells immortalized by the highly oncogenic HPV16, foreskin keratinocytes immortalized by HPV16, and keratinocytes grown from papillomas with an HPV etiology. We do not observe this effect on normal keratinocytes. The mechanism of this growth inhibition is consistent with lipid peroxidation.

Materials and methods

Cells and cell culture

HPV16 immortalized cervical and foreskin keratinocytes were kindly provided by Craig Woodworth, National Cancer Institute. Keratinocytes from explants of laryngeal papillomas (surgical discards) were derived as described by Steinberg et al. (24), and keratinocytes of explants of foreskin were derived as described by Rheinwald and Green (25). Medium for maintenance and growth of cells was F12-DMEM medium supplemented with epidermal growth factor (EGF), hydrocortisone, cholera toxin, insulin and 5% fetal bovine serum.

Reagents

Eicosapentaenoic acid (EPA), DHA and linoleic acid (>99% purity) were purchased from Sigma (St Louis, MO) and stored under nitrogen after opening. Fatty acids were prepared freshly by dissolving in 99% ethanol or dimethyl sulfoxide (DMSO). The final ethanol or DMSO concentration in media was 0.1%. α-α-Tocopherol (vitamin E) and indomethacin were also purchased from Sigma.

Proliferation assays

For cell counts, cells were plated to give a density of 10^4 cells/cm^2 in 16 mm wells. Treatment (e.g. medium with fatty acid) was started 1 day later unless otherwise indicated. Control cultures contained solvent (ethanol or DMSO) as used in experiment and at a final concentration in the medium of 0.1%. Medium was changed every 48 h. After washing cells three times in phosphate-
buffered saline (PBS) and separating cells with trypsin and vigorous pipetting, the number of cells was counted using an electronic cell counter (Coulter Electronics, Hialeah, FL). For bromodeoxyuridine (BrdU) incorporation, cells were incubated in medium containing 3 μg/ml of 5-bromo-2′-deoxyuridine (Sigma) for 24 h before fixing with 70% alcohol. Fixed cells were treated with mouse monoclonal antibody to BrdU (Becton Dickinson, Lincoln Park, NJ) followed by staining by the ABC method using diaminobenzidine (Vector Laboratories, Burlington, CA). The percentage of positive cells was determined by counting 500–1500 cells per individual condition.

**Cell viability assay**

Viability was measured by the ability to exclude trypan blue dye. Trypsinized cells were incubated with 0.2% trypan blue (Gibco, Grand Island, NY) and examined macroscopically. The percentage of cells that excluded trypan blue was determined by counting 500–1500 cells per individual condition.

**Results**

To begin to determine whether diets rich in omega-3-fatty acids inhibit growth of HPV immortalized cervical cells, we added DHA and EPA, the major omega-3-fatty acids found in fish oil from cold water fish, to the growth medium of these cells. Linoleic acid, an omega-6-fatty acid, found in corn oil and many other sources, was also evaluated. We had hypothesized that omega-6-fatty acids would enhance proliferation of HPV immortalized cervical cells whereas omega-3-fatty acids would be growth inhibitory.

**Omega-3-fatty acids inhibited growth of HPV immortalized cervical cells**

As shown in Figure 1, both DHA and EPS inhibited the growth of HPV16 immortalized cervical keratinocytes. DHA inhibited growth more than EPA. Linoleic acid neither stimulated nor inhibited growth. A growth curve was carried out over 11 days with replenishment of new growth medium every 48 h. The effect of DHA was further investigated. Figure 2 shows that growth inhibition was dependent on the concentration of DHA. Fewer cells replicated as the concentration of DHA increased over a 10-fold concentration. The cell numbers (Figure 2A) or BrdU incorporation (Figure 2B) were measured after 10 days of treatment with DHA, a time when maximum inhibition peaked. The cell numbers or percentage of replicating cells were decreased 3- to 4-fold using concentrations of DHA between $10^{-5}$ and $10^{-4}$ M.

**DHA caused growth arrest rather than acting as a cytotoxic**

The percentage of viable cells was virtually identical to that of control when cells were treated with DHA for 4 days (Figure 3). After 15 days of treatment, a trend toward lower viability, which was more apparent at higher concentrations, occurred with continued exposure to DHA. In a separate type of evaluation (Figure 4), we determined that growth of cells was reversible after removal of DHA. Growth of cells treated with DHA was compared between cells after removal, or not, of DHA. Cells immediately started growing at a faster rate after removal of DHA.

**DHA caused growth inhibition of other cells containing HPV**

DHA inhibited the growth of HPV16 immortalized foreskin keratinocytes similar to that of the HPV16 immortalized cervical keratinocytes (Figure 5). Additionally, DHA inhibited growth of keratinocytes grown from explants of laryngeal papillomas: benign tumors that contained HPV types 6 or 11. In fact, the effect was much greater with the papilloma cells than with immortalized cells. Cell counts were used for this assay.

**DHA did not inhibit growth of normal keratinocytes**

By using BrdU incorporation, we compared the effect of DHA on normal keratinocytes grown from explants of foreskin and from HPV16 immortalized foreskin keratinocytes (Figure 6). DHA inhibited HPV immortalized foreskin cells but had no effect on the normal cells. We compared the effect of DHA on keratinocytes grown from explants of normal larynx and laryngeal papillomas. DHA inhibited papilloma cells but had no effect on normal cells. Together, these data show that DHA has a profound growth inhibitory effect on HPV containing cells but not on normal cells.

**Evaluation of the mechanism of growth inhibition by DHA**

Omega-3-fatty acids exert growth inhibition by a number of mechanisms. We wanted to evaluate the mechanism of the growth inhibition of HPV immortalized cervical cells. The percentage of replicating cells was determined after 7 days of treatment. Since both indomethacin and DHA inhibit cyclooxygenase (26), we asked whether indomethacin had a growth inhibitory effect similar to that of DHA (data not shown). Indomethacin had no significant effect on the growth of HPV immortalized cervical cells. Additionally, the growth of cells was not different with DHA alone or DHA and indomethacin. Hence, inhibition of cyclooxygenase does not appear to account for the growth inhibition caused by DHA. Estradiol enhances growth of HPV immortalized cervical cells (27). As shown in Figure 7, growth inhibition occurred in the presence of estradiol, which means that estradiol did not antagonize or compensate for the DHA effect. If DHA has an effect on estradiol, such as modulating estrogen metabolism (22) or inhibiting receptor binding (23), the effect of DHA in this assay was much greater than abrogation of estrogen enhancement. DHA is subject to lipid peroxidation. Antioxidants prevent lipid peroxidation. Therefore, if DHA inhibited growth of HPV-containing cells via lipid peroxidation, we would expect that antioxidants would antagonize the effect of DHA growth. As shown in Figure 8, α-tocopherol (vitamin E) significantly abrogated the growth inhibiting effect of DHA. A time course was performed using α-tocopherol at 10 and 100 μM. The results confirmed that α-tocopherol prevented
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Fig. 2. Growth inhibition caused by DHA was dose-dependent. Parallel cultures of HPV16 immortalized cervical cells were grown in medium containing various concentrations of DHA. BrdU was added 24 h before assay (B). The number of cells per well (A) and percentage of cells that incorporated BrdU (B) were determined at 10 days.

Fig. 3. DHA did not alter the percentage of viable cells after 4 days in medium containing DHA. The effect of DHA after 15 days of treatment was minimal. Parallel cultures of HPV16 immortalized cervical cells were grown in medium containing various concentrations of DHA. The percentage of viable cells (able to exclude trypan blue) was determined at 4 and 15 days.

growth inhibition by DHA (Figure 9). Both concentrations of \(\alpha\)-tocopherol worked equally well. Additionally, \(\alpha\)-tocopherol appeared to have a slight inhibitory effect compared with the control at 7 days but not at earlier times (treatment for 3 and 5 days). None the less, \(\alpha\)-tocopherol abrogated the growth inhibitory effect of DHA at all time points. Therefore, we conclude that lipid peroxidation likely caused the growth inhibition of these cells by DHA.

**Discussion**

In cell culture, we determined that the omega-3-fatty acid, DHA, not only caused growth arrest of pre-cancerous cells (keratinocytes immortalized by the highly oncogenic HPV16) but also growth inhibition of keratinocytes from benign tumors with an HPV etiology. DHA did not inhibit growth of normal keratinocytes. This suggests a difference in the lipid content of keratinocytes affected by HPV pathology versus normal cells. The DHA effect was apparently the result of lipid peroxidation since \(\alpha\)-tocopherol, an antioxidant, abrogated this growth arrest. Our results parallel some results observed with breast cells. Both EPA and DHA cause growth arrest of breast cancer cells in culture, and this growth arrest is abrogated by vitamin E (6). The expectation is that lipid peroxidation would be lethal to cells. Both the breast culture studies and our studies indicate that the immediate effect of DHA is growth arrest. Killing of cells apparently takes time and continued exposure, and this possibility is supported by our data. Others report suppressive effects of lipid peroxidation products formed
Fig. 4. Cell growth resumed after removal of DHA. Medium that contained DHA (60 µM) was added to HPV16 immortalized cervical cells 5 days after plating 10^4 cells. In parallel cultures at 8 days, growth continued in medium with DHA (solid line) or without DHA (dotted line). Cell counts were determined at 3, 5, 8, 10 and 12 days.

Fig. 5. DHA inhibits other HPV containing cells. Medium that contained DHA (60 µM) was added to HPV16 immortalized cervical keratinocytes (HPV-CX), HPV16 immortalized foreskin keratinocytes (HPV-FS) and keratinocytes grown from the explants of laryngeal papillomas (LX-Papilloma). Cell counts were determined at 7 days in cells grown with and without DHA. P-values between DHA-treated and the control were <0.001, 0.01 and 0.001 for HPV-CX, HPV-FS and LX-Papilloma, respectively.

Fig. 6. DHA inhibits cells that contain HPV but not normal cells. DHA (60 µM) was added to the growth medium of normal foreskin keratinocytes (FS), HPV16 immortalized foreskin keratinocytes (HPV-FS), normal laryngeal keratinocytes (LX) or keratinocytes from laryngeal papillomas (LX-Papilloma). The incorporation of BrdU was determined at 7 days. The P-value (treated compared with control) was <0.001 for HPV-FS and LX-Papilloma, respectively.

from omega-3-fatty acids on breast cancer growth in immuno-suppressed mice. This is in spite of supplementing the dietary omega-3-fatty acid ethyl esters with vitamin E (28). Together, our results suggest that cells from HPV induced papillomas or HPV immortalized cells behave like cancer cells in response to omega-3 fatty acids and lack unsaturated essential polyunsaturated fatty acids.

The growth arrest we observed occurred in the presence of estradiol. The importance of this observation is that estrogen promotes the pathology of HPV lesions. Greater than 90% of HPV lesions and cancers in the genital epithelium occur in the most estrogen sensitive cells (transformation zone in the cervix), even though the rest of the genital tract of both men and women becomes infected with HPV (29–31). In a mouse model, estrogen exacerbates HPV pathology and malignant conversion (32). In cell culture, estrogen enhances growth of HPV16 immortalized cells including anchorage-independent growth (27).

In vivo, omega-3-fatty acids have additional effects that
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Fig. 7. Growth inhibition of HPV16 immortalized cervical cells is much greater than any estrogen enhancement. Estradiol (E\(_2\) (10\(^{-6}\) M), DHA (60 \(\mu\)M), or E\(_2\) and DHA was added to the growth medium for HPV16 immortalized cervical keratinocytes. The incorporation of BrdU was determined at 7 days. The P-value for DHA compared with the control was \(\leq 0.02\). The P-value for DHA and E\(_2\) compared with the control was \(\leq 0.02\).

Fig. 8. \(\alpha\)-Tocopherol abrogates the growth inhibition caused by DHA. \(\alpha\)-Tocopherol (VitE) (100 \(\mu\)M), DHA (60 \(\mu\)M) or vitamin E (100 \(\mu\)M) plus DHA (60 \(\mu\)M) were added to the growth medium for HPV16 immortalized cervical keratinocytes. The incorporation of BrdU was determined at 7 days. The P-value for DHA compared with control was \(\leq 0.02\). The P-value for DHA plus vitamin E compared with control was \(\leq 0.7\).

by HPV infection with highly oncogenic HPVs (35). The immortalized HPV cervical keratinocytes are considered to be equivalent to pre-cancerous cells since they simulate the pathology of pre-cancerous lesions (36). In these cells, HPV DNA becomes integrated in cellular DNA as usually occurs in cancers (37,38) and can become malignant after repeated passage or introduction of activated ras oncogene (39,40). Hence, the population of women with pre-cancerous lesions of the cervix might benefit from increased dietary omega-3-fatty acids. While not the focus of this study, DHA did inhibit growth of keratinocytes grown from explants of laryngeal papillomas: benign tumors caused by HPV with a low oncogenic potential. Since this disease has significant morbidity (41), the possibility of adjunct therapy with omega-3-fatty acids is worth investigating. Importantly, this growth inhibition was specific to the HPV infected and immortalized cells and not normal cells. Studies using animal models for HPV disease would be the next logical step to determine effectiveness of omega-3-fatty acids as a useful dietary supplement for prevention of HPV pathology and malignant conversion.

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References
