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

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Introduction

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.

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 (26).

Reagents
Eicosapentaenoic acid (EPA), DHA and linoleic acid (>99% purity) were purchased from Sigma (St Louis, MO) and stored under nitrogen after opening. 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-
The cell numbers or percentage of replicating cells or BrdU incorporation (Figure 2B) were measured after 10 days over a 10-fold concentration. The cell numbers (Figure 2A) 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 cytotoxin**

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. 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...
Fatty acids and HPV immortalized keratinocytes

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 α-tocopherol worked equally well. Additionally, α-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, α-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 α-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 immunosuppressed 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
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


Fig. 7. Growth inhibition of HPV16 immortalized cervical cells is much greater than any estrogen enhancement. Estradiol (E₂) (10⁻⁶ M), DHA (60 μM), or E₂ 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 P<0.02. The P-value for DHA and E₂ compared with the control was P<0.02.

Fig. 8. α-Tocopherol abrogates the growth inhibition caused by DHA. α-Tocopherol (VitE) (100 μM), DHA (60 μM) or vitamin E (100 μM) plus DHA (60 μM) 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 P<0.02. The P-value for DHA plus vitamin E compared with control was P<0.07.

Fig. 9. α-Tocopherol abrogates the growth inhibition caused by DHA over a range of time and concentrations. Vitamin E, DHA (60 μM) or vitamin E and DHA (60 μM) were added to the growth medium of HPV16 immortalized keratinocytes. Cell counts were determined at 3, 5 and 7 days.
relationship to peroxides and vitamin E. *Breast Cancer Res. Treat.*, 34, 199–212.


