n-3 Fatty Acids Decrease Colonic Epithelial Cell Proliferation in High-Risk Bowel Mucosa

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ABSTRACT: The n-3 fatty acids (C20:5, eicosapentaenoic acid; C22:6, docosahexaenoic acid) may be important in the development, growth, and metastasis of colon cancer, a leading cause of death in North America. Patients who have had a bowel neoplasm have a high risk of developing a second neoplasm, and this risk is associated with a high percentage of cells correspond to the S phase of bromodeoxyuridine (BrdUrd) labeling in mucosal epithelial cells. To determine the effect of n-3 fatty acid supplementation on DNA synthesis of rectal mucosa, patients with stage 1 or stage 2 colon carcinoma or adenomatous polyps were randomized to consume either 9 g/d n-3 fatty acid capsules or 9 g/d placebo capsules. Plasma phospholipid fatty acid analysis and proctoscopic mucosal biopsies were performed at baseline, 3, and 6 mon. Colonic crypts were isolated from the mucosa, disassociated with enzymes, and incubated with BrdUrd, and %S phase was measured by flow cytometry. The plasma phospholipid n-6/n-3 ratio was determined by gas chromatography. Supplement compliance was assessed by plasma phospholipid n-6/n-3 ratio. Mean capsule consumption in these two group was 82%. Prior to supplementation, there were no significant differences in the %S phase and the plasma n-6/n-3 ratio between these groups. Patients whose colonic epithelial cells indicated hyperproliferation at baseline showed a strongly positive correlation to the %S phase of BrdUrd uptake and the n-6/n-3 ratio. There was no significant change after n-3 treatment in patients with low baseline. Those in the placebo group showed no significant difference in n-6/n-3 ratio, although there was an increase in the %S phase of BrdUrd uptake at 6 mon. The n-3 group did not have significant side effects, and polyps were not found after completing 12 mon of n-3 fatty acid supplementation. This study suggests that n-3 fatty acid may be a useful chemopreventive agent in some patients as reflected in a plasma biomarker of colon tumor growth and metastasis. A low plasma phospholipid n-6/n-3 fatty acid ratio may serve as a nutritional marker that is associated with colonic epithelial cell hyperproliferation in the n-3–supplemented group as compared with the placebo group.Characteristics of mucosal proliferation at baseline may be a crucial factor for the effect of n-3 fatty acid supplementation.
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Abbreviations: BrdUrd, bromodeoxyuridine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FITC, fluorescein isothiocyanate; LI, labeling index.

An estimated 133,500 cases of colorectal cancer were diagnosed in the United States in 1996 (1). Colorectal cancer is the second leading cause of cancer mortality and will result in over 54,900 deaths in the United States. Epidemiological and laboratory animal studies appeared that linked high fat consumption with colorectal cancer (2). The low colon cancer rate among Alaskan and Greenland Eskimos has been associated with high consumption of fish (3,4). Animal studies indicated that the primary effect of fat is exerted on the promotion stage of carcinogenesis, with the n-6 fatty acid having been recognized as a major promoter (5–7), whereas fish oil containing the n-3 fatty acids eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) are found to reduce tumor incidence, growth, and metastasis (8–11). The proposed pathophysiological mechanisms of fish oil protective effects include influences on the mucosal prostaglandin synthesis, reducing ornithine decarboxylase and tyrosine protein kinase activities, which has been shown to exert a proliferative effect on the colonic mucosa (12,13).

Colorectal adenomatous polyps with abnormal cell–kinetics characteristics has been suggested to be putative precursor lesions to carcinoma (14). Bruce *et al.* (15) and Lippmann *et al.* (16) showed that hyperproliferation of colonic epithelium is an appropriate intermediate end-point for chemopreventive intervention.

By the support of prior evidence, we conducted this clinical trial by use of objective flow cytometric assay. The aim of our study was to investigate the tolerance and effect of long-term n-3 fatty acid supplementation on DNA synthesis in high risk bowel mucosa and the efficacy of the plasma phospholipid n-6/n-3 ratio as a nutritional marker on the prevention of colonic tumor development and metastasis.

EXPERIMENTAL PROCEDURES

Patients with resected Dukes's A or B adenocarcinoma of the colon or rectum or severely dysplastic adenomatous polyps were recruited. All patients were eligible only after complete excision of their cancers as well as of any associated polyps. Twenty-seven patients were (2:1) randomly assigned to double-blind treatment groups: 17 for the n-3 fatty acid group and 10 for the placebo group. The n-3 capsules were composed of IND #041821 (NAOO, Charleston, SC), an n-3 ethyl ester

concentrate prepared from vacuum-deodorized menhaden oil using transesterification, urea adduction, and short-path distillation. The concentrate contained approximately 80% n-3 fatty acid ethyl esters (44% EPA, 24% DHA, 10–12% other n-3 fatty acid ethyl esters). The placebo ethyl ester (NAOO) was prepared from corn oil. The preparation contained approximately 50% linoleic ethyl ester, 10% C_{16}, 15% other C_{18} fatty acid ethyl esters. Both capsules contained 0.2 mg/g 1,5-di-*tert*-butyl-1,4-benzohydroquinone as antioxidant, 2 mg/g tocopherols, and 2.0 mg/g cholesterol. Cholesterol was added to the placebo capsules to match the n-3 capsules. Patients were required to consume 9 capsules per day, 1 g per capsule.

Cell proliferation study: BrdUrd incorporation. Mucosal tissue obtained by proctoscopic mucosal biopsy was incubated for 20 min in 1.5 mM EDTA and 1.3 mM dithiothreitol in calcium-free, phosphate-buffered saline to separate crypt cells. After the suspension was precipitated on ice, crypt cells were prepared for enzymatic digestion. The cell suspension was filtered through a 0.22 μm sterile membrane. Single-cell suspensions were incubated at 37°C in RPMI (pH 7.2–7.4) containing 5% fetal calf serum, 20 μM fluorodeoxyuridine, and 30 μM BrdUrd for 1 h. After BrdUrd labeling, the cells were fixed in ice-cold 70% ethanol. Ethanol-fixed cells were added to 2 N HCl with 0.2% pepsin to denature the DNA for detection of BrdUrd by the fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody. After incubation for 40 min in the dark, the cells were centrifuged and neutralized with 0.1 M sodium tetraborate (pH 8.5). Cells were washed and incubated with 20 μl of FITC-conjugated anti-BrdUrd antibody (Becton Dickinson, San Jose, CA) for 45 min in the dark. Nuclei labeled with BrdUrd-anti-BrdUrd-FITC were then washed and resuspended in 0.01 mg propidium iodide. After 15 min of staining, cells were ready for analysis by flow cytometry.

Flow cytometry. Cytometric analysis was performed on a fluorescence activated cell sorter (FACS) (Becton Dickinson, Mountainview, CA). Cells were excited with a 15 mW laser at 488 nm. Red fluorescence was collected through a 585-nm bandpass filter and recorded as the total amount of DNA. Green fluorescence of the BrdUrd-labeled cells was collected through a 530-nm bandpass filter and recorded as the amount of incorporated BrdUrd. Data were collected in a 64 x 64 channel distribution showing the total DNA (red) against the logarithmic amount of incorporated BrdUrd (green).

Fatty-acid analysis. Plasma phospholipid fatty acid was extracted by use of a 2:1 mixture of chloroform/methanol. Hepatadecanoic acid (Nu-Chek-Prep, Elysian, MN) was added as an internal standard prior to extraction. The chloroform layer was dried, and thin-layer chromatography with LK5D silica gel plates (Whatman Chemical Separations, Inc., Clifton, NJ) was applied to separate various lipid fractions. The solvent system used to develop the plates was a mixture of petroleum ether/diethylether/glacial acetic acid (80:20:1). Phospholipid fractions were scraped and placed into teflon-lined, screw-cap glass tubes and hydrolyzed and methylated under nitrogen in a steam bath for 45 min by 14% boron/trifluoride in methanol (Alltech Associates, Deerfield, IL).

Gas–liquid chromatography. Fatty acid methyl esters were separated and quantified by use of a Hewlett Packard (Palo Alto, CA) 5890 Series II gas chromatograph equipped with a 30-M fused silica capillary column (0.32 mm I.D., 0.20 μm film thickness, Supelco, Bellefonte, PA) and a flame ionization detector. Helium was used as the carrier gas with a flow rate of 5 mL/min. The split ratio used was 14.3:1 with injector and detector temperatures at 240°C. Oven temperature was programmed to start at 70°C, then rise to 130°C at a rate of 10°C/min; then to 150°C at a rate of 2°C/min; then reach 195°C at a rate of 3°C/min. Peaks were identified by comparison to authentic standards (Nu-Chek-Prep) by a Hewlett Packard 7673 controller. Quantitation was based on the relative responses of the internal standard and fatty acid methyl esters in the sample as compared with the responses of standards. Results were expressed in absolute concentrations (μmol/L plasma) as well as a relative percent of the total fats analyzed.

RESULTS

Fatty acid analysis of the plasma phospholipid showed that EPA and DHA contents increased over 6 mon in the n-3–supplemented group, whereas levels of linoleic acid and arachidonic acid decreased (Figs. 1 and 2). The mean of the plasma n-6/n-3 ratio in the n-3 group decreased over 6 mo. Unlike the n-3 group, the placebo group showed no significant changes in the concentration and relative percent of EPA, DHA, linoleic acid, arachidonic acid, and the n-6/n-3 ratio (Figs. 3 and 4).

The BrdUrd labeling index (LI) of colonic mucosa in the n-3 group showed a strongly positive correlation to the plasma phospholipid n-6/n-3 ratio. It is interesting that there was a wide range of BrdUrd labeling index among subjects before starting this trial. From the pattern of mucosal proliferation at baseline, it can be classified into two phenotype categories: high percent of BrdUrd-labeled cells in the S phase relative to the total number of cells examined (>10%S phase of BrdUrd LI, n = 7) and low proliferation (<10%S phase of

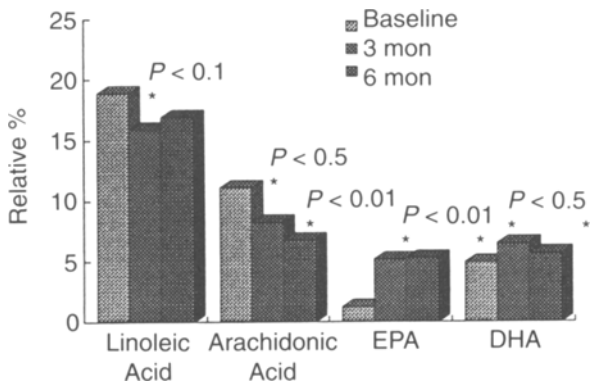

FIG. 1. Plasma phospholipid fatty acid profile of patients with high baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

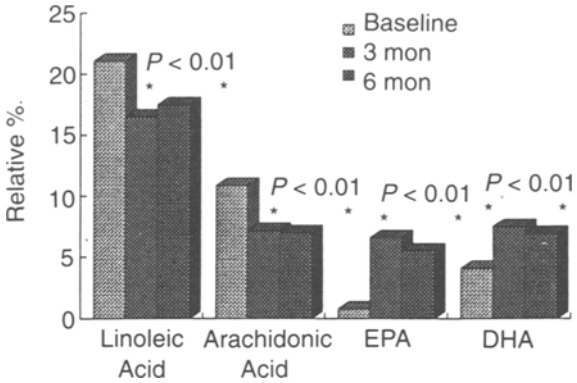

FIG. 2. Plasma phospholipid fatty acid profile of patients with low baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test. Abbreviations as in Figure 1.

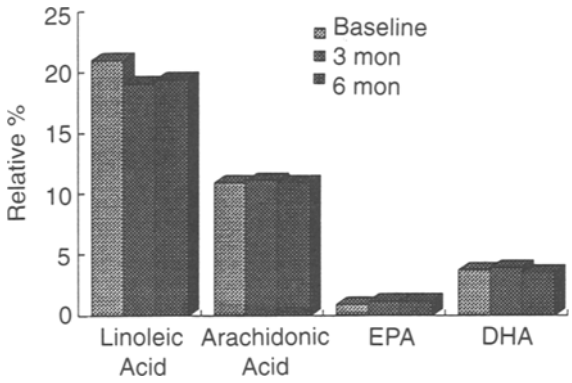

FIG. 3. Plasma phospholipid fatty acid profile of patients on placebo. Abbreviations as in Figure 1.

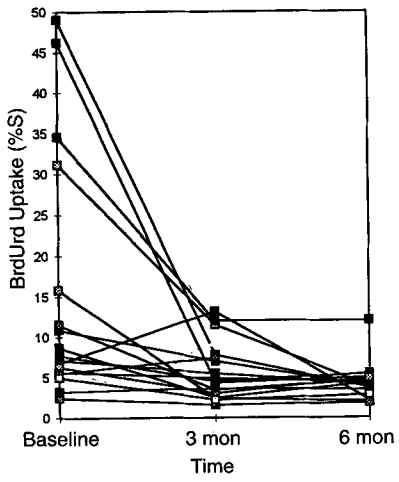

FIG. 4. Plasma n-6/n-3 and proliferation rate on placebo group.

BrdUrd LI, n = 10). It is important to note that the responses of decreasing BrdUrd LI to n-3 fatty acid supplementation mostly resulted from reductions of patients whose baseline patterns showed abnormal or hyperproliferation (Fig. 5). All

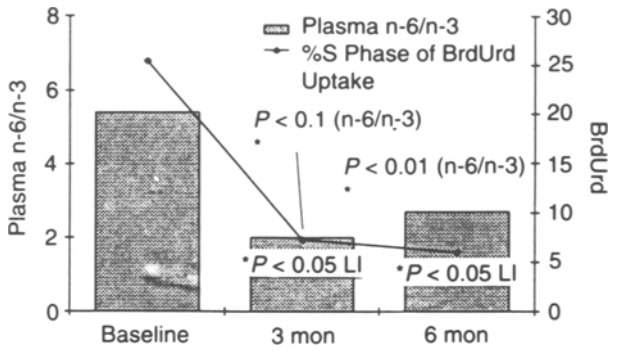

FIG. 5. Bromodeoxyuridine (BrdUrd) uptake in n-3 group.

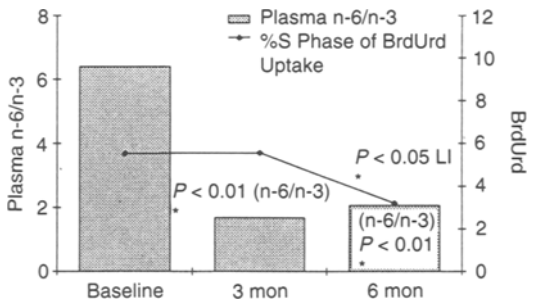

FIG. 6. Patients with high baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test. Abbreviation as in Figure 5.

hyperproliferative patients were found to have significantly decreased BrdUrd LI after 3 mon of n-3 treatment. In contrast, there was no dramatic change over time in those with low baseline LI in n-3. Subjects with high baseline LI decreased the mean proliferation rate of epitheial cells from 25.5 to 7.3 at 3 mo, along with the decline of the plasma n-6/n-3 ratio from 5.4 to 2.0. (Fig. 6). In contrast, other subjects with low baseline tended to maintain the proliferation rate, even the plasma n-6/n-3 ratio significantly decreased from 6.4 to 1.7 at 3 mon (Fig. 7). In the placebo group, the %S phase of BrdUrd uptake was increased from less than 6 to 9% at 3 mon, and to over 10% at 6 mon (Fig. 7). Despite the additional 4.5 g per d of linoleic acid consumption, the plasma n-6/n-3 ratio in the placebo group showed no significant difference. In addition, prior to supplementation, there were no significant differences in the plasma phospholipid n-6/n-3 ratio between these groups.

The fish oil supplement was well tolerated by study participants. There were no side effects, and no additional polyps were found in 12/12 patients who completed 12 mon of n-3 supplementation. Plasma fatty acid levels and capsule counts (82%) indicated good overall compliance. None of the patients experienced significant body weight gain or loss or any other physiological changes. Responses of the fatty acid profile and mucosal proliferation rate to n-3 was noticed as soon as three months after treatment started.

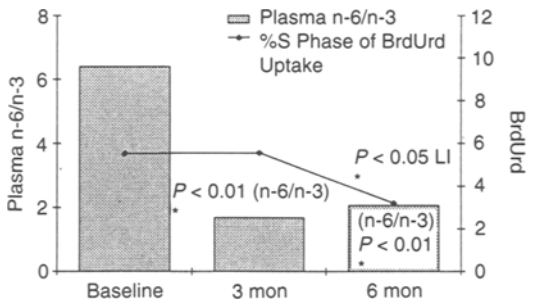

FIG. 7. Patients with low baseline proliferation on n-3 fatty acid supplement; *P* values were analyzed by Student's *t*-test. Abbreviation as in Figure 5.

DISCUSSION

In our study, the plasma phospholipid n-6/n-3 ratio successfully predicted responses of mucosal LI to fish oil supplementation. Increased incorporation of 22:6n-3 (DHA) and 20:5n-3 (EPA) and decreased incorporation of 20:4n-6 (AA) are reliable indices of n-3 supplement ingestion. Previous studies also showed that high fish oil intake altered the plasma fatty acid profile and incorporation of n-3 fatty acids into colonic mucosal cells (17). The plasma fatty acid profile has been shown to parallel the tissue status (18). Due to limitation of obtaining rectal biopsy specimens, we developed this plasma phospholipid n-6/n-3 ratio as a nutritional compliance marker. This new finding can be beneficial for the future design of fish oil chemoprevention trials.

Patients in the n-3 group experienced significant reductions in the BrdUrd LI. The effect of n-3 fatty acids was found exert only in patients with hyperproliferative patterns in this study. It seems that patients who had abnormal patterns of colonic cell proliferation benefited more from fish oil supplementation. Occurrence of adenomatous polyps in those patients who have normal baseline patterns may be not related to different types of fat intake. It has been suggested that more than one genetic pathway leads to the development of adenocarcinoma of the colon and rectum (19). It is possible that the effect of fatty acids on lowering abnormal proliferation is only capable in hyperproliferation. Anti *et al.* (18) previously reported a similar discrepancy among subjects' proliferation patterns at baseline. Our data shows that the LI of hyperproliferation could reach more than 30% BrdUrd LI, although previous data indicated that the highest percentage of LI was only in the low 20s by use of [^{3}H]thymidine autoradiography. Unlike Italian diets based on olive oil, which is low in linoleic acid, however, North American diets consume much more n-6 polyunsaturated fatty acid, which consists of more than 50% safflower, sunflower, soybean, corn, and cottonseed oils (19, 20). In our study, there was approximately 15 g/d of linoleic acid (18:2n-6) in the average diet of 30% fat content in both groups. Because of the competition between n-3 and n-6 for eicosanoid formation in the phospholipid of cell membranes, it is possible that chemoprevention studies conducted in North America need a longer period of treatment and a higher supplemental dose of n-3 fatty acids (21).

In conclusion, these cases suggest that n-3 fatty acids may be useful chemopreventive agents, as reflected in a plasma biomarker of colon proliferation tumor growth and metastasis. A decrease in the plasma phospholipid n-6/n-3 ratio can serve as a nutritional marker associated with colonic epithelial cell proliferation in the n-3–supplemented group compared with the placebo group. More studies must be undertaken, however, to test whether n-3 fatty acids are effective in inhibiting mucosa neoplastic proliferation in various genetic pathways.

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