Review article

n-3 Fatty acids and lipid peroxidation in breast cancer inhibition

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Long-chain *n*-3 fatty acids (FA) consistently inhibit the growth of human breast cancer (BC) cells both in culture and in grafts in immunosuppressed mice. Large cohort studies have, however, failed to confirm a protective effect for fish oils rich in *n*-3 FA against BC risk. The present review examines new evidence on biological mechanisms which may be involved in the inhibition of mammary carcinogenesis by long-chain *n*-3 FA, focusing on an apoptotic effect by its lipid peroxidation products. Dietary intake of *n*-3 FA leads to their incorporation into cell membrane lipids. Increased apoptosis in human BC cells following exposure to long-chain *n*-3 FA such as eicosapentaenoic and docosahexaenoic acids is generally ascribed to their inhibition of cyclooxygenase 2 which promotes mammary carcinogenesis. In addition however, long-chain *n*-3 FA are particularly likely to activate peroxisome proliferator-activated receptor (PPAR)-γ, a key regulator of lipid metabolism but also capable of modulating proliferative activity in a variety of cells including mammary cells. Expression of PPAR-γ in the nucleus is activated by second messengers such as J series prostaglandins and the latter have been shown to cause apoptosis *in vivo* in explants of human BC cells in immunosuppressed mice. In mammary tumours, it is observed that long-chain FA not only increase apoptosis, but also increase lipid peroxidation, and the apoptotic effect can be reversed by antioxidants. The rationale for use of *n*-3 FA dietary supplements in counteracting BC progression needs to be tested clinically in a phase 2 pilot study, while at the same time, the effect on whole-body lipid peroxidation needs to be monitored. Dietary supplements of fish oil rich in *n*-3 FA are proposed for premenopausal women over the age of 40 years who are shown to be at increased BC risk. Biological markers in breast tissue of BC progression will be monitored, and observed changes related to serial plasma levels of isoprostanes as a measure of whole-body lipid peroxidation.

Antioxidants: Apoptosis: Breast cancer: Dietary intervention: *n*-3 Fatty acids

Epidemiological studies in different countries have so far failed to identify which individual constituents of dietary fat might either enhance, or protect from, breast cancer (BC) risk in women. In the USA, a recent report from the Nurses' Health Study (Holmes *et al.* 1999) found no increase in risk associated with a higher intake of animal fat, polyunsaturated fat or transunsaturated fat. Nor was there any decrease in risk associated with a higher intake of vegetable fat, monounsaturated fat or *n*-3 fatty acids (FA) from fish oil. In the laboratory however, long-chain *n*-3 FA have consistently been shown to inhibit the growth of human BC cells both in culture and in explants in immunosuppressed mice (Wynder *et al.* 1997). α-linolenic acid is the parent *n*-3 FA and the long-chain group includes docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Dietary supplements of DHA and EPA have been shown to inhibit *in vivo* development of the carcinogen-induced rat mammary cancer model (Noguchi *et al.* 1997).

Dietary supplements of fish oil are widely taken in the west as a health aid by the general population. Fish oils are a rich source of long-chain *n*-3 FA, and international ecological studies have reported a higher *per capita* fish consumption to be associated with a lower BC incidence in the population (Kaizer *et al.* 1989; Hursting *et al.* 1990;

Abbreviations: BC, breast cancer; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; PPAR, peroxisome proliferator-activated receptor.

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Caygill *et al.* 1996). This was especially significant in populations with a high animal-fat intake (Caygill *et al.* 1996). However, large cohort studies in the USA (Toniolo *et al.* 1994) and Norway (Vatten *et al.* 1990) have failed to show a significant association between BC risk and fish consumption by individuals.

Clinical trials of long-chain *n*-3 FA have been delayed, partly because of lack of support from cohort studies, and also because of uncertainty as to the biological mechanisms involved. The present review examines: (1) effects of essential FA on mammary carcinogenesis; (2) recent evidence on the biological mechanisms involved; (3) role of lipid peroxidation in the induction of apoptosis in mammary cancer; (4) proposed clinical study.

Effect of essential fatty acids on mammary carcinogenesis

Essential FA comprise *n*-6 and *n*-3 types and both compete for incorporation into cell membrane phospholipids (Bartsch *et al.* 1999). In this way, the *n*-6:*n*-3 FA ratio can modulate membrane activity and thereby affect cell signalling and proliferative activity both in normal and neoplastic cells. Experimental studies show that the addition of *n*-6 FA stimulates the growth of human BC cells in culture, and that dietary supplements of corn oil (rich in *n*-6 FA) stimulate growth and metastasis in human mammary cancer explants in immunosuppressed mice (Rose *et al.* 1993). In contrast, the addition of long-chain *n*-3 FA inhibits the growth of human BC cells both in culture and in explants in immunosuppressed mice (Rose & Connolly, 1993). A similar contrast has been shown in the rat mammary cancer model (Karmali *et al.* 1984; Birt, 1990), where diets containing a high proportion of *n*-6 FA stimulate the growth of carcinogen-induced tumours, whilst the dietary addition of fish oils rich in long-chain *n*-3 FA causes an opposite effect.

The FA ratio in adipose deposits in the body reflects long-term intake of different types of FA. Since *n*-6 FA act as competitive inhibitors of *n*-3 FA in mammary carcinogenesis (Noguchi *et al.* 1995), the *n*-6:*n*-3 ratio in mammary adipose tissue has been studied in human BC specimens. A study of 642 cases in postmenopausal women (Simonsen *et al.* 1998) showed no positive association of BC with a higher *n*-6 FA content in adjacent adipose tissue, or negative association with a higher *n*-3 FA content. However, a higher *n*-6:*n*-3 FA ratio was significantly associated with a higher BC risk. The past 50 years has seen an increasing proportion of *n*-6 FA in the fat intake in industrialised western countries, and it has been suggested that this may be responsible for a large part of the increased risk of postmenopausal BC in western women (Yam *et al.* 1996).

Administration of dietary fish-oil supplements (10 g/d) has been shown to cause a significant increase in *n*-3 FA levels in breast adipose tissue and plasma in BC patients (Bagga *et al.* 1997). It has been reported that a higher *n*-3 FA level in adipose tissue is associated with a better prognosis in BC patients (Bougnoux *et al.* 1994) and also that a higher *n*-3 FA level in breast adipose tissue in women is associated with a lower BC risk (Klein *et al.* 2000).

Interaction between various types of FA in the aetiology of BC is suggested by an ecological study which reported an association between *per capita* fish consumption and a lower BC risk incidence, but particularly in populations with a higher animal-fat intake (Vatten *et al.* 1990). Also relevant is an epidemiological study showing that *n*-6 FA intake is associated with increased BC risk only when fat represents more than 30 % total energy intake, and another showing that *n*-3 FA protects against BC only when the main energy intake is from complex carbohydrates (Weisburger, 2000).

Biological mechanisms of effects by fatty acids on mammary carcinogenesis

A fat-rich diet increases the incidence of chemically-induced rat mammary cancer, and diets containing a high proportion of *n*-6 FA enhance the growth of normal rat mammary epithelium (Meade *et al.* 1999). The biological mechanisms are unclear but originally focused on the role of cyclooxygenase (COX) in the production of eicosanoids such as prostaglandin E_2. Tumour cells show a higher level of eicosanoids than do normal tissue cells (Cohen *et al.* 1986) and inhibition of eicosanoid production by *n*-3 FA is associated with growth inhibition of mammary cancer in animal models (Rose *et al.* 1996).

Linoleic acid is the parent *n*-6 fatty acid, and α-linolenic acid is the parent *n*-3 FA. Whereas linoleic acid is metabolised to arachidonic acid, α-linolenic acid is metabolised to EPA and DHA. Whilst α-linolenic acid and EPA are precursors of different prostaglandins, they compete with each other at the COX level, and ingestion of EPA can decrease production of prostaglandin E_2, which is an α-linolenic acid metabolite (Mantzioris *et al.* 2000).

The promotion of mammary carcinogenesis *in vitro* by *n*-6 FA is associated with enhanced expression of COX 2, whilst its inhibition by *n*-3 FA is associated with diminished production of COX 2 (Badawi *et al.* 1998). The observation suggests that COX 2 is involved in mammary carcinogenesis, but the molecular mechanism is uncertain. It is possible that COX-induced prostaglandin E_2 may enhance oestrogen synthesis in mammary stromal tissue thereby stimulating growth in hormone-dependent tumours. Human mammary cancer specimens show a strong correlation between oestrogen biosynthesis in the mammary stroma and the expression of COX 2 (Brueggemeier *et al.* 1999).

In addition to the COX-mediated pathway, an effect by dietary fatty acids on the cell membrane can influence signal transduction pathways in the cell. In this way, proliferative activity can be regulated through the protein kinase C transduction pathway, the *ras* gene or growth factor receptors (Bandyopadhyay *et al.* 1993). Protein kinase C activity is known to be higher in BC specimens than in normal breast tissue (Gordge *et al.* 1996) and changes in the cell membrane due to an altered FA ratio may thus influence the response of protein kinase C to stimulation by growth factors and hormones (Platet *et al.* 1998).

Recent research shows that in human BC cells which express the nuclear peroxisome proliferator-activated receptor (PPAR)-γ, FA can regulate gene expression through the PPAR pathway, in addition to other pathways (Sessler & Ntambi, 1998). There are at least three isotypes of PPAR (α, γ and β–Δ) which regulate different target genes. It is relevant that activation of PPAR-γ by natural ligands is either by a high dietary fat intake or else by high dietary content of long-chain *n*-3 FA such as DHA or EPA (Jump *et al.* 1997).

It is reported that β-oxidation of DHA can occur in peroxisomes (Singh *et al.* 1984), whereas in the case of shorter-chain *n*-3 FA, it occurs in the mitochondria. It is possible that peroxisomal enzymes are required for oxidation of long-chain *n*-3 FA (Martinez *et al.* 2000) and that this may explain the cytostatic activity of their peroxidation products. Troglitazone, a synthetic activator of PPAR-γ, has been shown to cause differentiation and apoptosis in human mammary cancer cells (Elstner *et al.* 1998; Mueller *et al.* 1998). The J series of prostaglandins are much more potent in this respect, and prostaglandin J_2 causes apoptosis in explants of human BC cells in immunosuppressed mice although the effect is dose-dependent (Clay *et al.* 1999).

The biological mechanisms which were described earlier and which attempt to explain *n*-3 FA-mediated inhibition of mammary carcinogenesis are not necessarily independent. A higher *n*-3:*n*-6 ratio in the cell membrane can alter its phospholipid profile and lead to changes in cell membrane fluidity (Welsch, 1987). These can influence cell signalling leading to changes in protein kinase C activity and in gene expression through PPAR-mediated pathways.

Lipid peroxidation and apoptosis in mammary cancer

Apoptosis in mammary cancer by *n*-3 FA is also likely to involve lipid peroxidation products (Welsch, 1995). FA, particularly long-chain *n*-3 FA, enhance the production of free oxygen radicals and lipid peroxides which can damage mammary epithelial cells by an effect on cell membrane fluidity, enzymes or receptors (Welsch, 1995). It is reported that apoptosis is most likely from the effect of DHA and EPA on tumour cells which over-express cytochrome P-450 (Das, 1999) and that it is associated in such cases with lipid peroxidation and depletion of antioxidants.

Feeding of DHA to rats causes reduction of plasma tocopherol levels to 21–73 % of that in controls (Song *et al.* 2000), presumably as a result of its interaction with products of increased peroxidation. In human subjects, vitamin E is an effective antioxidant comprising eight active constituents (Traber & Packer, 1995). Although γ-tocopherol is the main form in the diet, α-tocopherol is the only form which has been shown to prevent vitamin E deficiency in human subjects. However, case-control studies on the association between vitamin E concentration and BC risk show conflicting results, and cohort studies have shown no evidence of protection from the disease in association with higher concentrations (Kimmick *et al.* 1997).

A high concentration of *n*-3 FA lipid peroxidation products is likely to modify antioxidant concentrations in the vicinity of transformed or neoplastic mammary cells. A comparison of breast adipose tissue from BC patients with that from controls shows that BC is associated with a lower vitamin E level in the adjacent adipose tissue (Chajes *et al.* 1996; Zhu *et al.* 1996) and it may be due to increased concentrations of free oxygen radicals.

It may be relevant to BC risk that increased lipid peroxidation in human subjects is often found in association with hyperinsulinaemic insulin resistance and a predominantly abdominal distribution of obesity (Paolisso & Giugliano, 1996). All three metabolic changes may result from persistently high fatty acid levels in the portal system (Groop *et al.* 1992). Abdominal obesity is likely to be a biological marker of the metabolic abnormality and is reported to be associated with a lower circulating tocopherol level than is seen in lean individuals (Paolisso & Giugliano, 1996). Abdominal obesity in women is associated with higher circulating concentrations both of free oestradiol and free insulin-like growth factor-I. Both are markers of increased BC risk, and activation of their respective receptors may synergise in stimulating growth of BC (Stoll, 2002).

Proposed clinical study

Dietary supplements of *n*-3 FA might be used in a phase 2 clinical study to examine their effect on late-stage promotion of mammary carcinogenesis in premenopausal women who are at increased BC risk. The study would focus on premenopausal women over the age of 40 years, using biological markers in breast tissue to help in assessing decrease in cancer progression. The years leading up to the menopause are a window of BC risk from environmental and lifestyle factors which favour progression of precancerous lesions to invasive BC. The latter presents most frequently in western women after the menopause (Stoll, 1999).

Premenopausal women regarded as being at increased BC risk include those with a family history of the disease, those with histopathological evidence of carcinoma *in situ* at surgery, and those with either abdominal obesity or increased circulating concentrations of insulin-like growth factor-I (Stoll, 1999). After entry into the clinical trial of supplementary *n*-3 FA, serial monitoring of nipple aspirates or core aspirates of breast tissue will be carried out. These samples will be examined for genetic markers of malignant progression such as p53 or HER-2neu (Prietto *et al.* 1999) or for changes in expression of oestrogen receptor or insulin-like growth factor-I receptor (Stoll, 2002).

Pilot clinical studies of a similar nature have been carried out in patients with precancerous changes of colorectal cancer. Decrease in cell proliferative activity was shown following dietary supplements of capsules containing fish-oil rich in *n*-3 FA, at a dosage of 4 g EPA and 2·2 g DHA/d for 6 months (Huang *et al.* 1996). A similar response was noted with a dosage of 2·5 g EPA and DHA/d for 1 month in patients with colonic adenoma

(Bartoli *et al.* 1993; Anti *et al.* 1994). Similar dosage levels can be used in a pilot clinical study of supplementary *n*-3 FA in premenopausal women at increased risk of BC.

Concerns have been expressed that supplementary dietary intake of long-chain *n*-3-FA might contribute to atheroma by causing peroxidation of LDL-cholesterol (Steinberg & Lewis, 1997). Whilst it is still controversial as to whether increased *n*-3 FA intake is completely free of any cardiovascular danger, a large trial of *n*-3 FA and vitamin E supplements for a period of 42 months has been shown to reduce the risk from myocardial death and complications in patients with a history of myocardial infarction (GISSI Prevenzione Investigators, 1999). There is, however, no clear evidence that supplementary vitamin E at pharmacological doses can be safely recommended as an antioxidant in cancer prevention trials. In fact, some observations suggest that the addition of vitamin E can reverse the inhibitory effects of DHA and EPA on human BC cells in culture (Chajes *et al.* 1995) and on the growth of human BC explants in immunosuppressed mice (Gonzalez *et al.* 1993).

There is evidence that long-chain *n*-3 FA may enhance lipid peroxidation leading to apoptosis in transformed or malignant mammary epithelial cells, but evidence of a need for it to be associated with antioxidant therapy is uncertain (Cognault *et al.* 2000). Most assays of lipid peroxidation in the past have involved measurement of thiobarbituric acid-reactive substances or malonaldehyde metabolites. Increasing evidence points to members of the isoprostane family as the most reliable current biomarkers of total body peroxidation (Halliwell, 1999). They are the peroxidation products of arachidonic acid residues by a COX-independent pathway (Betteridge, 2000). Some foods contain F_2-isoprostanes, but the amounts are unlikely to alter assays of plasma levels (Gopaul *et al.* 2000). Different families of isoprostanes arise from the peroxidation of EPA and DHA (Roberts *et al.* 1998) so that MS measurement of F_2-, F_3- and F_4-isoprostanes would be needed to determine the relative contribution of EPA and DHA metabolites to the overall level of lipid peroxidation.

The proposed clinical study needs to incorporate serial monitoring of blood levels of isoprostane metabolites as a measure of whole-body lipid peroxidation. Measurements in each patient need to be examined for their relationship to the biological markers of progression in mammary carcinogenesis in breast tissue. Evidence of such correlation might lead to subsequent studies of similar dietary supplementation in patients with metastatic BC looking for evidence of DNA damage in the tumour, such as an increase in the apoptotic index. The scheme provides a logical scientific basis for a study of dietary intervention by *n*-3 FA supplements as one means of decreasing BC risk in women.

It is relevant that a recent study of preoperative cytotoxic therapy in patients with localised BC showed greater tumour regression in those with a higher concentration of DHA in the breast adipose tissue (Bougnoux *et al.* 1999). The researchers suggest that increased response might be associated with an increase in lipid peroxidation products in the tumour. A previous study by the group on human BC cells in culture showed that supplementary DHA added to the medium increased cytotoxicity by doxorubicin in the cells (Germain *et al.* 1998).

Conclusion

The rationale for use of dietary *n*-3 FA in counteracting BC progression needs to be tested clinically. Experimental evidence suggests that when it is incorporated into the cell membrane, *n*-3 FA enhances lipid peroxidation and that this can lead to increased apoptosis in transformed or malignant mammary epithelial cells. A phase 2 pilot study of fish oil rich in *n*-3 FA is proposed in premenopausal women over the age of 40 years who are at increased BC risk. Biological markers in breast tissue of progression in mammary carcinogenesis will be monitored, and observed changes related to serial plasma concentrations of isoprostanes as a measure of whole-body lipid peroxidation.

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