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Effects of polyunsaturated fatty acids on human breast cancer cell adhesion and signaling.

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EFFECTS OF POLYUNSATURATED FATTY ACIDS ON HUMAN BREAST CANCER CELL ADHESION AND SIGNALING

by

NINA SHARONE GERMAN

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
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ABSTRACT OF DISSERTATION
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Title Effects of Polyunsaturated Fatty Acids on Human Breast Cancer Cell Adhesion and Signaling

Epidemiological and experimental data suggest that polyunsaturated fatty acids (PUFAs) may modulate the growth and metastasis of human breast cancer cells. The mechanisms by which these fatty acids influence breast cancer cell progression are not clear; however, changes in cell adhesion may be involved. To examine the effects of PUFAs and epidermal growth factor (EGF) on cell adhesion, cell adhesion molecule and protein kinase expression in the human breast cancer cell line MDA-MB-231, we treated cells with either the ω-3 PUFAs eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or the ω-6 PUFA linoleic acid (LA). The combined effect of EPA and EGF was not additive in decreasing adhesion of cells to the 3 extracellular matrix (ECM) components studied, thus suggesting that the decreased adhesion to ECM substrates by combined EPA and EGF treatment may be the result of a common postreceptor signaling pathway. Our results also suggest that lipid peroxidation products are not solely responsible for EPA-induced decreases in MDA-MB-231 cell adhesion to ECM components. Treatment of cells with either EPA or LA over a period of 5 days resulted in a decrease in cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) activity (23.7 ± 0.63 and 24.7 ± 1.51 U/mg protein, respectively) compared to untreated controls (27.6 ± 0.62 U/mg protein, $p < 0.05$). Expression of the regulatory subunit of PKA ($PKA_{RI\alpha}$),

as measured by immunoblot analysis and scanning densitometry, was significantly lower in cells treated with ω-3 PUFAs but was unchanged in LA-treated cells. There was no significant difference in intracellular cAMP production in cells treated with PUFAs. Protein kinase C-α (PKC-α) expression was lowered in cells treated with 5 μg/ml EPA and DHA but not in cells treated with 2.5 μg/ml EPA and 5 μg/ml LA. Treatment with 2 and 4 μM H-89, a PKA inhibitor, decreased cell adhesion to both Matrigel and type IV collagen. Compared to untreated controls, cells treated with EPA for 5 days exhibited decreased expression of the β1 integrin subunit ($p < 0.01$). EPA and DHA, at 5 μg/ml, decreased tyrosine phosphorylation of several proteins, but many proteins were unaffected by this treatment. Immunoprecipitation studies revealed the absence of a protein band in the 120-125 kDa region, thus suggesting that focal adhesion kinase (FAK) is not phosphorylated in response to PUFAs in this cell line. However, treatment with 5 μg/ml EPA and DHA resulted in decreased FAK expression. There was also a decrease in FAK expression in cells treated with 4 and 8 μM H-89, thus suggesting that the FAK and PKA signaling pathways are somehow interrelated. Overall, these findings suggest that PUFAs, especially ω-3 PUFAs, have an effect on the activity and expression of several key signaling molecules in the MDA-MB-231 cell line and that these signaling molecules may, in turn, mediate PUFA-modulation of cell adhesion to ECM components.

DEDICATION

I dedicate this work to my parents, Joseph German and Joyce C. German, and grandparents, Mr. and Mrs. William F. Carter, Sr., and Rev. and Mrs. Porter German; who all taught me to trust in the Lord and to never give up.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
BSA	bovine serum albumin
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate (cyclic AMP)
COX	cyclooxygenase
DAG	diacylglycerol
dbcAMP	N6,O-2'-dibutyryladenosine 3',5'-cyclic monophosphate
DGLA	dihommogamma linolenic acid
DHA	docosahexaenoic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunoassay
EPA	eicosapentaenoic acid
FAK	focal adhesion kinase
FBS	fetal bovine serum
FFA	free fatty acid
G_s	stimulatory G protein

GLA	gamma linolenic acid
H-89	N-[2- (p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide • 2 HCl
Ig	immunoglobulin
IgG	immunoglobulin G
IMDM	Iscove's Modified Dulbecco's medium
LA	linoleic acid
LNA	linolenic acid
LT	leukotriene
MDA	malondialdehyde
OD	optical density
PBS	phosphate buffered saline
PG	prostaglandin
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PMSF	phenylmethylsulfonylfluoride
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBA	thiobarbituric acid
TBARS	thiobarbituric acid-reactive substances
TBS	tris-buffered saline
TX	thromboxane

INTRODUCTION

Cancer of the breast is the most common cancer among women in the United States and is second to lung cancer as the most frequent cause of cancer-related deaths (World Cancer Research Fund, 1997). In 1996, there were an estimated 910,000 new cases diagnosed worldwide (World Health Organization [WHO], 1997), accounting for 9% of all new cancers. There is a strong correlation between diet, nutritional deficiencies, or both, and many cancers. It has been estimated and widely accepted that a majority of all women's cancers are related to nutritional factors alone (World Cancer Research Fund, 1997).

Dietary fat consumption has been associated with breast cancer risk. This hypothesis has been the source of much scientific controversy and debate throughout the 1980s and 1990s. The indication that dietary fats are associated with mammary tumorigenesis has been provided on the basis of experimental models (Cave, 1991; Welsch, Oakley, Chang, & Welsch, 1993) and ecological correlations from epidemiological data (Godley, 1995). More recently, the type of fat in breast cancer development has been considered (Cave, 1991). The type of fatty acid can have a direct effect because, unlike protein and carbohydrates, fatty acids are incorporated directly into cell membranes. Studies have also shown that free fatty acids as well as their metabolites activate key components of major signal transduction pathways (Liu, B., Timar, Howlett, Diglio, & Honn, 1991; Murakami, Chan, & Routtenberg, 1986; Verkest, McArthur, & Hamilton, 1988). The essential fatty acids (ω-3 and ω-6), which mammals cannot synthesize, are

either incorporated intact or converted to other fatty acids of the same family. Studies suggest that ω-6 fatty acids found in vegetable oils may act as promoters, whereas ω-3 fatty acids found in fish oils may reduce the incidence and development of experimental mammary tumors (Rose & Connolly, 1990; Rose, Connolly, Rayburn, & Coleman, 1995; Welsch, 1995). These results may explain the increasing incidence of breast cancer in the United States, where linoleic acid ($18:2_{\omega-6}$)-rich vegetable oil consumption has steadily increased, as well as the lower incidence in countries where fish oil (rich in ω-3 fatty acids) constitutes a higher proportion of the dietary lipid intake (Hursting, Thomquist, & Henderson, 1990; Kaizer, Boyd, Kriukov, & Tritchler, 1989; Lund & Bonaa, 1993).

Previous studies in our laboratory have provided evidence that polyunsaturated fatty acids (PUFAs) and their metabolites influence adhesion of cultured human breast cancer cells to individual components of the basement membrane (Johanning & Lin, 1995). However, the causative mechanisms that are responsible are not known with certainty. Changes in adhesion of tumor cells to adjacent cells or to the nearby extracellular matrix (ECM) are likely involved in many of the steps in the chain of events leading to metastasis formation (Albelda, 1993). In the current study, the human breast cancer cell line MDA-MB-231 will be used. Previous studies demonstrated that ω-6 fatty acids stimulated and ω-3 fatty acids inhibited basement membrane invasiveness and adhesion of these cells (Palmantier, Roberts, Glasgow, Eling, & Olden, 1996; Rose, Connolly, & Liu, 1994a), as well as other cell lines (Connolly & Rose, 1993; Johanning & Lin, 1995; Reich, Royce, & Martin, 1989). MDA-MB-231 cells have also been shown to be highly invasive (Wang, Nohara, Olivera, Thompson, & Spiegel, 1999). The studies presented in this paper are an attempt to further understand the mechanisms responsible for the actions

of polyunsaturated fatty acids on human breast cancer cell adhesion to ECM basement membrane components.

REVIEW OF LITERATURE

Biochemistry of Fatty Acids

Most dietary and body fats consist of free fatty acids esterified with glycerol (triglycerides or neutral fat), the fatty acids being an aliphatic chain of carbon atoms with terminal methyl ($-CH_3$) and carboxyl (-COOH) groups. The total number of carbon atoms in the molecule is in most cases even, although fatty acids containing odd-numbered carbon atoms are also found (Sardesai, 1992). Fatty acids have the basic formula $CH_3\ [CH_2]_n$ COOH, where n can be any number from 2 to 24. One method of fatty acid classification is according to their chain length. Those containing 2 to 4 carbon atoms are called short-chain fatty acids, those with 6 to 12 carbon atoms are called medium-chain fatty acids, and those with 14 to 24 carbon atoms are called long-chain fatty acids (Sardesai, 1992). Fatty acids are designated by a prefix according to the number of carbon atoms they contain, for example, eicosa (20 carbon), docosa (22 carbon), et cetera. They are also classified as saturated, with no double bonds; monounsaturated, with one double bond; or polyunsaturated, with two or more double bonds (Linscheer & Vergroesen, 1994). The double bonds found in PUFAs have a cis-conformation, in which the two hydrogen atoms are on the same side of the carbon-chain plane and are separated by one methylene group ($-CH_2-CH=CH-CH_2-CH=CH-CH_2-$). Trans-isomers have the hydrogen atoms on opposite sides of the carbon-chain plane; they are formed in the rumen of cattle, sheep, and other ruminants (and therefore appear in butter and beef fat), and often two-thirds of the cis-isomers in oils are converted to trans during partial hydrogenation as in manufacture of

margarine (Sinclair, 1984). Trans fatty acids are also formed as a result of deep-frying foods. Depending on the number of double bonds, 2, 3, 4, 5, or 6, fatty acids are called dienoic, trienoic, tetraenoic, pentaenoic, or hexaenoic, respectively (Linscheer & Vergroesen, 1994).

The carbon atoms of the fatty acid are numbered from the carboxyl group (Δ numbering system) or from the terminal methyl group (ω or n numbering system). Fatty acids are abbreviated in the Δ nomenclature by listing the carbon number and position of double bonds ($C_{a}\Delta_{b}$). The number after the Δ in this classification system signifies the position of the double bond relative to the carboxyl end. In the ω numbering system, fatty acids are abbreviated as C_{a}: b, ω_{c}, where b represents the number of double bonds, and c represents the location of the first double bond (i.e., number of carbon atoms away from the ω carbon). Most fatty acids in the blood and tissues of humans have 16, 18, or 20 carbon atoms, but there are a few with longer chains that exist in the lipids of the central nervous system (Sardesai, 1992).

Nonesterified (Free) Fatty Acid Metabolism

Free fatty acid (FFA) is the form in which fat is released into the blood by adipose tissue (Gordon & Cherkes, 1958). The concentration of FFA and its flux through the circulation vary widely from hour to hour, reflecting nutritional state and physical activity (Frayn, 1998). At usual physiologic concentrations, most of the circulating FFA is bound to albumin (Goodman, 1958). Studies have shown that, in spite of the relatively low concentration of FFA, it is of great importance in supplying fat to tissues because of its rapid turnover rate (Dole, 1956; Gordon & Cherkes, 1956). Essentially, all of the fatty acid re-

quired for oxidation in the fasting state is derived from the plasma FFA (Frederickson & Gordon, 1958).

It is highly unlikely that tumor cells can synthesize sufficient fatty acid de novo (e.g., from glucose or low-molecular-weight precursors) to satisfy metabolic and growth needs, and at least a portion of the fatty acid requirement is thought to be derived from the circulating fat of the host (Henderson & LePage, 1959). In vitro studies demonstrate that tumor cells readily utilize long-chain FFA (e.g., palmitate, stearate, oleate, and linoleate) when this substrate is available as the physiologic albumin complex (Spector & Steinberg, 1965, 1967; Spector, Steinberg, & Tanaka, 1965). Spector (1967) showed that FFA was available to Ehrlich ascites tumor cells throughout its growth and that FFA could be incorporated into esterified lipids and catabolized by the tumor cells in vivo.

Biosynthesis of Polyunsaturated Fatty Acids

Mammalian tissues contain four families of PUFAs. The precursors of two of these groups are the monounsaturated fatty acids palmitoleic (16:1, ω7) and oleic acids (18:1, ω9), which can be synthesized endogenously from saturated fatty acids; thus, these precursors are not essential. The precursors for the other two families, linoleic (LA; 18:2, ω6) and linolenic (LNA; 18:3, ω3) acids, must be derived from dietary sources; thus, these precursors are essential because mammals do not possess the required desaturase that is needed for their formation (Bézard, Blond, Bernard, & Clouet, 1994; Sardesai, 1992). These four precursors are alternately desaturated (two hydrogens are removed to create a double bond) and chain elongated (the addition of two carbons). The desaturations are catalyzed by Δ_6, Δ_5, and Δ_4 desaturases to form the principal PUFAs found in

animal tissues. The critical enzyme in these reactions is Δ_6 desaturase, which exhibits the greatest affinity for species with the greatest number of double bonds in the C_{18} fatty acids (Brenner, 1971). Therefore, the desaturation of LA is inhibited by the presence of LNA or higher members of the ω-3 family. In the presence of LA and LNA, little desaturation of oleic acid occurs. However, in the absence of ω-3 and ω-6 (essential) fatty acids, oleic acid is desaturated via Δ_6 desaturase, elongated via an elongase enzyme, then desaturated once more to form eicosatrienoic acid (20:3, ω9), which accumulates in tissues (Holman, 1960). The effects of this unusual highly desaturated and elongated ω9 metabolite are not well known, but it may play a role in the increased incidence of tumorigenesis related to essential fatty acid deficiency (Eynard et al., 1997).

Each PUFA family has characteristic end products that accumulate in tissue lipids, whereas the intermediates are usually found in much smaller, often trace, amounts. For oleic and palmitoleic acids, the major PUFAs are the trienes 20:3, ω7 and 20:3, ω9, respectively (Sprecher, 1989). For LA, the major PUFAs are arachidonic acid (AA; 20:4, ω6) and some dihomogamma linolenic acid (DGLA; 20:3, ω6; Marcel, Christiansen, & Holman, 1968). For LNA, the main end products of metabolism are eicosapentaenoic acid (EPA; 20:5, ω3) and docosahexaenoic acid (DHA; 22:6, ω3; Sprecher, 1989). The 22-carbon hexaenoic acid is the most unsaturated fatty acid commonly found in the lipids of higher animals (Sardesai, 1992).

Dietary Sources of Essential Fatty Acids

The most prevalent ω-6 fatty acid is LA. It is present in almost all vegetable oils, such as corn, soybean, safflower, and sunflower oils. AA, a product of LA, is found in

foods of animal origin. LNA is present in only small amounts in soybean and rapeseed oils and in leafy vegetables, such as spinach, kale, and romaine lettuce. The only plant source that is rich in LNA is linseed oil, but it has a highly unpleasant varnish-like taste. LNA-derived fatty acids make up a large part of PUFAs in marine fish oils and fat. Varieties of fish and seafoods rich in ω-3 fatty acids include salmon, mackarel, sardines, scallops, oysters, and red caviar. Cold-water plants, such as phytoplankton and algae, synthesize LNA, and the fish that feed on these marine plants convert this fatty acid to the higher members, including the two most abundant components of this class, EPA and DHA (Sardesai, 1992).

Functions of Essential Fatty Acids

The PUFAs derived from essential fatty acids serve as cellular membrane phospholipid components that can influence the physicochemical characteristics of the lipid bilayer (Murphy, 1990). Changes in membrane lipids can modify the mobility and function of a variety of membrane proteins, which may result in altered cell/organ functions (Sinclair, 1984). LA is specifically required in the skin to maintain the integrity of the epidermal water barrier (Elias & Brown, 1978; Wertz, Swartzendruber, Abraham, Madison, & Downing, 1987). DGLA, AA, and EPA are the precursors of eicosanoids, which influence many cell processes and organ functions (Piomelli, 1996; Thaler-Dao, Crastes de Paulet, & Paoletti, 1983). DHA is found in high concentrations in the structural lipids of the brain and retina and has a role in the development of the brain and visual function (Neuringer, Anderson, & Connor, 1988).

Louw, Engelbrecht, and Cloete (1998) investigated the possible role of essential total fatty acids and their metabolites during cervical carcinogenesis. Results showed a significant decrease in LA and AA when carcinoma was compared with normal tissue, thus reflecting essential fatty acid deficiency. This deficiency might lead to the activation of Δ_9 desaturase, which converts palmitic acid to palmitoleic acid and stearic acid to oleic acid. The decrease in LA levels might be a reason for the increased levels of DGLA in cancer cells of the cervix. Increased levels of DGLA might also be indicative of a very low activity or even loss of Δ_5 desaturase activity (enzyme responsible for the conversion of DGLA to AA) in cancer cells. The high concentrations of DGLA and lower concentrations of 20:2n-6, a DGLA precursor, in cervical cancer cells may indicate that Δ_8 desaturase (enzyme responsible for the conversion of 20:2n-6 to DGLA) is very active in cancer cells. Desaturation at C8 is not common; however, evidence for Δ_8 desaturase has been obtained in MCF-7 human breast cancer cells (Grammatikos, Subbaiah, Victor, & Miller, 1994). Thus, alternative pathways may enable the desaturation of LA when Δ_6 desaturase is not active.

Eicosanoid Biosynthesis

The eicosanoids are the oxidation products of 20-carbon (eicosa) PUFAs derived from the essential fatty acids DGLA, AA, and EPA (Thaler-Dao et al., 1983). Because AA-derived eicosanoids have a higher concentration in tissue phospholipids in most species, these eicosanoids dominate over those formed from the other two 20-carbon acids (Sardesai, 1992). The rate-limiting step in eicosanoid formation is the liberation of PUFA from membrane phospholipid by phospholipase A_2, making free fatty acids available for

metabolism. By the action of cyclooxygenase (COX), AA forms prostaglandin (PG) G_2, which quickly converts to PGH_2 (Figure 1). After this point, most cells become highly selective in their metabolism of PGH_2 to the biologically active product. As a group, these active metabolites of PGH_2 are known as prostanoids (Sinclair, 1984). Aspirin and other nonsteroidal antiinflammatory agents block the COX enzyme (Vane, 1971). Prostaglandins are potent messenger substances that regulate a wide variety of processes, including stomach acid secretion, uterine contraction, reproduction, blood pressure control, and inflammation (Samuelsson, 1981). They exert their effects at or near their site of synthesis and are then rapidly inactivated. PGH_2 forms thromboxane (TX) A_2 in platelets, via the action of thromboxane synthase (Arita, Nakano, & Hanasaki, 1989). TXA_2 aggregates platelets and is also a potent vasoconstrictor. In endothelial cells, PGH_2 is converted to PGI_2 via the action of prostacyclin synthase. The action of PGI_2 is the opposite of TXA_2 (Moncada & Vane, 1978). Because of these effects, PGI_2 has been successfully used in the treatment of a number of thrombotic disorders (Sardesai, 1992).

In addition to being converted to PG, AA can also be converted to leukotriene (LT) A_4 via the action of 5-lipoxygenase (Figure 2). LTA_4 can either form LTB_4 or LTC_4, which can be converted to the smaller molecules LTD_4 and LTE_4. LTB_4 is a potent chemotactic agent that attracts neutrophils and macrophages to and causes aggregation at sites of infection or injury (Ford-Hutchinson, Bray, Doig, Shipley, & Smith, 1980). It is also associated with plasma exudation, swelling, and edema.

Lipid Peroxidation

Lipid peroxidation is an oxidative deterioration of unsaturated fatty acids that gives rise to a variety of oxidation products with the potential of interfering with cell replication, cell survival, or both. The mechanism by which tumor cells may be killed in vitro by exogenous PUFAs may involve lipid peroxidation (Cantrill, Ells, DeMarco, & Horrobin, 1997). Several mechanisms by which lipid peroxidation products retard or inhibit tumor growth are cell membrane damage and changing the cellular composition or cytoskeleton assembly, which may lead to inactivation of membrane transport systems or membrane-bound enzymes (Spector & Burns, 1987), which may, in turn, adversely affect the entrance of cells into the cell cycle or accelerate their exit, inactivate polymerase reactions (Roubal & Tappel, 1966), and lead to formation of intermolecular or intramolecular linkages between amino acid sulfhydryl groups and biomolecules (e.g., DNA, RNA, proteins: Reiss & Tappel, 1973).

The presence of higher concentrations of highly unsaturated fatty acids in certain dietary fats (e.g., fish oil) offers the possibility that oxidation products of these fatty acids may play an important role in the inhibitory or growth-rate-limiting action exhibited by these types of fat (Gonzalez et al., 1991). When cells in culture are supplemented with PUFA, the fatty acid is rapidly removed from the medium and incorporated into both neutral lipids and phospholipids, depending upon the fatty acid used and its concentration (DeAntueno et al., 1993). Incorporation of PUFA into cell lipids provides substrate for both eicosanoid synthesis and lipid peroxidation (Cantrill et al., 1997).

Gonzalez et al. (1991) and Gonzalez, Schemmel, Dugan, Gray, and Welsch (1993) showed that the type of dietary fat can clearly affect the growth of human breast

carcinomas maintained in athymic nude mice. There was a consistent and significant inhibition of growth of these carcinomas by dietary fish oil that appeared to be due to increased accumulation of tumor lipid peroxidation products. Addition of high levels of antioxidants to the fish oil diet substantially reduced the level of tumor lipid peroxidation products and simultaneously caused a substantial increase in tumor growth, as previously shown by Gavino, Miller, Ikharebha, Milo, and Cornwell (1981). Other studies have shown that the extent of lipid peroxidation may be fatty-acid specific (Begin, Ells, & Horrobin, 1988; Cantrill et al., 1997; Chow, Sisfontes, Björkhem, & Jondal, 1989).

Lipid peroxidation may be crudely evaluated by the thiobarbituric (TBA) assay for malondialdehyde (MDA). Cells in tissue culture undergo lipid peroxidation and generate an MDA precursor. Cells challenged with a PUFA generate increased amounts of an MDA precusor (Gavino et al., 1981). The TBA assay involves measuring a fluorescent red 1:2 adduct that is formed between MDA and TBA via an acid-catalyzed nucleophilic-addition mechanism (Liu, J., Yeo, Doniger, & Ames, 1997). The TBA adduct has an absorption maximum at 532 nm with a very high molar absorptivity of 1.53×10^5. Because of the nonspecificity of the TBA adduct, the absorbance at 532 nm measures TBA-reactive substances (TBARS), rather than MDA (Liu et al., 1997).

Relationship Between Dietary Fat and Breast Cancer

Scientists have discovered an increasing number of links between the environment, diet, and the health of the individual. There is evidence from both epidemiologic (Carroll, 1975) and experimental (Cave, 1991; Welsch, 1992) studies that dietary fatty acids, particularly ω-6 and ω-3 fatty acids, influence the development and subsequent

progression of breast cancer. In addition, specific fatty acids (e.g., ω-6 and ω-3) may exert opposing effects so that the net result is dependent on their relative concentration in the diet (Rose, 1997).

Ecologic (correlational) studies have shown that populations migrating from low-incidence to high-incidence countries acquire breast cancer rates similar to those in their new homeland within two or three generations (Buell, 1973; Ziegler et al., 1993). This is most likely due to changes in patterns of dietary fat intake because, according to Armstrong and Doll (1975), the rates of breast cancer are positively correlated internationally with per capita total fat consumption. However, the quality of the data on average fat consumption, which is usually based on food-disappearance estimations of the amount of fat in the food supply, is not adequate to describe a definite relationship between fat consumption and cancer. Willett and Stampfer (1990) report that the average fat intake of persons in the United States calculated from food-disappearance data is at least 50% greater than actual measured intake. A major difficulty in interpreting ecologic comparisons of diet and breast cancer is the potential for confounding by known and suspected risk factors for breast cancer (e.g., family history, age at menarche, age at menopause, excess body weight, and parity; Hunter & Willett, 1996). Country-specific per capita fat consumption is highly correlated with level of economic development (Armstrong & Doll, 1975). Prentice et al. (1988) have demonstrated that the ecologic relation between fat disappearance and breast cancer incidence rates is still statistically significant after adjustment for per capita Gross National Product and average age at menarche.

The relation between dietary fat and breast cancer risk can be examined in more detail in case-control and cohort (prospective) studies because potential risk factors can

be measured and controlled in analysis. However, case-control studies do have their weaknesses: (a) These studies of diet are based on the reports of recalled diets of people with and without cancer, (b) there exists a possibility of differential recall of diet by case subjects compared with control subjects, and (c) random misclassification due to errors in estimating diet may lead to bias effect estimates toward the null value. Although no significant association between total fat intake and risk of breast cancer has been seen in most individual case-control studies, a meta-analysis of 12 case-control studies of breast cancer showed a weak positive association with dietary fat intake (Howe et al., 1990); however, the effect was primarily restricted to postmenopausal women and appeared to be stronger for saturated and monounsaturated fat intake than for polyunsaturated fat intake. Studies included in this meta-analysis consisted of all case-control studies of diet and breast cancer that had been completed by the end of 1986 and that included specific estimates of fat intake. Nevertheless, several other studies have found an association between polyunsaturated fat consumption and breast cancer risk (Hursting et al., 1990; Prentice & Sheppard, 1990; Sasaki, Horacsek, & Kesteloot, 1993).

Cohort studies have an important advantage over case-control studies because the assessment of diet in cohort studies is made before diagnosis and, thus, is unbiased by the cancer experience. These studies, however, do have limitations: (a) Diet is usually assessed by a self-administered questionnaire because of the large number of subjects that must be followed, and (b) studies are expensive because they usually require several years to accrue enough cases for analysis. Findings from pooled cohort studies by Hunter et al. (1996) suggest that dietary fat plays no role in breast cancer. There was little overall association between the percentage of energy intake from fat and the risk of breast can-

cer, even among women whose energy intake from fat was less than 20%. In contrast, a meta-analysis conducted by Wu, Pike, and Stram (1999) suggested that the possibility of reducing fat consumption below 20% of calories would reduce breast cancer risk. Because studies show that estrogens influence cancer risk (Bernstein & Ross, 1993; Pike, Spicer, Dahmoush, & Press, 1993), Wu et al. (1999) conducted a meta-analysis of 13 dietary fat intervention studies that investigated serum estradiol levels. There were statistically significant reductions in serum estradiol levels among both premenopausal and postmenopausal women. The greatest reductions occurred in two studies in which dietary fat was reduced to 10%-12% of calories compared with 18%-25% of calories in the other studies. Boyd, Martin, Noffel, Lockwood, and Tritchler (1993) conducted a meta-analysis of 16 case-control and 7 cohort studies. This meta-analysis examined the association of breast cancer risk with specific types of fat and found associations with saturated and monounsaturated fat but not with polyunsaturated fat.

In cohort studies, the consistent absence of a significant positive association between dietary fat intake and breast cancer has been attributed to misclassification of the measures of dietary fat intake, thus causing relative risks to be biased toward the null (Hunter & Willett, 1996). Inconsistencies in cohort studies of dietary fat and disease may result from differences in study population characteristics; differences in the mechanistic pathways for specific diseases; or the presence or lack of threshold levels, bias, and chance (Wynder et al., 1997). Wynder et al. (1997) suggest that the "validation" of the food-frequency questionnaire in the meta-analysis of cohort studies by Hunter et al. (1996) is a misrepresentation of the accuracy of dietary data. True validity would require nonintrusive observation of the respondent's total diet over a long time. No such study

has ever been or likely will be done. However, studies have examined the concordance of food-frequency questionnaire responses with multiple food records or recalls. This is not a true validation method because recalls and records do not represent the time period of interest, they contain error, and they inaccurately represent intake (Livingstone et al., 1990). A recent cohort study by Holmes et al. (1999) found no evidence that lower intake of total fat or specific major types of fat was associated with a decreased risk of breast cancer.

Credibility for the dietary fat hypothesis has been provided by animal studies in which increases in total fat and polyunsaturated fat, in particular, increased rates of mammary carcinogenesis in some but not all animal models (Birt, 1986; Boissonneault, Elson, & Pariza, 1986; Freedman, Clifford, & Messina, 1990). Several experimental studies have indicated that various types of dietary fat differ in their ability to affect mammary tumorigenesis. ω-6 PUFAs have been shown to promote breast cancer tumorigenesis (Hubbard & Erikson, 1987; Katz & Boylan, 1987), possibly because of increased synthesis of PGs (Rose et al., 1994a; Rose & Hatala, 1994; Wynder et al., 1997). However, ω-3 PUFAs (namely EPA and DHA) have been shown to reduce breast cancer growth (Cave, 1991; Karmali, Adams, & Trout, 1993; Karmali, Marsh, & Fuchs, 1984), perhaps because they inhibit the oxidative metabolism of AA via the COX pathway of PG synthesis (Cave, 1991; Ferretti & Flanagan, 1990). The exact mechanisms by which fatty acids exert stimulatory and inhibitory effects on tumors remain controversial; however, AA-derived eicosanoids are believed to play a role in the metastatic process (Noguchi, Rose, et al., 1995; Rose & Connolly, 1990).

In summary, the hypothesis that a high-fat diet is associated with the development of breast cancer is supported by ecologic studies and animal model studies and somewhat by case-control studies in humans. The evidence from cohort studies does not support this association; however, the possibility of a weak association cannot be excluded. There are a number of factors that may be responsible for the inability of human clinical trials to consistently show a significant association between dietary fat intake and breast cancer risk: (a) The intake of specific fatty acids, rather than that of total fat, may influence breast cancer risk (Godley, 1995; Lund & Bonaa, 1993); (b) there may be inter-individual and intra-individual variation in fat metabolism and fat synthesis (Kuhajda et al., 1994); (c) the dietary information from large clinical studies may be inaccurate (Cuzick, 1992); (d) the stringent conditions under which fat promotes mammary carcinogenesis in animal models may be difficult to duplicate for use in human clinical trials (Ip, 1993); and (e) the length and timing of the studies is also an important factor (Bradlow & Fishman, 1993). A considerable latency period could exist between fat intake and its effect on disease.

Linoleic Acid

Dietary LA provides the source of AA, which is incorporated into cell membrane phospholipids and is the substrate for eicosanoid biosynthesis. AA is metabolized either through the COX pathway, resulting in PG and TX production, or through the lipoxygenase pathway, resulting in LT and hydroxyeicosatetraenoic acid. Eicosanoid production is controlled in most normal cells, but it often reaches exaggerated levels in malignant tissues (Hubbard, Chapkin, & Erickson, 1988). Miyamoto-Tiaven, Hillyard, and Abraham (1981) showed that the growth-enhancing effect of LA was inhibited by indo-

methacin, a COX inhibitor, and was partially reproduced by PGE_2, which bypasses the metabolic block. However, other studies have shown that indomethacin at low concentations (1 μg/ml) results in stimulation of thymidine incorporation or a failure to reverse LA-stimulated adhesion of MDA-MB-231 cells in vitro, whereas at higher concentrations (40-50 μg/ml), it suppresses cell growth, thymidine incorporation, and cell adhesion (Johanning & Lin, 1995; Noguchi, Earashi, et al., 1995; Rose & Connolly, 1990). Other studies have shown that lipoxygenase products (e.g., hydroxy and hydroperoxy fatty acids rather than COX metabolites (e.g., PGs and TXs), play a role in fatty acid-stimulated growth of tumor cells (Buckman, Hubbard, & Erickson, 1991; Johanning & Lin, 1995; Rose & Connolly, 1990).

COX-1 and -2 are two of the targets of nonsteroidal antiinflammatory agents, and treatment with nonsteroidal antiinflammatory agents is associated with a decrease in COX-2 activity in colon tumors. Studies suggest that ω-6 PUFAs promote colon and mammary tumorigenesis by upregulating COX-2 and p21ras expression (Badawi, El-Sohemy, Stephen, Ghoshal, & Archer, 1998; Singh, J., Hamid, & Reddy, 1997).

Eicosapentaenoic Acid and Docosahexaenoic Acid

Animal research suggests that ω-3 fatty acids serve as competitive inhibitors of the cyclooxygenase and lipoxygenase pathways and generate an alternative set of eicosanoids that tend to suppress tumor growth rather than favoring it (Ferretti & Flanagan, 1990). ω-3 PUFAs may exert their antitumor effect by inhibiting COX-2 expression and AA metabolism (Badawi et al., 1998). Metabolic products of the action of COX on AA are reduced in mammary tumors of animals fed diets high in ω-3 long-chain PUFAs (e.g.,

EPA and DHA; Abou-el-ela et al., 1988; Karmali et al., 1984). Other mechanisms have also been examined. Christiansen, Lund, Rørtveit, and Rustan (1991) found that rats fed a diet high in fish oil had reduced liver microsomal fatty acid desaturase activities (Δ5 and Δ6 desaturase). Both desaturases are important in the metabolism of LA to AA. Gonzalez et al. (1993) proposed that dietary fish oil inhibits mammary gland tumorigenesis by the generation of secondary lipid peroxidation products.

Metastasis

Metastasis is the process by which malignant cells from a primary tumor are disseminated to other areas of the body to form secondary growth. The major cause of death in patients with cancer is usually not from the primary tumor but rather from the inability to control metastasis. Metastasis is a complicated series of sequential processes dependent on various tumor cell-host interactions (Stracke & Liotta, 1992). Figure 3 illustrates the major steps in the pathogenesis of metastasis. Initial neoplastic transformation of susceptible cells gives rise to a small population of tumor cells. Vascularization of this early neoplastic lesion allows further proliferation of tumor cells and enlargement of the primary tumor. Malignant cells within the primary tumor next begin to invade the surrounding host tissue(s). Entry of invading tumor cells into lymphatics or blood vessels serves to transport them to distant sites in the body where they lodge and arrest in the capillary beds of a specific organ. The arrested cells then exit from capillaries into the surrounding organ parenchyma where, subject to provision of a suitable environment, they proliferate to form metastases.

Invasion. Invasion involves penetration of host tissue and the ECM. There are physical barriers between circulating tumor cells and normal extravascular tissues: the layer of endothelial cells that lines the interior of blood vessels and lymph vessels, and the ECM (Liotta, 1992). The binding of tumor cells to the endothelial layer causes it to retract and expose the tissue beneath. However, the ECM, the barrier beneath the endothelial cells, is more substantial. The ECM is a dense meshwork of diverse proteins and carbohydrate molecules. It consists of the basement membrane and interstitial stroma (located just beneath the basement membrane). The basement membrane does not usually contain pores or channels large enough for tumor cells to passively traverse it. Thus, metastatic tumor cells must find another way to penetrate the basement membrane. Invasion of the basement membrane barrier consists of three distinct but interlocking steps: adhesion of the tumor cell to the basement membrane; activation of destructive enzymes that cleave or unravel basement membrane molecules immediately beneath the tumor cell; and protrusion of the tumor cell's pseudopodia (amoeboid false feet) into the zone of lysis, followed by migration of the entire tumor cell (Liotta, 1992). These three steps must be tightly coordinated and properly timed. Metastatic cancer cells penetrate the basement membrane via the production of various proteases. Important proteases are the type IV collagen-specific collagenases, which degrade the type IV collagen network that forms the basic structure of the basement membrane (Dano et al., 1985). A major focus has been on the family of proteases called the matrix metalloproteinases (Bosman, 1994; Deryugina, Luo, Reisfeld, Bourdon, & Strongin, 1997; Van Noorden, Meade-Tollin, & Bosman, 1998), which are regulated by tissue inhibitors of matrix metalloproteinases. Matrix metalloproteinases are a family of zinc-requiring matrix-degrading enzymes that

includes the collagenases, gelatinases, and the stromelysins, all of which have been implicated in invasive cell behavior (Hynes, 1992; Stetler-Stevenson, Liotta, & Kleiner, 1993). Two other protease families are also under investigation for activity during metastasis: cysteine proteases, such as cathepsin-B; and serine proteases, such as urokinase-type plasminogen activator (Van Noorden et al., 1998). Urokinase-type plasminogen activator converts plasminogen to plasmin, which can activate proenzymes by proteolytic cleavage (Dano et al., 1985).

Angiogenesis. Angiogenesis plays a critical role in various pathological conditions, such as solid tumor formation and metastasis, and in inflammation-related diseases (Stromblad & Cheresh, 1996). The expansion of solid tumors beyond a minimal size (~2mm) is critically dependent on the formation of new blood vessels to supply oxygen, nutrients, and growth factors (Folkman, 1995). Angiogenesis is also crucial for the formation of metastases at secondary sites, and, accordingly, a high degree of vascularization in certain tumors indicates a poor clinical prognosis and an increased risk of metastasis (Weidner, 1995).

The mechanism of angiogenesis can be divided generally into three phases: initiation, proliferation/invasion, and maturation. First, angiogenic stimulators, such as prostaglandins, hydroxy fatty acids, growth factors, and cytokines (e.g., 12-hydroxyeicosatetraenoic acid, vascular endothelial growth factor, fibroblast growth factor, and interleukin-8) are released from tumors, inflammatory cells, or both. These factors stimulate vascular cell proliferation and invasive behavior, thereby promoting blood vessel growth and invasion of the tumor. These angiogenic signals trigger the prolifera-

tion/invasion phase of angiogenesis, which is characterized by secretion of both components of the ECM and proteolytic enzymes, which serve to remodel the extracellular microenvironment. Endothelial cells invade the remodelled ECM and proliferate at the leading edge of a migrating column, thereby forming a sprout. Eventually, the vascular sprouts begin to mature as cell differentiation and lumen formation ensue. The new vessel secretes basement membrane components, which serve to induce and maintain the endothelial cells in a differentiated and quiescent state. The sprouts also fuse into loops, thereby enabling blood to circulate in the new vessels (Stromblad & Cheresh, 1996). There is some evidence that angiogenesis is probably initiated because cells in the mass, especially those in the interior, become starved for oxygen (Van Noorden et al., 1998). Once the blood supply is in place, the cell mass can import the oxygen and the nutrients it needs to keep growing. Furthermore, the cells have a passageway through which they can escape and invade other tissues.

Extravasation/proliferation of metastatic cells. Cancer cells that survive the trip through the bloodstream ultimately seek out a new tissue. The selection of the new target is often quite specific to the type of cancer cell. The choice of target is generally determined by very specific interactions between molecules on the cancer cell surface and molecules on the surfaces of the endothelial cells that line the blood vessels in the new host tissue (Van Noorden et al., 1998). It is likely that carbohydrates protruding from the cancer cell surface become bound to a type of carbohydrate receptor on the endothelial cells called a selectin (McEver, 1997). Normally, the carbohydrate-selectin interactions are used by white blood cells that need to identify particular tissues to combat local infec-

tion; however, cancer cells can use this system as well. Once the cancer cell contacts a surface to which it can adhere, it rolls along the blood-vessel wall because the carbohydrate-selectin bonds are relatively weak (Walcheck et al., 1996). The cancer cell then comes to a complete stop as integrin-mediated bonds form between the cells. The cancer cell then migrates into the host tissue by passing through the blood-vessel wall and degrading the connective-tissue matrix with proteases. The cancer cell is now ready to proliferate and form a new tumor in its new host tissue.

Effects of Fatty Acids on the Metastatic Cascade

Metastasis. High levels of dietary LA (12% w/w) increased metastasis of 4526 mouse mammary tumor cells in BALB/cAnN mice via influences on lodgement, implantation, and survival of these cells (Hubbard & Erickson, 1989). Karmali et al. (1993) also found that high dietary levels of LA enhanced experimental metastasis of 13762MAT:B mammary adenocarcinoma cells in Fischer 44 rats. Likewise, Rose, Hatala, Connolly, and Rayburn (1993) showed that nude mice fed a diet high in LA (8 and 12% w/w) exhibited a higher incidence of grossly visible MDA-MB-435 cell pulmonary metastatic nodules than those fed a 2% LA diet ($p < 0.05$). In a study of MDA-MB-231 cell tumor-bearing mice, only 1 in the 12% LA dietary group and 2 in the 2% LA dietary group had macroscopic nodules, but the incidence of microscopic metastases was 68 and 42%, respectively (Rose, Connolly, & Liu, 1994b). This study confirmed a report by Price, J. E., Polyzos, Zhang, and Daniels (1990) that, in contrast to MDA-MB-435 cells, the MDA-MB-231 cell line exhibits a low propensity for the development of macroscopic metastases from tumors located in the mammary fat pad of nude mice.

In contrast, studies have shown that diets high in EPA and DHA (ω-3 fatty acids) can reduce the severity of lung metastases of MDA-MB-435 cancer cells in the nude mouse model, Ncr-nu/nu (Rose & Connolly, 1993; Rose et al., 1995). Likewise, Reich et al. (1989) found that malignant B16-F10 murine melanoma and HT-1080 human fibrosarcoma cells cultured in media containing 0.1-10 μM EPA had reduced ability to metastasize to the lung after intravenous injection into the tail vein of C57BL/6 mice. Ip, Singh, Thompson, and Scimeca (1994) found that dietary conjugated LA, between 0.05 and 0.5%, produced a dose-dependent inhibition in mammary tumor yield when fed chronically to rats treated with a low dose (5mg) of 7,12-dimethylbenz(a)anthracene .

Adhesion. R. K. Singh et al. (1995) showed that 0.1 mM stearate (a saturated fatty acid) inhibited human fibrosarcoma HT-1080 tumor cell adhesion to Amgel (a new biologi-cally active human ECM) and laminin; however, similar treatment with 0.1 mM oleic acid, a monounsaturated fatty acid, for 2-4 hr did not alter tumor cell adhesiveness. In contrast, Johanning and Lin (1995) showed that treatment with < 0.1 mM oleic acid (for 5 days) increased adhesion of MDA-MB-231 cells to Matrigel and fibronectin. The differences in the concentrations of oleic acid used in these studies, as well as differences in length of incubation period and cell type, may be the reasons for differences in the results. Jiang et al. (1995) found that gamma linolenic acid (GLA), at concentrations up to 100 μM, markedly reduced attachment of human colon cancer cell lines HT115, HT29, and HRT18 to ECM components (Matrigel and fibronectin). Johanning and Lin (1995) showed that 0.75 μg/ml LA and 2.5 μg/ml EPA increased adhesion of MDA-MB-231 cells to Matrigel and type IV collagen, whereas 2.5 μg/ml EPA decreased adhesion of a

less metastatic cell line, SK-BR-3, to these two basement membrane substrates. Likewise, German and Johanning (1997) found that 4.5 μg/ml EPA decreased adhesion of MDA-MB-231 cells to Matrigel, fibronectin, and type IV collagen. Both of these studies involved treating the cells with or without EPA for 5 days. It appears that low levels of EPA stimulate adhesion of MDA-MB-231 cells to the ECM, whereas higher levels are inhibitory.

In summary, these studies indicate that both saturated and unsaturated fatty acids modify the ability of cancer cells to adhere to protein components of the basement membrane in vitro.

Invasion. Connolly and Rose (1993) found that, at concentrations of 0.25 and 0.5 μg/ml, LA stimulated and EPA and DHA inhibited MDA-MB-435 tumor cell invasion through Matrigel. Singh et al. (1995) showed that 0.3 mM stearate inhibited the ability of highly invasive human fibrosarcoma HT-1080 cells to traverse Amgel by 59.4 ± 8%. In contrast, treatment with 0.3 mM myristate or palmitate (saturated fatty acids) had no effect. GLA, at concentrations up to 100 μM, markedly reduced invasion of human colon cancer cell lines HT115, HT29, and HRT18 (Jiang et al., 1995).

Growth. Several studies have shown that high levels of fish (menhaden) oil, as well as other ω-3 fatty acids, significantly suppress growth of tumor cells in vitro and in vivo (Gonzalez et al., 1991, 1993; Grammatikos et al., 1994; Rose & Connolly, 1990; Welsch, Oakley, Chang, & Welsch, 1993). In contrast, members of the ω-6 family (namely LA and AA) have been shown to stimulate growth in various tumor cell lines

(Holley, Baldwin, & Kiernan, 1974; Hussey & Tisdale, 1994; Rose & Connolly, 1989, 1990). Various studies have shown that rodents on high fat diets, either saturated or unsaturated, experience increased mammary gland tumorigenesis (Welsch, 1994). In addition, the enhancing effect of increased dietary fat on mammary gland tumorigenesis was most often observed during the promotional stage of this tumorigenic process. In contrast, several studies have shown that altering the types of dietary fat influences the development of mammary tumors in mice and rats (Abou-el-Ela et al., 1988; Boissoneault et al., 1986; Braden & Carroll, 1986; Hubbard et al., 1988; Hubbard & Erickson, 1987; Karmali, et al., 1984; Katz & Boylan, 1987).

The relevance of these experimental studies to human breast cancer risk and disease progression is unclear; however these studies do present a plausible biological basis for such a relationship in human populations.

Involvement of Membrane Signaling Pathways in the Metastatic Cascade

Malignant transformation of normal cells is characterized by dysregulation of cellular proliferation and differentiation, which has been associated with aberrations in cell signaling systems (Cho-Chung, 1990). Protein kinases A and C (PKA and PKC) are critical intracellular components of two second messenger systems activated by the binding of extracellular ligands to cell-surface receptors. Both play a critical role in signal transduction pathways involved in the control of cell proliferation (Gordge, Hulme, Clegg, & Miller, 1996; Starzec et al., 1994). Focal adhesion kinase (FAK) is implicated in the regulation of cytoskeletal organization, cell adhesion, and motility (Schaller & Parsons, 1994). Starzec et al. (1994) found that alterations in the PKA pathway are early events and are probably important to cell immortalization, but do not necessarily lead to

malignant development. In contrast, changes in the PKC pathway are later events associated with advanced malignant transformation. Studies have also shown that the activities and expression of PKA, PKC, and FAK signaling pathways are significantly higher in malignant compared with normal breast tissue (Gordge et al., 1996; O'Brian, Vogel, Singletary, & Ward, 1989; Owens et al., 1995).

Cyclic adenosine monophosphate-dependent protein kinase (PKA). Adenosine 3', 5'-cyclic monophosphate (cAMP) functions as a second messenger to transduce and amplify the action of hormones, neurotransmitters, and other effectors through a G-protein-coupled adenylate cyclase signaling pathway. Adenylate cyclase is activated by a stimulatory G protein (G_s). G_s is composed of three polypeptides: an α chain ($G_{s\alpha}$), which binds and hydrolyzes guanosine triphosphate and activates adenylate cyclase; and a tight complex of a β chain and a γ chain ($G_{\beta\gamma}$), which anchors G_s to the cytoplasmic face of the plasma membrane. When activated by binding to a receptor-ligand complex, the guanyl-nucleotide-binding site on $G_{s\alpha}$ is altered, allowing guanosine triphosphate to bind in place of guanosine diphosphate. The binding of guanosine triphosphate is thought to cause $G_{s\alpha}$ to dissociate from $G_{\beta\gamma}$, allowing $G_{s\alpha}$ to bind tightly to an adenylate cyclase molecule, which is activated to produce cAMP. Within less than a minute, the $G_{s\alpha}$ hydrolyzes its bound guanosine triphosphate to guanosine diphosphate, causing $G_{s\alpha}$ to dissociate from the adenylate cyclase and reassociate with $G_{\beta\gamma}$ to reform an inactive G_s molecule. The signal produced by cAMP is transduced by binding to its receptor protein, the regulatory subunit of PKA (Alberts et al., 1994). All actions of cAMP that contribute to

the regulation of a variety of cellular functions are related to protein phosphorylation of PKA targets via activation of PKA.

The PKA holoenzyme contains two catalytic subunits bound to a regulatory subunit dimer. The binding of two cAMP molecules to each of the regulatory subunits leads to the dissociation of the regulatory-catalytic subunit complex into a regulatory subunit dimer and two catalytic subunits. The catalytic subunit is an active protein-serine/threonine kinase that regulates activities of other cellular proteins via phosphorylation (Corbin et al., 1978). In mammalian cells, PKA exists as two isoforms, type I and type II, distinguishable by their different regulatory subunits, R-I and R-II (Taylor, Buechler, & Yonemoto, 1990). It is likely that there are functional differences between types I and II PKA isoforms due to their different affinities for cAMP and different turnover rates (Weber & Hilz, 1986). The synthesis of R-I and R-II and the relative abundance of PKA-I and PKA-II isoforms are differentially regulated during differentiation, cell growth, and neoplastic transformation (Cho-Chung & Clair, 1993). Predominant expression of PKA-II is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKA-I are steadily detected in tumor cells and transiently detected in normal cells exposed to mitogenic stimuli (Cho-Chung, Pepe, Clair, Budillon, &Nesterova, 1995). In this respect, PKA-I and its regulatory subunit, RIα, are generally overexpressed in human cancer cell lines and primary tumors and are induced following transformation by certain oncogenes, such as ras (Cho-Chung & Clair, 1993; Cho-Chung et al., 1995). Overexpression of RIα and PKA-I has been correlated with poor prognosis in breast cancer patients (Miller, Watson, Jack, Chetty, & Elton, 1993). Several studies have shown that use of an RIα antisense oligodeoxynucleotide efficiently

depleted the growth stimulatory RIα and induced apoptosis and differentiation in a variety of human breast cancer cell lines (Cho-Chung, 1996; Srivastava, Srivastava, Park, Agrawal, & Cho-Chung, 1998; Srivastava, Srivastava, Seth, Agrawal, & Cho-Chung, 1999).

Studies have demonstrated the importance of PKA activation in the regulation of cancer cell motility, adherence, and cytoskeletal organization (Lozano, Taitz, Petruzzelli, Djordjevic, & Young, 1996; Maier et al., 1996). Lozano et al. (1996) showed that the motility and adhesiveness of human head and neck squamous cell carcinoma cultures to type I collagen, fibronectin, vitronectin, and laminin are dependent on production of PG E_2 and on PKA activation. Studies by Maier et al. (1996) showed that Lewis lung carcinoma tumors have significantly more PKA activity than do nonmetastatic clones, and these cells exhibited a higher capacity to penetrate through filters coated with laminin, vitronectin, and type I collagen and were less adherent to these same ECM components. These studies suggest that PKA signaling is important for modulating the tumor cell-ECM interaction.

Protein kinase C (PKC). PKC is a family of serine/threonine protein kinases that plays a critical role in signal transduction, tumor promotion, and cell regulation (Blobe, Khan, & Hannun, 1995; Epand, 1994). Diacylglycerol (DAG), a product of phosphatidylinositol turnover, as well as tumor promoting phorbol esters, is a potent activator of PKC (Castagna et al., 1982; Hannun, Loomis, & Bell, 1986). The PKC activation by phospholipids and DAG occurs in a two-step process (Blobe et al., 1995). In the initial step, calcium and phosphatidylserine recruit inactive PKC from the cytosol to the membrane,

where it remains inactive, but has increased affinity for substrates. This inactive PKC then interacts with DAG at the membrane. DAG serves to increase the affinity of PKC for calcium and phophatidylserine so that physiological levels of these compounds are then able to activate PKC.

PKC does not exist as a single enzyme but involves 12 isoenzymes characterized to date (Dekker & Parker, 1994). These isoenzymes are closely related in structure: each is composed of a single polypeptide chain divided into two domains, a regulatory domain at the amino terminus and a catalytic domain at the carboxyl terminus (Blobe, Obeid, & Hannun, 1994). These isoenzymes have been divided into three groups based on biochemical properties and sequence analysis: the calcium-dependent (conventional) group comprised of PKC α, βI, βII, and γ; the calcium-independent (novel) group, including PKC δ, ε, η, θ, and μ; and the atypical PKC isoenzymes PKC ζ, ι, and λ (which do not respond to DAG or phorbol esters in vitro or in vivo). The individual isoenzymes are expressed in a tissue-specific and developmentally regulated manner, suggesting that the individual PKC isoenzymes have distinct roles in vivo (Blobe et al., 1995).

PKC has been shown to be activated by free fatty acids, both in vitro and in vivo (Holian & Nelson, 1992; McPhail, Clayton, & Snyderman, 1984). These studies showed that 50 μM AA was able to activate PKC in the presence of calcium. However, there were several reports that were contradictory with respect to the role of calcium and DAG in fatty acid-induced activation (Murakami et al., 1986; Seifert, Schachtele, Rosenthal, & Schultz, 1988; Sekiguchi, Tsukuda, Ogita, Kikkawa, & Nishizuka, 1987; Verkest et al., 1988). Khan, Blobe, and Hannun (1992) presented a model for the activation of PKC by free fatty acids and phosphatidylserine/DAG. They showed that phospholipase A_2-

induced lipid hydrolysis causes the release of AA, which partitions between the membrane and cytosol, perhaps aided by fatty acid binding proteins. The cytosolic AA then interacts with cytosolic PKC to activate PKC. Johanning and Lin (1995) showed that calphostin C, a PKC inhibitor, suppresses the increase in adhesion observed when MDA-MB-231 cells are incubated in media with added LA. Thus, LA appeared to increase human breast cancer cell adhesion to ECM components by activating the PKC pathway. Holian and Nelson (1992) showed that stearic acid (a saturated fatty acid) and oleic acid are weak activators of PKC in the absence of phosphatidylserine and diolein. Fish oils, which are rich in ω3 fatty acids, may impede tumor angiogenesis and invasiveness by downregulating PKC (McCarty, 1996).

PKC has been implicated in the metastatic process (Gopalakrishna & Barsky, 1988). Studies have shown a positive correlation between PKC activity and the ability of tumor cells to form metastases as well as the ability of inhibitors of PKC or the down-regulation of PKC to inhibit metastasis (Gopalakrishna & Barsky, 1988; Liu, B. et al., 1992; Palmantier et al., 1996). One mechanism of PKC action in cancer cell adhesion is thought to be by modulation of cellular adhesion to the ECM in response to PKC (Liu et al., 1992; Palmantier et al., 1996). In addition, many cell adhesion receptors are PKC substrates (Herbert, 1993). Studies using various protein kinase inhibitors have also provided evidence that PKC may be important in certain phases of metastasis (Liu et al., 1992; Schwartz et al., 1990). PKC is an attractive target in cancer therapy. Several compounds that inhibit PKC expression have exhibited promising antitumor activity in vitro (Basu, 1993) and in vivo (Yuen et al., 1999). A Phase I study of an antisense oligonucleotide to PKC-α (ISIS 3521/CGP 641284) provided evidence of antitumor activity in

patients with ovarian cancer (Yuen et al., 1999), thus providing the rationale for Phase II studies in ovarian cancer and other malignancies.

Padma and Das (1999) showed that the cis-PUFAs AA, GLA, EPA, and DHA activated phospholipase C and enhanced diacylglycerol formation in an AK-5 macrophage cell line. These fatty acids also enhanced PKC activity but decreased PKA activity, thus suggesting that cis-PUFAs can alter activities of second messenger systems such as diacylglycerol and protein kinases. Roberts et al. (1998) suggest that cis-PUFAs do not stimulate cell adhesion by direct activation of PKC or by perturbation of membrane fluidity; rather, cis-PUFA-induced cell adhesion and cell spreading are PKC dependent. This study also suggested that an increase in intracellular calcium stimulates AA metabolism through a lipoxygenase pathway resulting in, or conjunction with, PKC activation. Activated PKC then leads to a rapid and specific increase in adhesion to type IV collagen and vitronectin.

Collectively, these studies suggest that PKC may be a key regulatory enzyme in tumor-cell adhesion to ECM components and that inhibition of PKC reduces tumor-cell metastasis.

Focal adhesion kinase (FAK). Cancer cell invasion is a complex process that includes alterations in cell adhesion and motility (Liotta, Steeg, & Stetler-Stevenson, 1991). Some of these alterations develop at focal adhesions, which serve as transmembrane junctions between the ECM and cytoskeleton and consist of three distinct components: (a) an extracellular component made up of ECM proteins; (b) a transmembrane region composed of integrin proteins, particularly members of the β1 family of integrins; and (c)

an array of intracellular cytoskeletal proteins (Burridge, Fath, Nuckolls, & Turner, 1988; Owens et al., 1995). The counterbalance between the formation of new focal adhesions and disruption of old focal adhesions is not only dictated by whether cells need to undergo motile transformation but also dramatically influenced by various external stimuli, including growth factors, hormones, cytokines, and bioactive lipids (Burridge et al., 1988).

FAK is a widely expressed and highly conserved nonreceptor protein tyrosine kinase of molecular mass 125 kDa that is localized to focal adhesions and is implicated in the regulation of cytoskeletal organization, cell adhesion, and migration (Schaller & Parsons, 1994). FAK is a structurally distinct kinase (Schaller et al., 1992). The catalytic domain is centrally located between similarly sized N-terminal and C-terminal noncatalytic domains. The N-terminal domain is proposed to interact with integrin cytoplasmic domains and may provide a direct link through which FAK is activated upon integrin clustering (Schaller, Otey, Hildebrand, & Parsons, 1995). In addition, the C-terminal domain contains several regions that appear to regulate FAK activity. The C terminus also contains potential binding sites for the src homology-2 domains of the cell-signaling molecules Grb2 and phosphatidylinositol 3-kinase (Hildebrand, Taylor, & Parsons, 1996; Schaller et al., 1995).

FAK plays a central role in integrin-mediated signal transduction (Clarke & Brugge, 1995). Binding of integrins to the ECM stimulates FAK to undergo phosphorylation on Tyr^{397} (Schaller et al., 1994), which, in turn, serves as a binding site for the src homology-2 domains of Src and Src-family tyrosine kinases (e.g., $pp60^{src}$, $pp51^{csk}$, $pp59^{fyn}$; Cobb, Schaller, Leu, & Parsons, 1994; Schlaepfer, Hanks, Hunter, & van der

Geer, 1994; Xing et al., 1994). A specific tyrosine residue within another focal adhesion protein, paxillin, has been identified as a primary target for phosphorylation by FAK (Parsons et al., 1994). FAK phosphorylation is coincident with adhesion of cells to ECM and is maximal prior to cell spreading; it has been suggested that activation of FAK may be necessary for cell spreading (Kornberg, L., Earp, Parsons, Schaller, & Juliano, 1992). Using an in vitro binding assay, Schaller et al. (1995) reported a direct association of the β1 integrin subunit with FAK. FAK bound to the peptide KLLMIIHDRREFA, which represents the membrane proximal part of the β1 integrin subunit. Danker, Gabriel, Heidrich, and Reutter (1998) also reported a possible association of FAK with the β1 integrin subunit in a human keratinocyte derived cell line.

Based on studies of elevated FAK in invasive and metastatic colonic lesions and breast tumors (Owens et al., 1995) and in adenomatous, invasive, and metastatic human tissue (Weiner, Liu, Craven, & Cance, 1993), the level of FAK expression might be a marker for determining the invasive potential of a tumor. Thus, FAK expression levels may be a useful marker of occult invasion in premalignant conditions. FAK might be a rational therapeutic target to interrupt the invasive and metastatic process. It would be useful to study the effects of attenuating FAK expression or interrupting its signal transduction pathway in tumor cells.

Epidermal growth factor (EGF). Interaction between tumor cells and host environment is precisely and sequentially regulated in vivo by growth factors and ECM components. Growth factors and systemic hormones play pivotal roles in hormone-regulated cancers such as breast cancer. The epidermal growth factor receptor (EGFR), which acts

as a signal transduction molecule in a variety of physiological and pathological states, has been shown to play a role in tumor progression (Zhang, Singh, Wang, Wells, & Siegal, 1996). EGF, transforming growth factor-α, and a growing family of other structurally related polypeptides bind to and activate the EGFR. The EGFR is a 170-kDa transmembrane tyrosine-kinase molecule that becomes activated when the ligand binds to the extracellular domain (Martinez-Lacaci & Dickson, 1996). After binding of its ligand, the EGFR becomes activated, undergoes a conformational change, dimerizes, and is internalized. Upon activation, the receptor can autophosphorylate and, in turn, phosphorylate other proteins, thus triggering a signal transduction cascade event (Eling & Glasgow, 1994).

Overexpression of EGFR and c-erbB2 (a member of the EGFR tyrosine kinase family) frequently occurs in human breast cancers, correlating with poor prognosis (Davidson, Gelmann, Lippman, & Dickson, 1987; Fujii, Nakajima, & Imamura, 1995; Sheikh et al., 1994). A common mechanism for c-erbB2 overexpression in breast cancer is gene amplification, but increased c-erbB2 protein is also found in some cancers with normal gene copy number (Slamon et al., 1989). However, EGFR overexpression is usually due to increased receptor synthesis (Earp, Dawson, Li, & Yu, 1995). EGFR expression is inversely correlated to estrogen receptor expression (Chrysogelos & Dickson, 1994; Lee, C. S. L., deFazio, Ormandy, & Sutherland, 1996). Estrogen receptor-negative breast tumors are more aggressive and metastatic than the estrogen receptor-positive tumors (Sheikh et al., 1994). EGF is potentially mitogenic for a variety of cultured cells, including mammary epithelial cells (Davidson et al., 1987; Dong, Berthois, & Martin, 1991). However, the mitogenic activity of EGF in vitro does not appear to necessarily

relate to the level of EGFR because EGF fails to stimulate the growth of some human breast carcinoma-derived cell lines with high numbers of EGFR (Veber, Prévost, Planchon, & Starzec, 1994). The MDA-MB-231 cell line possesses large numbers of EGFR on its cell surface (3×10^4 EGF binding sites per cell; Mueller et al., 1994), but does not respond to EGF (0.5 pM to 5 nM) under standard culture conditions (Davidson et al., 1987). In contrast, EGF (10 – 100 ng/ml) has been shown to be growth inhibitory to MDA-MB-468 cells, which also overexpress EGFRs (Armstrong, D. K. et al., 1994).

Yudoh et al. (1994) showed that EGF is capable of modulating cancer cell adhesion and invasiveness. EGF increased adhesion, as well as in vitro invasiveness, of high-metastatic RTC sarcoma cells to Matrigel, fibronectin, and laminin. Hamada et al. (1995) also showed that EGF enhanced the metastatic capacity and in vitro invasiveness into Matrigel of a weakly malignant cell line derived from a highly metastatic rat mammary adeno-carcinoma cell line, c-SST-2. In contrast, German and Johanning (1997) showed that EGF caused a dose-dependent decrease in adhesion of MDA-MB-231 cells to Matrigel; however, Long and Rose (1996) showed that EGF did not affect the invasive potential of MDA-MB-231 cells. Fujii et al. (1995) showed that the effect of EGF on adhesion and spreading of human cutaneous squamous carcinoma cells was dependent on the ECM component being studied. EGF decreased spreading on fibronectin and increased spreading on type I collagen by selectively up-regulating $\alpha_2\beta_1$ integrin expression in HSC-1 cells. Lichtner, Wiedemuth, Noeske-Jungblut, and Schirrmacher (1993) and Rohde-Schulz and Lichtner (1995) showed that EGF exhibited rapid effects on adhesion of the rat mammary adenocarcinoma cell line MTLn3 to fibronectin and collagen, respectively.

Xie et al. (1998) report that EGF-treated NR6 fibroblasts exhibit decreased adhesiveness to Amgel, presumably due to focal adhesion disassembly.

The interaction of growth factors with their receptors on breast cancer cells can lead to the hydrolysis of phospholipids and release of fatty acids, such as AA, which can be further metabolized by the lipoxygenase pathway (Natarajan et al., 1997). Arachidonic acid and LA metabolites serve to modulate growth factor signaling pathways that lead to cell proliferation, transformation, or both (Eling & Glasgow, 1994). Wickramasinghe, Jo, McDonald, and Hardy (1996) suggest that long-chain saturated fatty acids inhibit EGF-induced growth of Hs578T breast cancer cells via a mechanism involving disruption of an EGFR-G-protein signaling pathway. Based on studies by Bandyopadhyay, Hwang, Imagawa, and Nandi (1993), it is proposed that, although both EGF and essential fatty acids evoke multiple pathways in cells, PKC and PKA activity, as well as tyrosine kinase activity of EGFRs, may converge in the phosphorylation of a 40-42 kDa protein which could be a downstream messenger of EGF and essential fatty acid action.

Several studies have reported that PKA-I expression and function are correlated to ras and EGFR-dependent mitogenic signaling (Burgering, 1993; Ciardiello et al., 1993). Tortora, Damiano, Bianco, Baldassarre, and Bianco (1997) recently provided direct evidence of interaction of PKA-I with the activated EGFR macromolecular signaling complex. This in vitro study, using human normal epithelial MCF-10A cells, showed that this interaction is due to the binding of the RIα subunit to the src homology-3 domain(s) of the Grb2 adaptor protein, thus allowing the recruitment of the PKA-I holoenzyme to the activated EGFR. The Grb2 adaptor protein is composed of one src homology-2 and two –src homology-3 domains that couple EGFR to the ras pathway. Tortora et al. (1997)

also showed that both RIα regulatory and Cα catalytic subunits coprecipitated with the activated EGFR and that these subunits were present in the macromolecular signaling complex as an activatable PKA-I holoenzyme.

In breast cancer, EGFR expression is inversely proportional to the level of expression of estrogen receptor (Chrysogelos & Dickson, 1994: Lee, C. S. L., et al., 1996), suggesting that the loss of hormone responsiveness may be associated with altered responses to ligand binding of the EGFR. Because EGFR overexpression is seen in breast cancer, the EGFR on the cancer cells represents a potential therapeutic target.

Role of Cell Adhesion Molecules in Metastasis

Cell-cell and cell-ECM adhesive interactions are clearly important for a wide range of phenomena in normal and aberrant cells (Albelda, 1993). The molecules that mediate adhesion of eukaryotic cells to ECM components and to other cells fall into four major classes: integrins, cadherins, selectins, and the immunoglobulin (Ig) superfamily. Cell adhesion molecules (CAMs) play an important role in morphogenesis, the inflammatory response, and metastasis (Tang & Honn, 1994; Weiss, 1994-1995). As a result, mechanisms of cell-cell and cell-ECM adhesion and the molecules that mediate these interactions have received increasing attention. Of particular current interest is the regulation of cell adhesion molecule expression, interactions of all cell adhesion molecules with each other, with ligands, and with intracellular proteins, and intracellular signaling interactions with ligands.

Integrins. Integrins, probably the best characterized CAMs, are noncovalently associated heterodimeric receptors consisting of an α and a β subunit (Hynes, 1992). Each subunit contains a transmembrane domain and a short cytoplasmic segment. Specificity of integrin binding to ECM components and to other cell adhesion factors appears to depend primarily on the extracellular portion of the α subunit, although both subunits are required for functional activity. The integrin α and β subunits can associate with one another in binding to a variety of ligands. ECM components that serve as ligands for integrins include fibronectin, laminin, collagens, tenascin, vitronectin, and fibrinogen (Akiyama, Nagata, & Yamada, 1990). The binding of ligands to integrins is usually mediated by arginine-glycine-aspartic acid sequences for various numbers of adhesive proteins (Ruoslahti & Pierschbacher, 1987). However, in most cases, different sequences other than arginine-glycine-aspartic acid (e.g., aspartic acid-glycine-glutamic acid-alanine, glutamic acid-isoleucine-leucine-aspartic acid-valine, glycine-proline-arginine-proline, and lysine-glutamine-alanine-glycine-aspartic acid-valine) mediate binding. Table 1 summarizes integrins that bind primarily to basement membrane components. Integrins are divided into three subfamilies, based on their cell-binding activity: (a) those that function as cell-cell adhesion molecules (primarily found on leukocytes); (b) those that bind primarily to the major constituents of the basement membrane (i.e., collagen and laminin); (c) and those that bind primarily to the ECM proteins found during early development, inflammation, and wound healing (i.e., fibronectin, fibrinogen, vitronectin, thrombospondin; Albelda & Buck, 1990; Hynes, 1992).

Table 1

Integrins That Bind Primarily to Basement Membrane Components

Integrins	Ligands
$\alpha 1\beta 1$ / $\alpha 2\beta 1$	Laminin, type I/IV collagen
$\alpha 3\beta 1$	Laminin, collagen, fibronectin
$\alpha 4\beta 1$ / $\alpha 5\beta 1$	Fibronectin
$\alpha 6\beta 1$ / $\alpha 7\beta 1$ / $\alpha 6\beta 4$	Laminin
$\alpha v\beta 1$	Vitronectin, fibronectin
$\alpha v\beta 3$	Vitronectin, fibronectin, tenascin, collagen, thrombospondin
$\alpha v\beta 5$	Vitronectin
$\alpha v\beta 6$ / $\alpha 4\beta 7$	Fibronectin

Each cell type has its own pattern of integrin distribution (Albelda et al., 1990; Virtanen et al., 1990). The cells of most tissues express $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ integrins, which are primarily required for adhesion to collagen and laminin (Albelda, 1993). The $\alpha_5\beta_1$ fibronectin receptor, however, is poorly expressed in most tissues, with the exception of large vessel endothelial cells (Cheresh, Smith, & Cooper, 1989).

In general, transformed and malignant cells express reduced levels of $\alpha_5\beta_1$ integrin (Feldman, Shin, Natale, & Todd, 1991; Tang & Honn, 1994; Zutter, Mazoujian, & Santoro, 1990), thus explaining a general reduction in the adhesion of malignant tumor cells to fibronectin. In contrast, most tumor cells demonstrate enhanced expression of integrin $\alpha_6\beta_1$ or $\alpha_6\beta_4$ (Lin, Zhang, & Kramer, 1993). Expression of many other integrins,

such as $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_v\beta_3$, may either remain unaltered or increase in some tumor types, but decrease in others (Mechtersheimer, Barth, Quentmeier, & Moller, 1994; Pignatelli, Hanby, & Stamp, 1991; Zutter et al., 1990). The pattern of integrin expression on tumor cells in situ, as well as that in cultured cells, appears to be complex and dependent on the tumor type. Malignant melanomas have a consistent upregulation of specific integrins during tumor progression (McGregor et al., 1989). Epithelial malignancies, however, have an inconsistent alteration in their integrin expression, although they generally tend to express fewer integrins than normal epithelia (Koukoulis et al., 1991; Zutter et al., 1990). A study by Gui, Puddefoot, Vinson, Wells, and Carpenter (1995) revealed that invasion of Hs578T and MDA-MB-231 human breast cancer cells is regulated by integrin receptors to the ECM. The invasive potential of these cell lines was inhibited by monoclonal antibodies directed against β_1 and β_5 integrins. Chiang, Yang, and Huang (1996) found that treating SW-480 human colon adenocar-cinoma cells with thrombin resulted in an upregulated cell-surface expression of β_3 integrins, which resulted in enhanced adhesion and migration on fibronectin. Antibodies against β_3 integrins blocked both the enhanced adhesion and migration. This upregulation of β_3 integrins also involved activation of PKC because thrombin-enhanced adhesion was diminished by PKC inhibition. EGF has also been shown to enhance adhesion and migration of human cutaneous squamous carcinoma on type I collagen via upregulation of $\alpha_2\beta_1$ integrin expression (Fujii et al., 1995). Hirasawa, Shijubo, Uede, and Abe (1994) showed that lung cancer cells adhere to ECM and endothelial cells through integrins, especially the β_1 subfamily.

In addition to functioning as transmembrane linkers between intracellular and extracellular proteins, integrins are also ideally placed to transmit signals across the cell

membrane. Therefore, integrins are also important signaling molecules (Hynes, 1992). Signal transduction through integrins occurs in two directions--moving from the extracellular microenvironment into the cell cytoplasm (outside-in-signaling) and from the cytoplasm out to the extracellular domain of the receptor (inside-out-signaling) (Hynes, 1992).

Outside-in integrin signaling has profound effects on many cellular functions, including cell-cycle progression, apoptosis, secretion of matrix-degrading proteinases, cell spreading, cell activation, cell differentiation, and gene expression (Clarke & Brugge, 1995; Schwartz, M. A., & Ingber, 1994). There is also evidence that the cytoplasmic domains play a role in the activation of intracellular signal transduction pathways resulting from ligand-receptor interactions (Hellberg, Eierman, Sjolander, & Anderson, 1995; Kanner, Grosmaire, Ledbetter, & Damle, 1993).

Inside-out signaling is the chief mechanism by which cells control integrin function (Faull & Ginsberg, 1996). The short cytoplasmic domains of integrins are topographically accessible to intracellular events and may, therefore, mediate inside-out signal transduction (Ginsberg, Du, & Plow, 1992). Much of what is known about integrin activation comes from studies of the platelet receptor αIIbβ3 (Marguerie, Plow, & Edgington, 1979). Thus, platelet aggregation is the best characterized example of a dependency on integrin affinity modulation (Bennett, Hoxie, Leitman, Vilaire, & Cines, 1983; Marguerie et al., 1979).

In their resting (inactive) state, integrins have low affinity for their ligands, and little or no detectable binding occurs under physiological conditions. The integrins must be activated to undergo adhesion and binding to the ECM. Activation of integrins occurs

by local stimuli, such as soluble agonists (hormones, cytokines, growth factors, etc.), or by solid interfaces, such as the ECM or other cells. These agonists do not act directly on the integrins but bind to cellular receptors, which leads to engagement of classical signal transduction systems. G proteins and tyrosine kinases usually transduce these signals, which results in activation of phospholipase C, changes in cytosolic calcium, and activation of cellular protein kinases (Schwartz, M. A., Schaller, & Ginsberg, 1995). Integrin activation results in a high affinity for its ligands, thus enabling cells to quickly adjust their adhesive phenotypes without changing the type or number of adhesion molecules that they express. As integrins bind to ECM, they become clustered in the plane of the cell membrane and associate with a cytoskeletal and signaling complex that promotes the assembly of actin filaments. These actin filaments form larger stress fibers, which causes more integrin clustering. As a result, aggregates consisting of ECM proteins, integrins, and cytoskeletal proteins assemble on each side of the membrane. Well-developed aggregates can be detected by immunofluorescence microscopy and are known as focal adhesions (Burridge et al., 1988).

Singh et al. (1995) found that stearate (a saturated fatty acid) inhibited human fibrosarcoma HT-1080 cell adhesion to Amgel via a mechanism involving a laminin integrin receptor. Tang, Grossi, Tang, Diglio, and Honn (1995) reported that a lipoxygenase metabolite of LA, 12(S)-hydroxyeicosatetraenoic acid, enhanced adhesion of Walker 256 carcinosarcoma cells to subendothelial matrix prepared from rat aortic endothelial cell monolayers via upregulation of $\alpha IIb\beta 3$ integrin expression. Roberts et al. (1998) suggest that cis-PUFAs selectively lead, probably via a specific pathway, to an activation of ad-

hesion molecules (e.g., integrins) perhaps utilizing PKC-dependent signal transduction pathways.

In summary, integrins are involved in cell migration, invasion, intravasation, and extravasation. Thus, a role for integrins in tumor growth and metastasis is obvious. Tumor progression leading to metastasis appears to involve equipping cancer cells with the appropriate integrin phenotype for interaction with the ECM. Interference with integrin signaling could provide a basis for the development of treatments for the various stages of cancer metastasis.

Cadherins. Cadherins are a family of calcium-dependent mediators of cell-cell adhesion important in forming and maintaining intercellular junctions (Takeichi, 1991). Therefore, alterations in cadherin expression may be expected to be associated with loss of normal cell-cell integrity. Cadherins bind homotypically (i.e., binding to another molecule of the same cadherin on a neighboring cell) in an interaction apparently mediated by an histidine-alanine-valine motif in the extracellular region of the cadherin (Nose, Tsuji, & Takeichi, 1990). All of the cadherins have a similar primary structure consisting of 723 to 748 amino acids. Each contains a putative signal peptide, an extracellular region with three repeated domains, a highly hydrophobic region that functions as a transmembrane anchor, and a relatively long cytoplasmic tail (Hatta, Nose, Nagafuchi, & Takeichi, 1988). Cadherins are divided into three subclasses: the E (epithelial)-cadherins, also known as uvomorulin, or L-CAM; the N (neural)-cadherins, also known as A-CAM or N-Cal-CAM; and P (placental)-cadherins (Takeichi, 1991). Cadherin function is dependent on interactions between the cytoplasmic domain and the elements of the cellular cyto-

skeleton. This interaction, however, is not direct. Cadherins are linked to the cytoskeleton by catenins (α, β, and γ; Ozawa, Baribault, & Kemler, 1989).

Expression of cadherins is developmentally regulated (Takeichi, 1991). In the adult, almost all epithelia express both E-cadherin and P-cadherin (Shimoyama et al., 1989). E-cadherin tends to be distributed uniformly throughout the epithelial layer, whereas P-cadherin is concentrated at the more basal layers. N-cadherin is found primarily on neural tissues and in the lens of the eye (Takeichi, 1991). It is also present in cardiac and skeletal muscles, where it plays an important role in maintaining the structure of the intercalated discs between adjacent muscle cells (Volk & Geiger, 1986).

Frixen et al. (1991) showed that carcinoma cell lines with an epithelioid phenotype were noninvasive and expressed E-cadherin, whereas, carcinoma lines with a fibroblastoid appearance were invasive and had lost E-cadherin expression. Downregulation of E-cadherin expression is involved in the loss of cell-cell contact, an initiating step in tumor progression and generation of metastatic variants (Tang & Honn, 1994). Decreased E-cadherin expression has also been correlated with increased grade of human prostate cancer and with poor prognosis in the prostate cancer patient (Umbas et al., 1994).

Jones, Royall, and Walker (1996) found a significant association between reduced membrane levels of E-cadherin and the presence of lymph node metastasis of infiltrating lobular carcinomas. There was also a highly significant correlation between the presence of cytoplasmic E-cadherin and metastasis. This study also demonstrated a link between reduced E-cadherin reactivity and EGFR expression, thus providing evidence of a possible link between membrane signaling pathways and modulation of E-cadherin expres-

sion. Siitonen et al. (1996) provided clinical evidence that loss of normal E-cadherin expression is an indicator of increased invasiveness and dedifferentiation in breast carcinoma. In a comparison of E-cadherin expression in normal breast specimens, fibroadenomas, and primary breast carcinomas, 70% of the primary tumors showed reduced expression of E-cadherin, suggesting that downregulation of this cell adhesion molecule is a common event in breast carcinoma (Guriec et al., 1996). This study also showed that patients exhibiting poorly expressed E-cadherin in tumor tissue had a worse prognosis.

Jiang et al. (1995) showed that treatment of various cell lines with GLA (an ω-6 fatty acid) for 24 hr increased E-cadherin expression in lung, colon, breast, melanoma, and liver cancer cells. LA and AA (ω-6 fatty acids) failed to induce these changes. This increased expression of E-cadherin was correlated with reduced in vitro invasion and increased aggregation.

Selectins. Selectins utilize carbohydrates as their recognition ligands (Rosen & Bertozzi, 1994; Varki, 1994). Selectins normally function as lymphocyte-homing and leukocyte enrollment receptors or as activation-induced surface proteins on platelets, endothelial cells, or both. Selectins have a common structure characterized by an N-terminal lectin-like domain, an area homologous to the EGFR, a variable number of complement-like repeats, and a short cytoplasmic domain. There are three subfamilies of selectins: L (leukocyte)-selectin, E (endothelial)-selectin (also known as ELAM-1), and P (platelet)-selectin (also known as GMP140 or PADGEM; Albelda, 1993). L-selectin is constitutively expressed on lymphocytes and neutrophils, whereas E- and P-selectins are

present on activated endothelial cells and are inducible by cytokines (Rosen & Bertozzi, 1994).

Lauri, Needham, Martin-Padura, and Dejana (1991) showed that adhesion of various colon carcinoma cell lines to cytokine-activated endothelial cells was inhibited by antibodies against E-selectin. Immunohistochemical studies revealed that, in contrast to normal tissues, a series of breast tumor cells showed increased intensity of staining for E- and P-selectins (52 and 67% of cases, respectively; Fox, Turner, Gatter, & Harris, 1995). In an analysis of 15 benign and 22 malignant estrogen-receptor-negative and estrogen-receptor-positive breast specimens, Nguyen et al. (1997) found that vascular expression of E-selectin was increased in the malignant breast tumors compared with their benign counterparts. In addition, interleukin-1α stimulated E-selectin expression more strongly in the estrogen-receptor-negative breast cancer cells (e.g., MDA-MB-231, Bt-549, Hs-578T) than in the estrogen-receptor-positive breast cancer cells (e.g., MDA-MB-435, MCF-7, T-47D).

Studies have shown that DHA and, to a lesser extent, oleic acid are able to reduce cytokine-induced endothelial expression of E-selectin in human adult saphenous vein endothelial cells (DeCaterina, Cybulsky, Clinton, Gimbrone, & Libby, 1995; DeCaterina & Libby, 1996). Collie-Duguid and Wahle (1996) also found that EPA and DHA were able to attenuate the induction of E-selectin expression in interleukin-1β-activated human umbilical vein endothelial cells.

Immunoglobulin (Ig) superfamily. The Ig superfamily encompasses a wide variety of molecules that share a common structural feature, the Ig homology unit. This struc-

ture consists of 70 to 110 amino acids organized into 7 to 9 β-pleated sheets. Each unit is stabilized by a constant disulfide bridge formed between two of the β strands (Hunkapiller & Hood, 1989). Most members of the Ig superfamily are involved in cell-cell recognition (Williams & Barclay, 1988) and include molecules that function in cellular immunity (i.e., major histocompatibility antigens, CD4, CD8, and the T-cell receptor), neural development (neural cell adhesion molecule), and leukocyte trafficking (intercellular adhesion molecule-1, platelet endothelial cell adhesion molecule, vascular cell adhesion molecule-1; Edelman & Crossin, 1991).

Members of the Ig superfamily are present on virtually every cell of the body (e.g., major histocompatibility antigens; Albelda, 1993). Cells of the immune system are particularly abundant in Ig-superfamily molecules (Springer, 1990). Other Ig superfamily members have more restricted distributions (e.g., carcinoembryonic antigen, neural cell adhesion molecule, and vascular cell adhesion molecule; Edelman & Crossin, 1991; Elices, Osborn, & Takada, 1990).

Because a number of the members of the Ig superfamily can function as cell-cell adhesion molecules, it is reasonable to postulate that these molecules may be involved in tumor invasion and metastasis. In some cases, a clear functional role of Ig superfamily members has been identified. In others, only an association with malignancy has been established. Because of the widely divergent nature of the Ig superfamily, it is more difficult to make generalizations than with the integrin, cadherin, or selectin families.

Some studies have shown that DHA and, to a lesser extent, oleic acid are able to reduce cytokine-induced endothelial expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (DeCaterina & Libby, 1996; DeCaterina et al., 1995).

In contrast, Collie-Duguid and Wahle (1996) showed that neither EPA nor DHA affected expression of intercellular adhesion molecule-1 or vascular cell adhesion molecule-1 in resting primary human umbilical vein endothelial cells. However, 65 μM EPA or DHA did attenuate the induction of these adhesion molecules in interleukin-1β-activated human umbilical vein endothelial cells.

Although our understanding of the biology of cell adhesion is rapidly advancing, it is difficult to propose one simple scheme in which CAMs can be related to tumor growth and metastasis. This difficulty can be related to a number of factors: (a) Conflicting experimental results that demonstrate both enhanced or diminished tumor cell adhesion may be attributed to the experimental systems used; (b) tumor heterogeneity and the phenotypic instability of metastatic cells over relatively short periods of time (Nicolson, 1987); and (c) the process of successful metastasis is complex, requiring tumor cells to have decreased and increased adhesive interactions with surrounding cells and ECM at some points in the cascade.

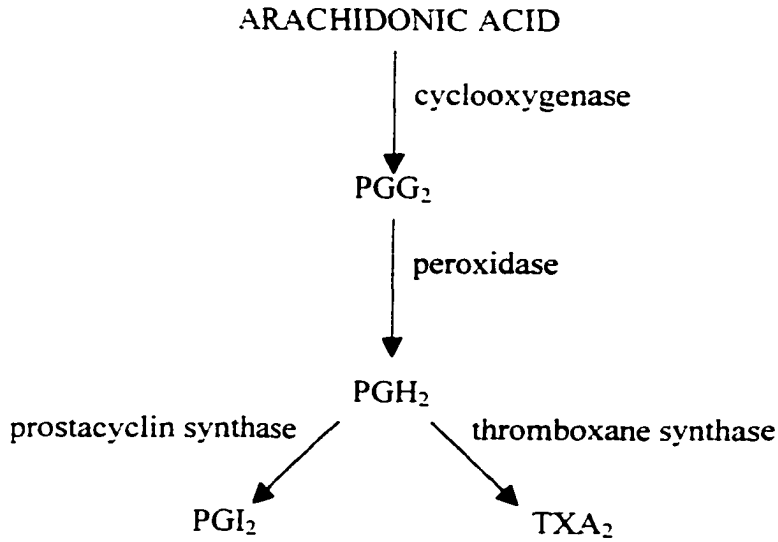


Figure 1. Synthesis of prostaglandins and thromboxanes from arachidonic acid. PGG_2 = prostaglandin G_2; PGH_2 = prostaglandin H_2; PGI_2 = prostaglandin I_2; TXA_2 = thromboxane A_2.

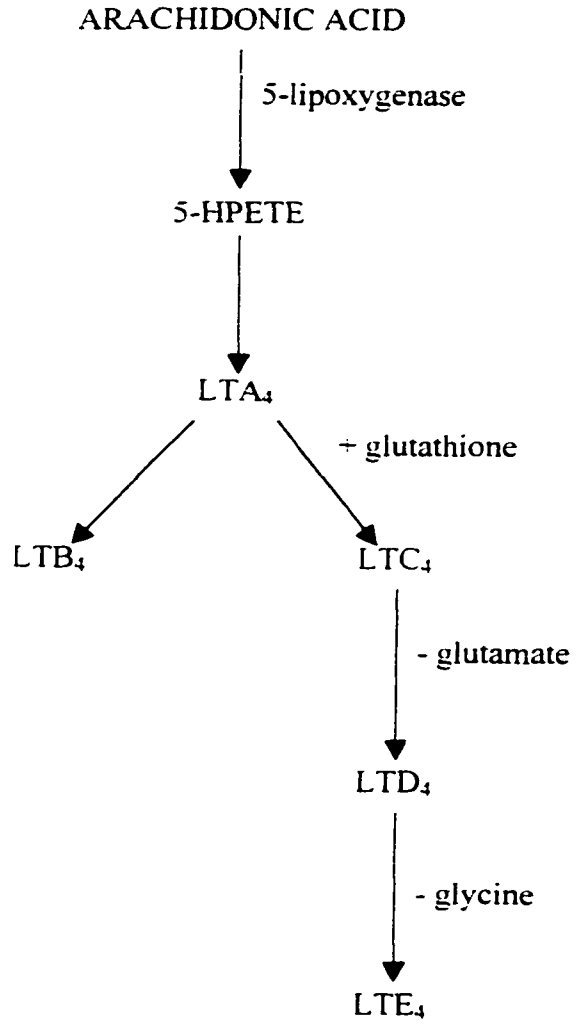


Figure 2. Synthesis of leukotrienes from arachidonic acid. 5-HPETE = 5-hydroperoxy-eicosatetraenoic acid; LTA_4 = leukotriene A_4; LTB_4 = leukotriene B_4; LTC_4 = leukotriene C_4; LTD_4 = leukotriene D_4; LTE_4 = leukotriene E_4.

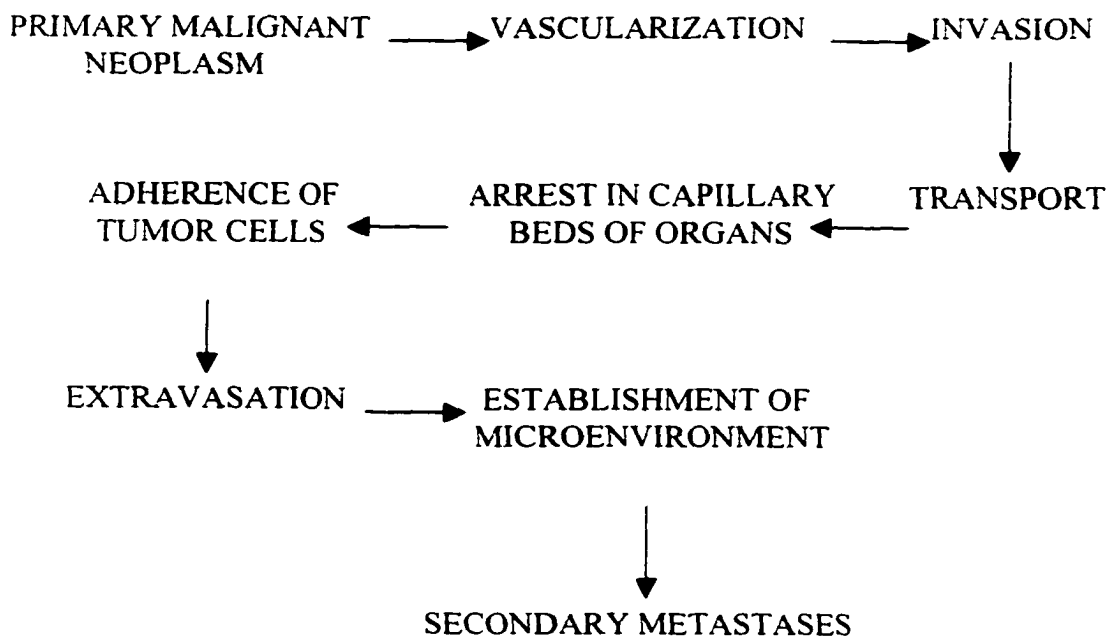


Figure 3. Schematic of the major steps in the pathogenesis of metastasis.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

The effects of PUFAs on breast cancer cell adhesion are mediated by the cell signaling molecules PKA, PKC, FAK, and β_1 integrin. In addition, the ω-3 PUFAs inhibit the EGF-induced increase in cell adhesion.

Specific Aims

In order to test the hypothesis, the following specific aims were established: (a) assess the influence of PUFAs and EGF on the adhesive response of MDA-MB-231 cells: (b) assess the effect of PUFAs on PKA activity, intracellular cAMP production, $PKA_{RI\alpha}$ subunit, PKC-α, and FAK expression in MDA-MB-231 cells; and (c) assess the effect of PUFAs on β1 integrin expression in MDA-MB-231 cells.

MATERIALS AND METHODS

Cell Line and Culture

The human breast carcinoma cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC: Rockville, MD). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in Iscove's modified Dulbecco's medium (IMDM: Fisher Scientific, Norcross, GA) supplemented with 5% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT), and an antibiotic mixture of 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone (Irvine Scientific, Santa Ana, CA). Media was changed every 2 days, and, before reaching confluence, cells were subcultured using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA; Irvine Scientific, Santa Ana, CA).

Fatty Acids

EPA, DHA, and LA (Cayman Chemical Co., Ann Arbor, MI) were prepared as stock solutions containing 0.1% butylated hydroxytoluene in an ethanol vehicle and stored at –20 °C. The ethanol vehicle was added at the same concentration to controls, and the final vehicle concentration was always < 0.2%.

Standard Assays

Cell adhesion assay. Assays of adhesion of breast cancer cells to basement membrane substrates were performed in 96-well polystyrene plates (Fisher Scientific, Norcross, GA) that were precoated with substrate according to an adaptation of a previously described method (Frandsen et al., 1992). Basement membrane substrates used in these assays were human type IV collagen (Sigma, St. Louis, MO), human fibronectin, and Matrigel, a soluble basement membrane extract of the Engelbreth-Holm-Swarm mouse tumor (Collaborative Biochemical Products, Bedford, MA). The major components of Matrigel are laminin, type IV collagen, entactin, and heparan sulfate proteoglycan (Vukicevic, 1992). Stock solutions of these substrates were prepared as recommended by the suppliers and stored at –20 °C. To prepare plates for the adhesion assay, substrates were diluted in phosphate-buffered saline (PBS) to a concentration of 200 μg/ml and added at 50 μl/well to plates that were then incubated for 2 hr at 37 °C. After the plates were washed twice with PBS, 100 μl of heat-denatured bovine serum albumin (BSA) was added to block nonspecific binding sites. The plates were incubated for 30 min at room temperature followed by two washes with PBS.

For adhesion assays, cells were seeded at 1×10^6/100-mm dish and grown for 5 days (with replacement of media after 3 days) in IMDM supplemented with antibiotics, 1% FBS, and 1.25 mg/ml delipidized BSA (Collaborative Biochemical Products, Bedford, MA). Delipidized BSA was included in the cell culture media to bind free fatty acids and prevent their cytotoxicity. Fatty acids were allowed to bind delipidized BSA for 15 min prior to adding cell culture medium. On the day of the adhesion assay, cells were washed twice with PBS and incubated in cell adhesion assay medium for 2 hr at 37 °C in

a humidified atmosphere of 5% CO_2, 95% air. The cell adhesion assay medium consisted of IMDM supplemented with antibiotics, 1.25 mg/ml delipidized BSA, and 20 μg/ml cycloheximide. Cells were then harvested by brief trypsinization, resuspended in their respective cell adhesion assay medium, washed once with PBS, and resuspended in their respective medium to a cell density of 3.5×10^4 cells/ml. An aliquot of the cell suspension was added to each well containing substrate (or to control wells lacking substrate), and plates were incubated at 37 °C for 90 min. Nonadherent cells were removed, and attached cells were stained with 0.5% crystal violet for 20 min at room temperature. Wells were gently rinsed three times with water and allowed to dry. Incorporated dye was dissolved in 100 μl/well of 0.1 M sodium citrate in 50% ethanol for 45 min, and the optical density (OD) was measured at 540 nm on a multiwell plate reader (Molecular Devices, Menlo Park, CA) with use of dual filters (540 nm for absorbance and 405 nm as a reference filter). A linear plot of OD versus number of adherent cells per well was made for each basement membrane substrate studied. The number of adherent cells per well was determined by counting a separate well of adherent cells on a ZM Coulter counter (Coulter Electronics, Ltd., Luton Beds, England). This plot was used to convert OD readings into number of adherent cells per well. The number of adherent cells per well was then divided by the number of cells added to the well at the beginning of the assay (3.5×10^4 cells) to calculate the percentage of adherent cells. Frandsen et al. (1992) demonstrated that the correlation between MDA-MB-231 cell number and OD was linear up to 5×10^4 cells/well, and the correlation between incubation time and OD was linear up to 90 min.

Immunoprecipitation. Fatty acid-treated cells were washed with ice-cold PBS and lysed with 0.5 ml boiling lysis buffer (2 M Tris, pH 7.4, 1 mM sodium orthovanadate, and 10% sodium dodecyl sulfate [SDS]). Supernatants were assayed for protein concentration using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL), according to manufacturer's instructions. Four hundred micrograms of whole cell lysate were adjusted to equal volume and precleared with 10 µl of Protein G-sepharose (Zymed Laboratories, Inc., San Francisco, CA) for 1 hr at 4 °C and then incubated with 5 µg of monoclonal mouse anti-FAK antibody (Transduction Laboratories, Lexington, KY) or goat anti-mouse immunoglobulin G (IgG: Sigma, St. Louis, MO) overnight at 4 °C. Subsequently, the immune complexes were incubated with 20 µl Protein G-sepharose for 1 hr at 4 °C. After extensive washing (6X) with ice-cold modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 µg/ml aprotinin and leupeptin, and 1 mM each of phenylmethylsulfonylfluoride, sodium orthovanadate, and sodium fluoride), the sepharose beads with the immune complexes were directly dissolved in sample buffer (0.5 M Tris-HCl, pH 6.8, 25% glycerol, 10% w/v SDS, 5% β-mercaptoethanol, and 1% bromphenol blue), and immunoprecipitates were separated on 7.5% denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Following transfer, the blot was blocked with 0.2% BSA in tris-buffered saline (TBS) and then immunoblotted with an anti-phosphotyrosine antibody (Transduction Laboratories, Lexington, KY). The protein bands were detected by chemiluminescence using the Immune Star goat anti-mouse IgG detection kit (Bio-Rad Laboratories) according to the manufacturer's protocol.

Immunoblotting. Fatty acid-treated cells were washed and lysed as described above. Whole cell lysates were centrifuged to remove insoluble cell debris. Equal amounts of whole cell lysates were separated on either 7.5% or 10% SDS-PAGE under reducing conditions. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon, Millipore Corp., Bedford, MA), blocked with 0.2% nonfat dry milk or BSA in TBS, and incubated with the appropriate antibody depending upon the experiment. Immunoreactive bands were detected by chemiluminescence using the Immune-Star goat anti-mouse IgG detection kit. Relative amounts of protein were determined by densitometry using a UMAX Supervista S-12 scanner.

Enzyme-linked immunoassay (ELISA). This procedure was performed as previously described by Fujii et al. (1995), with some modifications. After 5 days of culture with or without fatty acids, as previously described, cells grown in 96-well plates (2.5×10^4 cells/well) were washed twice with PBS and fixed in 4% paraformaldehyde. Nonspecific protein binding sites on the wells were saturated by incubating for 1 hr at room temperature with 2% nonfat dry milk in PBS. A primary antibody (depending on the experiment) was added to each well for 2 hr at room temperature. After extensive washing (6X) with 0.05% Tween 20 in PBS, a 1:1,000 dilution of horse radish peroxidase-conjugated goat anti-mouse IgG (Dako Corp., Carpinteria, CA) was added to each well for 1 hr at room temperature. Extensive washing of plates was followed by color development using 2, 2'–azinobis (3–ethylbenzthiazoline–b–sulfonic acid) chromagen reagent (Sigma, St. Louis, MO). After 1 hr, the peroxidase reaction was quantified by measuring the OD on a multiwell plate reader at 405 nm.

Statistical Analysis

Data are shown as mean ± SEM of the indicated number of separate observations. Each experiment described was repeated at least two times. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by a least significant difference multiple comparison test. A value of $p < 0.05$ was taken to indicate statistical significance.

Experiment 1

This study was designed to investigate the effect of EGF and the ω-3 PUFA EPA on adhesion of the human breast cancer cell line MDA-MB-231 to subendothelial matrix ECM components. Human recombinant EGF (CalBiochem, LaJolla, CA) was prepared as a stock solution in IMDM and added to media at indicated concentrations. A dose-response relationship was established between EGF and cell adhesion by treating cells with graded concentrations of EGF (0-10 nM) for 5 days. Cells were subsequently treated with either 4.5 μg/ml EPA, 1 nM EGF, or both for 5 days. An adhesion assay was performed as previously described. The cell adhesion medium was supplemented with or without 4.5 μg/ml EPA and 1 nM EGF.

Experiment 2

To examine the contribution of lipid peroxides to the decrease in breast cancer cell adhesion in cells treated with EPA, cells were treated with or without 5 μg/ml EPA and 10 μM α-tocopherol (Kodak, Rochester, NY) for 5 days. Alpha (α)-tocopherol was

prepared as a 10-μM solution in ethanol and added to media at indicated concentrations. An adhesion assay was performed as previously described.

Thiobarbituric acid-reactive substances (TBARS) assay. This assay was used to compare TBARS production in cells treated with or without fatty acids and α-tocopherol. Cells were treated with or without fatty acids and α-tocopherol for 5 days. Media and cells were separated and assayed for TBARS according to the method of Gavino et al. (1981), with some modifications. One milliliter of 20% trichloroacetic acid and 2 ml of 0.67% thiobarbituric acid were added to cells resuspended in PBS (pH 7.0), and cells were incubated for 20 min at 90 °C. The cell suspension was centrifuged at 12,000 × g for 10 min at 4 °C. The absorbance of the supernatant was measured at 532 nm using PBS as a reference. The absorbance values obtained were compared with a standard curve of known concentrations of MDA generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane and were normalized to the protein content of each sample.

Experiment 3

This study was designed to examine the effects of PUFAs on the activity or expression of PKA and PKC and intracellular cyclic AMP production. Whole cell lysates were separated on a 10% SDS-PAGE gel, transferred to PVDF membranes, blocked with 0.2% nonfat dry milk, and incubated with a 1:5,000 dilution of a monoclonal mouse anti-PKCα antibody (Chemicon International, Temecula, CA) or a 1:250 dilution of a monoclonal mouse anti-$PKA_{RIα}$ antibody (Transduction Laboratories, Lexington, KY). Human endothelial cell lysate, derived from an aortic endothelium cell line, was used as a posi-

tive control. Immunoreactive bands were detected by chemiluminescense using the Immune-Star goat anti-mouse IgG detection kit.

Analysis of cyclic AMP-dependent protein kinase (PKA) activity. After 5 days of culture with or without fatty acids, cells were washed with cold PBS. Pelleted cells were suspended in extraction buffer (20 mM Tris, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10 μg/ml leupeptin, and 2 mM PMSF) and lysed via three alternating freeze-thaw (-80 °C/ 37 °C) cycles. Cell lysates were assayed for PKA activity by incubating with the substrate, Kemptide, which was labeled with a fluorescent dye. The reaction mixture was then applied to the affinity membrane of a Spinzyme Affinity Separation Unit. The membrane specifically binds the phosphorylated substrate while nonphosphorylated substrate passes through the support when it is washed with binding buffer (0.1 M sodium acetate, 0.5 M sodium chloride, and 0.02% sodium azide, pH 5.0). The phospho-rylated product was then eluted from the membrane with elution buffer (0.1 M ammo-nium bicarbonate, pH 8.0, containing 0.02% sodium azide) and assayed spectrophoto-metrically by measuring its absorbance at 570 nm. The protein concentration of the lysates was determined by the method of Bradford (1976). PKA activity was expressed on the basis of units per milligram of protein. One unit of PKA activity will transfer 1 pmole of phosphate to dephosphorylated substrate per minute per mg of kinase.

Analysis of intracellular cyclic AMP. The Biotrak cAMP direct enzymeimmunoassay (Amersham Pharmacia Biotech, Piscataway, NJ) was used to directly measure intracellular cAMP in cell lysates. This assay is based on competition between unlabeled

cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific antibody. The amount of peroxidase-labeled ligand bound by the antibody will be inversely proportional to the concentration of added unlabeled ligand. The peroxidase ligand that is bound to the antibody is immobolized on-to polystyrene microtitre wells precoated with second antibody. Any unbound ligand is removed from the well by washing. The amount of peroxidase-labeled cAMP bound to the antibody is determined by addition of tetramethylbenzidine/hydrogen peroxide single- pot substrate. The reaction is stopped by adding an acid solution. and the resultant color is read at 450 nm in a microtitre plate spectrophotometer. After 5 days of culture with or without fatty acids. cells were lysed with lysis reagent (Amersham Pharmacia Biotech. Piscataway. NJ). Aliquots of cell lysate were transferred to a donkey anti-rabbit Ig-coated plate for assay. Rabbit anti-cAMP antiserum was added to each well and incubated for 2 hr at 4 °C. cAMP-horseradish peroxidase was then added and incubated for 1 hr at 4 °C. All wells were washed four times with wash buffer (0.01 M phophate buffer. pH 7.5. containing 0.05% Tween 20). Enzyme substrate was dispensed into each well and incubated for 1 hr at room temperature. Sulfuric acid (1.0 M) was added to each well. and the OD was determined in a plate reader at 450 nm. A standard curve was generated by plotting the percent bound (%B/B_0) as a function of the log cAMP concentration. The percent bound was calculated using the following relationship:

$$\%B/B_0 = \frac{[\text{standard or sample OD} - \text{nonspecific binding (NSB) OD}]}{\text{zero standard } (B_0) \text{ OD} - \text{NSB OD}} \times 100$$

The cAMP concentration (pmol/well) of samples was read directly from the graph.

Experiment 4

The fourth experiment was designed to examine the effect of PKA inhibition on cell adhesion. MDA-MB-231 cells were treated with 0-4 μM of the PKA inhibitor N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide•2HCl (H-89) for 1 hr. Cells in control groups were treated with diluent dimethylsulfoxide. An adhesion assay was performed as previously described.

Experiment 5

To examine expression of human β1 integrin in cells treated with PUFAs, cells were treated with or without PUFAs for 5 days. An ELISA assay was performed, as previously described. A 1:1,000 dilution of a monoclonal mouse anti-human β1 integrin antibody was used as the primary antibody.

Treatment with dbcAMP and H89. This assay was performed to determine a possible direct influence of second messenger signaling on β1 integrin expression. Cells were grown in 96-well plates (2.5×10^4 cells/well) for 5 days as previously described without fatty acids. Cells were then treated with graded concentrations (0-2 mM) of the cAMP derivative N6,O-2'-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP) (Sigma, St. Louis, MO) or with the PKA inhibitor H-89 (0-50 μM) (Biomol, Plymouth Meeting, PA) for 1 hr. β1 integrin expression was detected using the ELISA assay previously described.

Experiment 6

Experiment 6 was designed to investigate the effects of PUFAs on the phosphorylation and expression of FAK, as well as other phosphotyrosine-containing proteins. Cells treated with fatty acids were incubated on fibronectin-coated 100-mm cell culture dishes for 1 hr. Cell lysates were then subjected to either immunoprecipitation or immunoblotting using either a monoclonal anti-FAK or anti-phosphotyrosine antibody.

RESULTS

Experiment 1

Previous studies have shown that EGF influences cell adhesion to basement membrane ECM components (Fujii et al., 1995; Lichtner et al., 1993; Rohde-Schulz & Lichtner, 1995; Yudoh et al., 1994). We initially examined the effect of EGF on adhesion of the MDA-MB-231 human breast cancer cell line to Matrigel, a soluble basement membrane extract of the EHS mouse tumor. When treated with EGF, MDA-MB-231 cells exhibited a dose-dependent decrease in adhesion to Matrigel (Figure 4). EGF also decreased adhesion to the ECM substrates fibronectin and type IV collagen (Figure 5). EPA decreased adhesion of MDA-MB-231 cells to ECM substrates (Figure 5). This experiment was performed on three separate and independent occasions. Because results from the experiments were similar, data from only one experiment are shown.

Experiment 2

We examined the hypothesis that lipid peroxidation products are responsible for the EPA-induced decrease in MDA-MB-231 cell adhesion to ECM components. Lipid peroxidation was measured by the TBARS assay. EPA generated substantial amounts of TBARS (4.72 nmol/mg protein) when added for 5 days to cultures of MDA-MB-231 cells, relative to controls with no added EPA (0.26 nmol/mg protein; Figure 6). When added to EPA-treated cells, the antioxidant α-tocopherol (Vitamin E), at 10 μM

concentration, greatly inhibited TBARS formation (Figure 6). However, α-tocopherol addition had no substantial effect on adhesion of EPA-treated cells to either Matrigel (9.92 ± 0.18% adhesion of EPA-treated cells vs. 10.17 ± 0.28% adhesion of EPA + α-tocopherol-treated cells) or type IV collagen (7.93 ± 0.28% adhesion of EPA-treated cells vs. 9.77 ± 0.14% adhesion of EPA + α-tocopherol-treated cells; Figure 7). Thus, it appears that peroxidation products are not responsible, or at least not entirely responsible, for EPA-induced decrease of MDA-MB-231 cell adhesion to ECM components.

Experiment 3

Because studies have implicated PKA and PKC as important regulators in metastasis (Gopalakrishna & Barsky, 1988; Liu, B., et al., 1991), we hypothesized that the increase or decrease in MDA-MB-231 cell adhesion was parallelled by a respective increase or decrease in PKA and PKC activity or expression.

To test our hypothesis, we first examined the effects of PUFAs on the activity of PKA. Treatment of cells with 5 μg/ml EPA or LA over a period of 5 days resulted in a decrease in PKA activity (23.7 ± 0.63 and 24.7 ± 1.51 U/mg protein, respectively) compared to untreated controls (27.6 ± 0.62 U/mg protein, $p < 0.05$; Table 2). This effect of PUFAs on PKA activity is similar to that observed in other studies where PUFAs were found to alter PKA activity (Speizer, Watson, & Brunton, 1991; Tappia, Man, & Grimble, 1995). We also measured the expression of the PKA regulatory subunit ($PKA_{RI\alpha}$) as well as the α isoform of PKC (PKC-α). Expression of the α isoenzyme of PKC has been shown to increase when MDA-MB-231 cells are treated with phorbol 12-myristate 13-

Table 2

cAMP-Dependent Protein Kinase Activity of MDA-MB-231 Cells Treated With Polyunsaturated Fatty Acids

Treatment[a]	PKA activity[b]
Control[c]	27.6 ± 0.62
EPA	23.7 ± 0.63*
LA	24.7 ± 1.51*

Note. MDA-MB-231 cells were treated with or without 5 μg/ml PUFAs for 5 days. Values represent the mean ± SEM of three different experiments. cAMP = cyclic adenosine monophosphate; EPA = eicosapentaenoic acid; LA = linoleic acid; PKA = cyclic AMP-dependent protein kinase.
[a]n = 6 for each treatment. [b]units/mg protein. [c]No fatty acid.
*p < .05.

acetate (Borner, Filipuzzi, Wartmann, Eppenberger, & Fabbro, 1989). In the current study, $PKA_{RI\alpha}$ subunit expression, as measured by immunoblotting and scanning densitometry, was significantly lower in cells treated with ω-3 PUFAs (EPA and DHA), whereas treatment with LA (an ω-6 PUFA) resulted in no significant difference in expression (Figure 8). PKC-α expression was significantly lowered only in cells treated with DHA and the higher but physiological level of EPA. Treatment with LA resulted in no significant difference in PKC-α expression (Figure 9). There was no significant difference in intracellular cAMP production in cells treated with PUFAs (data not shown). These results suggest that dietary fatty acids may affect major signal-transducing second-messenger systems in breast cancer cells via modulation of protein kinase activity and subunit/isoenzyme expression.

Experiment 4

Studies have shown that PKA and PKC inhibition results in decreased cell adhesion to various substrates (Johanning & Lin, 1995; Liu, B., et al., 1992; Lozano et al., 1996; Young, Young, Lozano, & Bagash, 1992). To investigate whether PKA inhibition would result in decreased MDA-MB-231 cell adhesion to ECM components, we used a selective PKA inhibitor, H-89. Cells were treated with H-89 and tested for adhesion to Matrigel and type IV collagen. Cells were treated with 2-4 μM H-89 for 60 min at 37 °C. As shown in Figure 10, H-89 pretreatment, at each level tested, decreased cell adhesion to both Matrigel and type IV collagen.

Experiment 5

Studies have shown that alterations in cell adhesion molecule expression may strongly influence tumor invasion and metastasis by affecting cell-ECM interactions (Albelda, 1993; Tang & Honn, 1994-1995; Weiss, 1994-1995). We examined the expression of human β1 integrin in MDA-MB-231 cells treated with PUFAs. Our results show that, compared to untreated controls, cells exposed to 5 μg/ml EPA for 5 days exhibited decreased expression of the β1 integrin subunit ($p < 0.01$; Table 3). Treatment with 5 μg/ml LA resulted in a small but nonsignificant increase in β1 integrin expression (Table 3). These effects on integrin expression may, in part, account for the changes in the adhesion patterns of cells exposed to PUFAs that have been observed in previous studies (German & Johanning, 1997; Johanning & Lin, 1995). Because ω-3, but not ω-6 PUFAs, were effective in decreasing expression, the decrease in β1 integrin expression is not a generalized effect of PUFAs on expression of this integrin subunit.

Table 3

β1 Integrin Subunit Expression in MDA-MB-231 Cells Treated With Polyunsaturated Fatty Acids

Treatment[a]	Specific absorbance
Control[b]	0.151 ± 0.005
EPA	0.126 ± 0.004**
LA	0.168 ± 0.007

Note. MDA-MB-231 cells were treated with or without 5 μg/ml PUFAs for 5 days. Values represent the mean ± SEM of the specific absorbance at 405 nm from three different experiments. EPA = eicosapentaenoic acid; LA = linoleic acid.
[a]n = 6 for each treatment. [b]No fatty acid.
**p < .01.

Studies have demonstrated the ability of dbcAMP to alter integrin expression (Halvorson & Coligan, 1995; Jung et al., 1994) as well as cell adhesion (Halvorson & Coligan, 1995). Because our data suggest that PUFAs modulate both PKA activity and β1 integrin expression, we evaluated the influence of PKA effectors on β1 integrin expression. In order to examine the role of cAMP, through its effect on PKA, in the regulation of β1 integrin function, we treated MDA-MB-231 cells either with a cAMP derivative, dbcAMP, or with the PKA inhibitor H-89. dbcAMP increased the expression of β1 integrin in a dose-dependent manner (Figure 11), whereas H-89 decreased β1 integrin expression in a dose-dependent manner (Figure 12). These results suggest a possible interaction between PKA and the β1 integrin subunit.

Experiment 6

Burridge, Turner, and Romer (1992) showed that cellular adhesion to fibronectin stimulates the tyrosine phosphorylation of a number of focal adhesion-associated proteins, including FAK and paxillin. To investigate the effect of PUFAs on the pattern of tyrosine phosphorylation of cellular proteins, we prepared lysates from MDA-MB-231 cells seeded onto fibronectin and analyzed the lysates by SDS-PAGE and immunoblotting. Figure 13 shows an immunoblot of cell lysates probed with an antiphosphotyrosine antibody. The molecular masses of the major phosphorylated proteins were estimated to be 39, 45, 62, 73, 98, 122, 148, and 185 kDa. Proteins with molecular masses of 122, 73, and 45 were chosen for densitometric analyses because several studies have shown that these proteins may correspond to FAK, paxillin, and FAK-related nonkinase, respectively (Burridge et al., 1992; Richardson, Shannon, Adams, Schaller, & Parsons, 1997; Tang, Tarrien, et al., 1995). In some cells, the C terminus of FAK is expressed as a separate protein, pp41/43. Cells treated with PUFAs showed no significant change in phosphotyrosine staining of the 73-kDa protein band when compared to control cultures (Figure 13). However, there was a trend toward decreased phosphotyrosine staining of the 122- and 45-kDa protein bands in cells treated with 5 μg/ml EPA and DHA.

We investigated the possibility that the 122-kDa band tyrosine-phosphorylated in response to PUFAs in MDA-MB-231 cells might be the 125-kDa protein FAK. Anti-FAK immunoprecipitates were prepared from MDA-MB-231 cells that had been treated with different PUFAs, and the immunoprecipitates were subsequently immunoblotted with an antiphosphotyrosine antibody. As shown in Figure 14, there was little or no detection of a 120-125-kDa protein band. Likewise, there was no detection of a 120-125-

kDa protein band in response to treatment with PUFAs for 1 hr (Figure 15). It is reasonable to suggest that FAK may not have been immunoprecipitated because our results showed no evidence of FAK phosphorylation in control cell lysates or PUFA-treated cell lysates. Because our results showed no evidence of FAK phosphorylation, we chose to investigate the effects of PUFAs on FAK expression. Lysates were prepared from MDA-MB-231 cells that had been treated with PUFAs, and the lysates were analyzed by SDS-PAGE and immunoblotting with an anti-FAK antibody. As shown in Figure 16, FAK expression was significantly decreased in cells treated with DHA for 5 days, whereas treatment with EPA and LA showed no significant difference. Figure 17 shows that treatment of MDA-MB-231 cells with PUFAs for 1 hr results in a different pattern of FAK expression than that seen in cells treated with PUFAs for 5 days. Treatment with ω-3 PUFAs resulted in no change in FAK expression. In contrast, treatment with LA decreased FAK expression.

Our study suggests that a PKA inhibitor has an effect on cell adhesion, and other studies have shown that serine/threonine protein kinases may affect FAK phosphorylation (Richardson et al., 1997). We tested the effects of H-89, a PKA inhibitor, on FAK expression rather than on FAK phosphorylation, because our results did not show an effect of PUFAs on FAK phosphorylation. Figure 18 shows the effect of H-89 on FAK expression. Treatments with 4 and 8 μM H-89 resulted in decreased FAK expression relative to the control, but 4 and 8 μM treatments were not significantly different from each other.

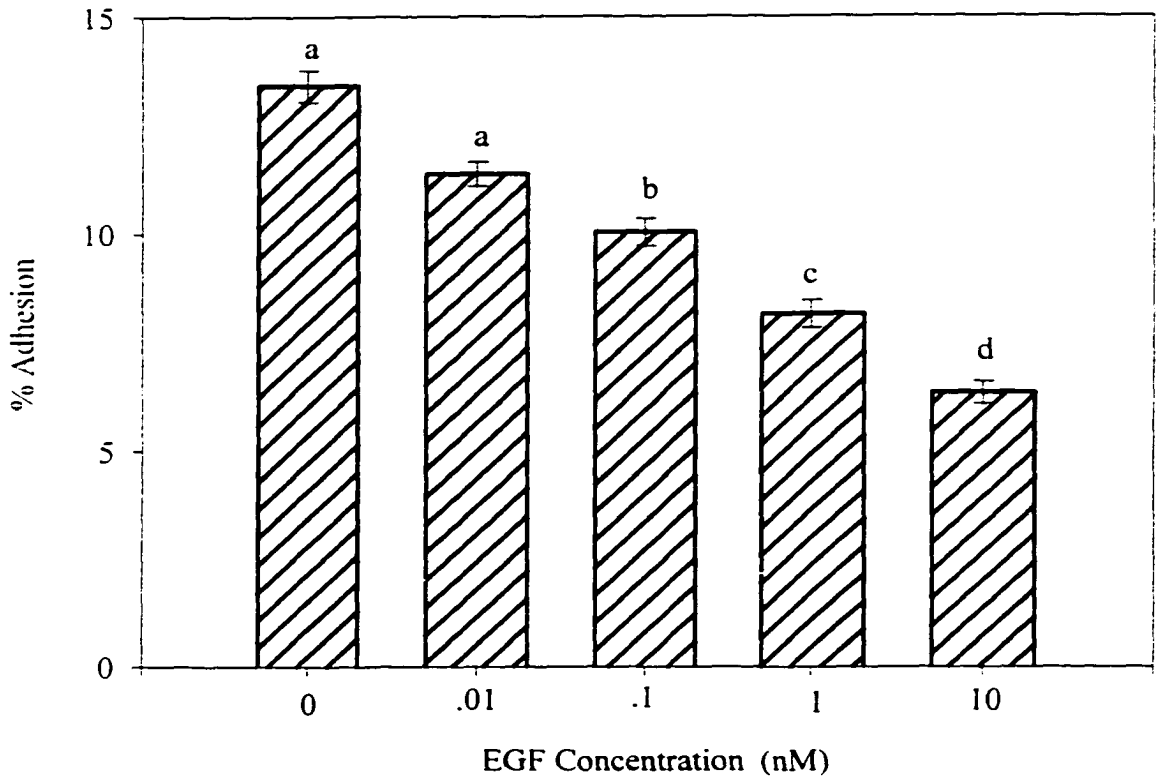


Figure 4. Effect of graded concentrations of epidermal growth factor (EGF)(0-10 nM) on MDA-MB-231 cell adhesion to Matrigel. Cells were seeded at 1 x 10^6/100-mm dish and grown for 5 days in Iscove's modified Dulbecco's medium supplemented with 1% fetal bovine serum, delipidized bovine serum albumin (1.25 mg/ml), and EGF. Values represent mean ± SEM ($n = 6$). Treatments not sharing a common letter are different ($p < 0.05$). This figure represents one of two repeated experiments.

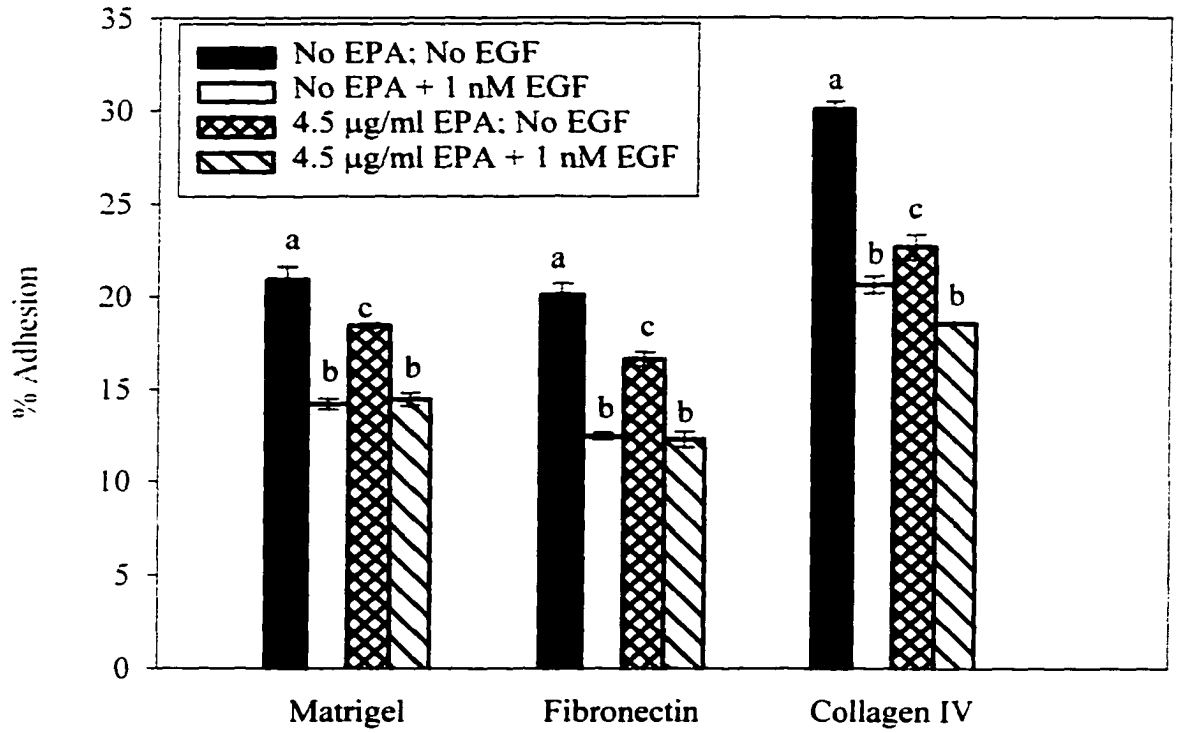


Figure 5. Effect of eicosapentaenoic acid (EPA; 4.5 µg/ml) and epidermal growth factor (EGF; 1 nM) on MDA-MB-231 cell adhesion to Matrigel, fibronectin, and type IV collagen. Cells were seeded, grown, and assayed as described in Figure 1. Values represent mean ± SEM ($n = 6$). For each substrate, treatments not sharing a common letter are different ($p < 0.05$). This figure represents one of three repeated experiments.

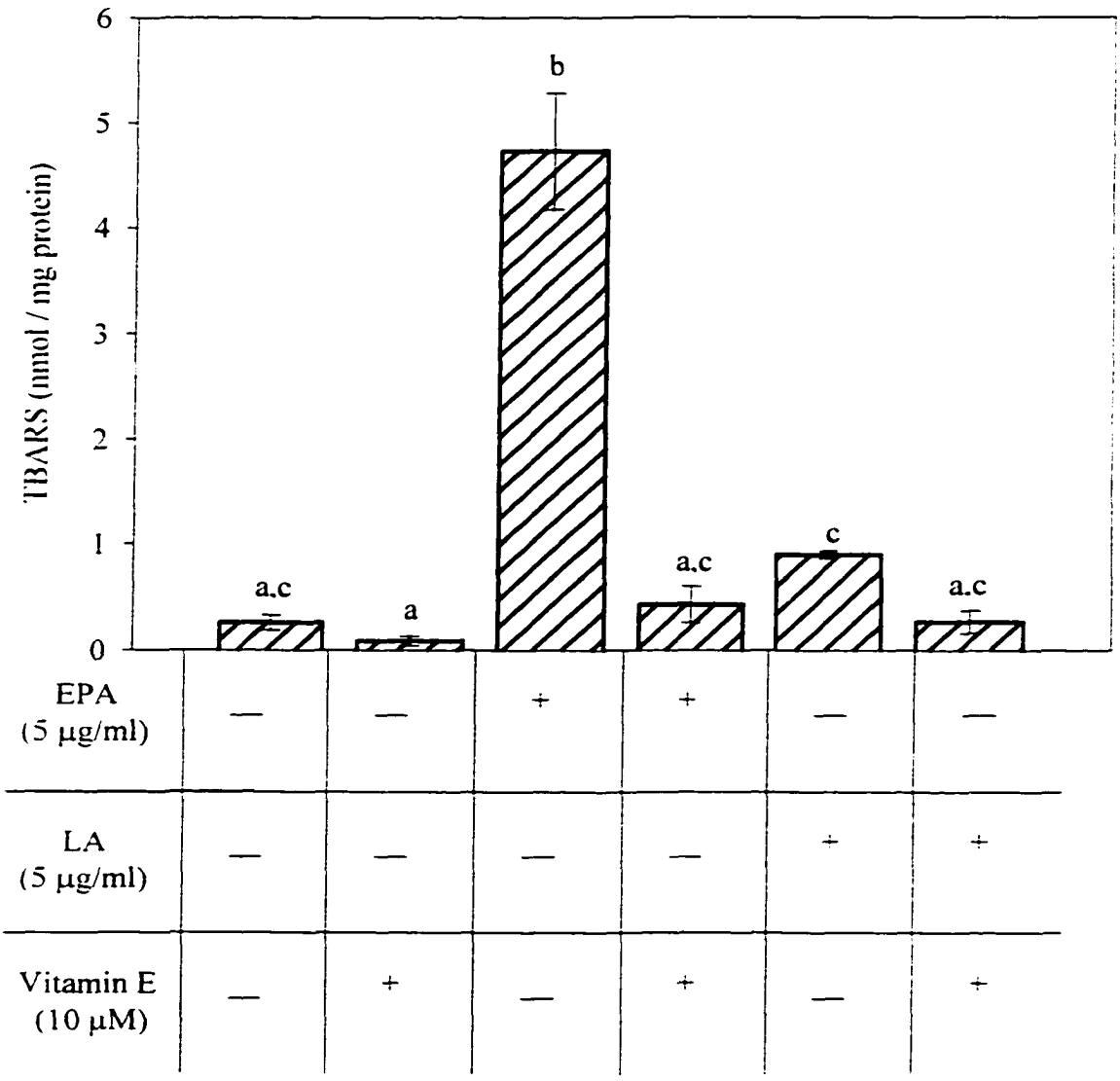


Figure 6. TBA-reactive substances (TBARS) production by MDA-MB-231 cells treated with EPA or LA. Values represent mean ± SEM (n = 3). Values not sharing a common letter are different ($p < 0.05$). This figure represents one of two repeated experiments. EPA = eicosapentaenoic acid; LA = linoleic acid.

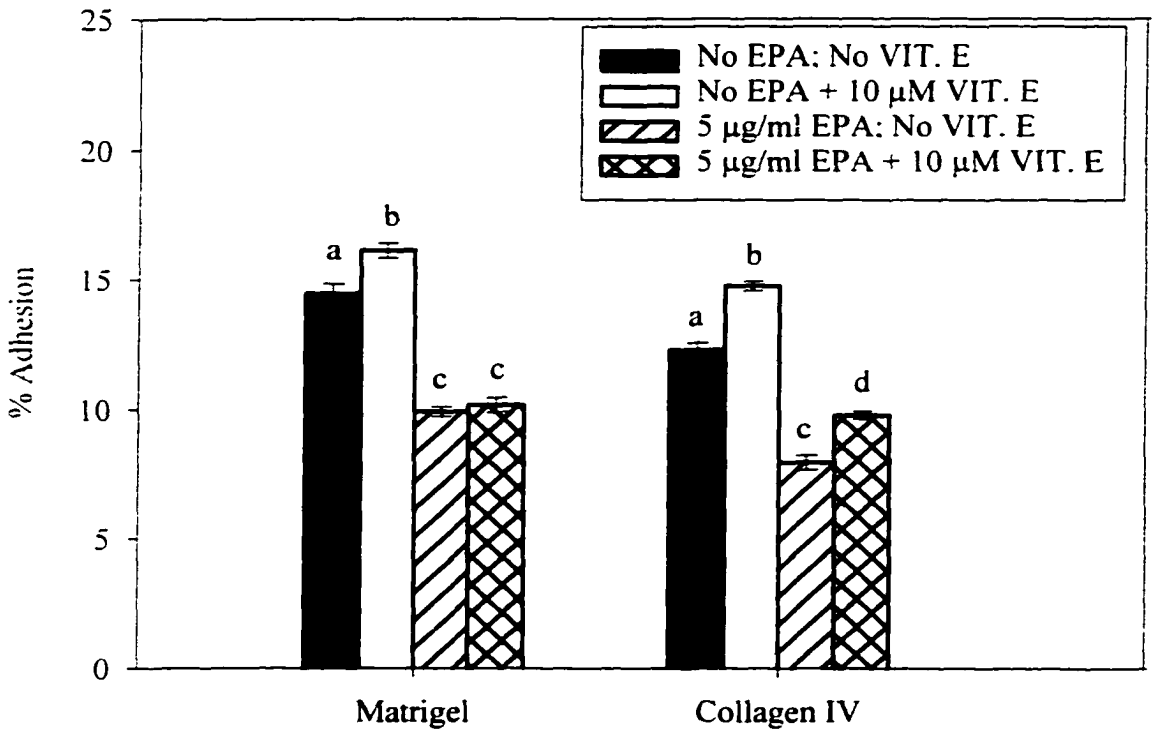
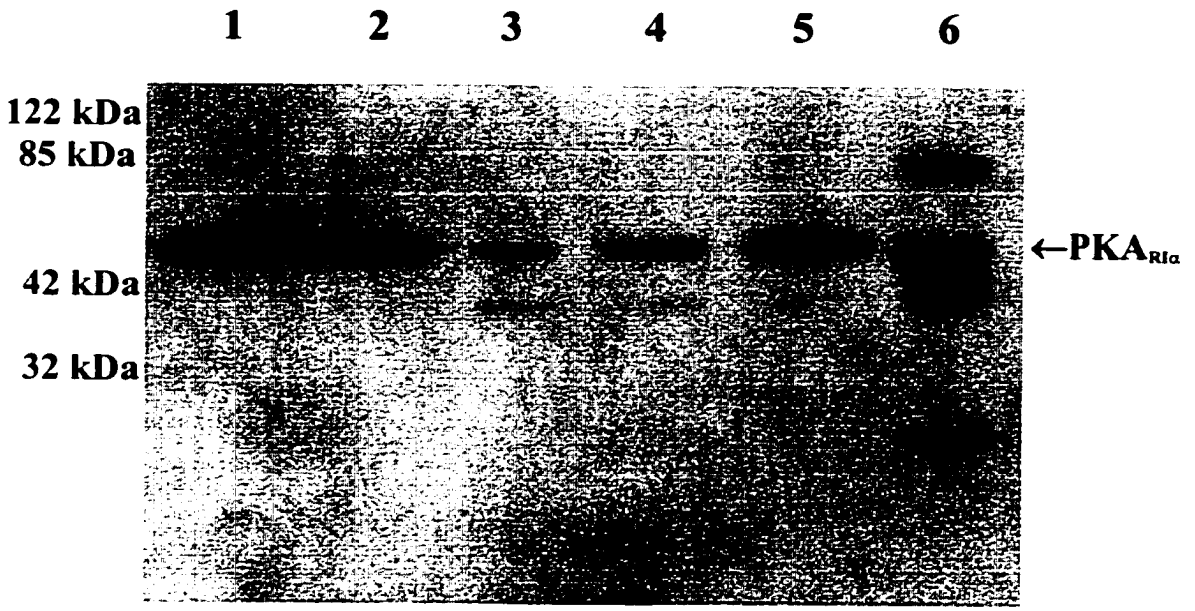


Figure 7. Effect of lipid peroxides on MDA-MB-231 cell adhesion to Matrigel and type IV collagen. Cells were seeded and grown for 5 days in Iscove's modified Dulbecco's medium supplemented with 1% fetal bovine serum and delipidized bovine serum albumin (1.25 mg/ml), and with or without EPA and vitamin E. Values represent mean ± SEM (n = 6). For each substrate, treatments not sharing a common letter are different ($p < 0.05$). This figure represents one of two repeated experiments. EPA = eicosapentaenoic acid; VIT. E = vitamin E.

Figure 8. Effect of PUFAs on $PKA_{RI\alpha}$ subunit expression in MDA-MB-231 cells. A. Total cell protein was isolated from MDA-MB-231 cells treated with 2.5 and 5 μg/ml EPA (lanes 2 and 3, respectively), 5 μg/ml DHA (lane 4), or 5 μg/ml LA (lane 5) for 5 days, separated (25 μg lysate) on a 10% SDS-PAGE gel, and transferred to PVDF membrane. Lane 1 represents cells treated with no PUFAs. An endothelial cell lysate was used as a positive control (lane 6). The $PKA_{RI\alpha}$ subunit was recognized by using a monoclonal mouse anti-$PKA_{RI\alpha}$ antibody (1:250 dilution). Visualization was performed by using the chemiluminescence detection system. B, graph of ratio of PUFA-treated cells to control calculated from densitometric scanning of A. A represents one of three repeated experiments, whereas B represents the average of the three experiments. Those not sharing letters are significantly different ($p < 0.05$). DHA = docosahexanenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid; $PKA_{RI\alpha}$ = protein kinase A regulatory subunit; PUFAs = polyunsaturated fatty acids; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

A.



B.

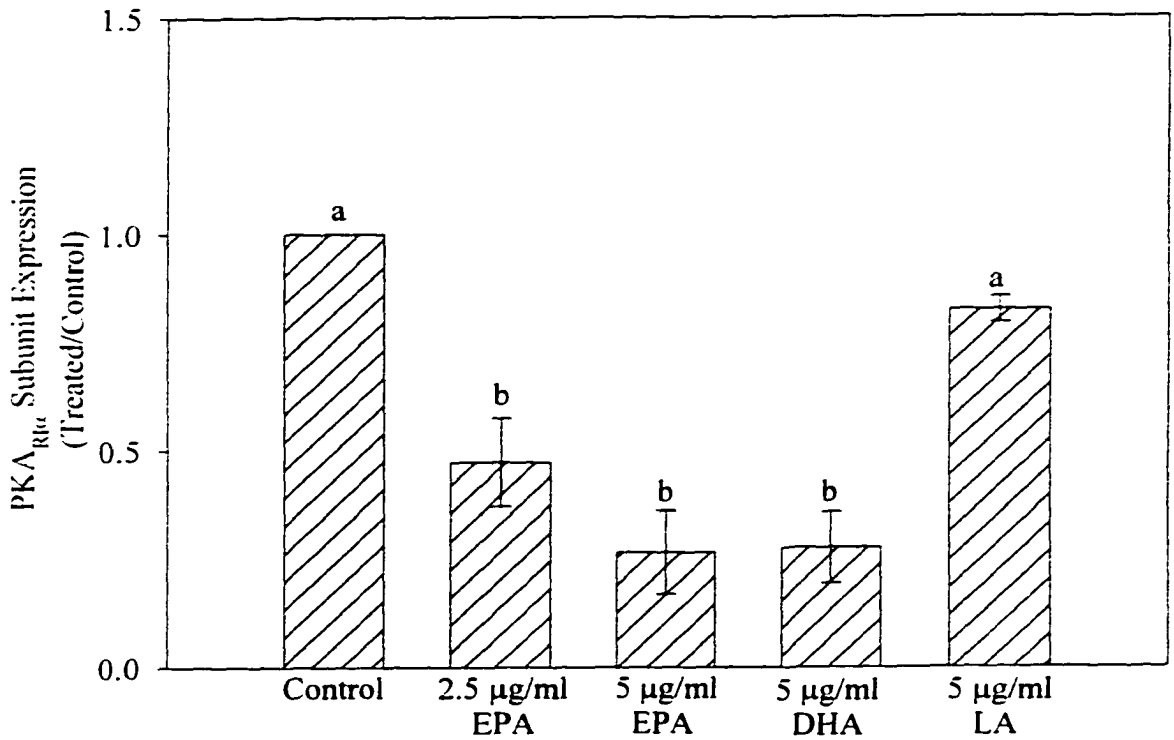
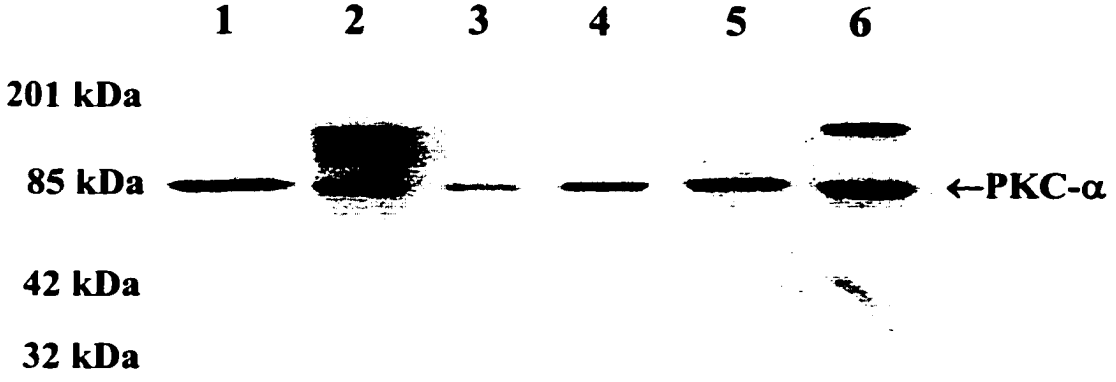
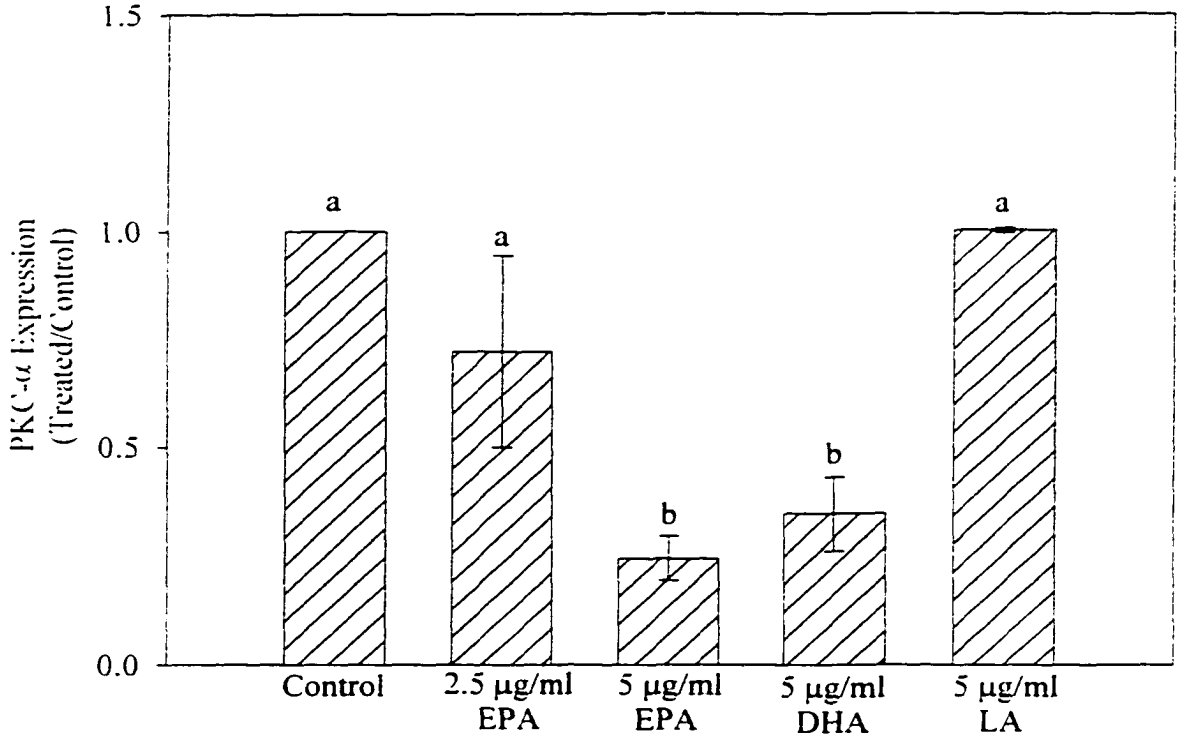


Figure 9. Effect of PUFAs on PKC-α isoenzyme expression in MDA-MB-231 cells. A. Total cell protein was isolated from MDA-MB-231 cells treated with 2.5 and 5 μg/ml EPA (lanes 2 and 3, respectively), 5 μg/ml DHA (lane 4), or 5 μg/ml LA (lane 5) for 5 days, separated (25 μg lysate) on a 10% SDS-PAGE gel, and transferred to PVDF membrane. Lane 1 represents cells treated with no PUFAs. An endothelial cell lysate was used as a positive control (lane 6). The PKC-α isoenzyme was recognized by using a monoclonal mouse anti-PKC-α antibody (1:5,000 dilution). Visualization was performed by using the chemiluminescence detection system. B, graph of ratio of PUFA-treated cells to control calculated from densitometric scanning of A. A represents one of three experiments, whereas B represents the average of the three experiments. Those not sharing letters are significantly different ($p < 0.05$). DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid; PKC-α = protein kinase C-alpha; PUFAs = polyunsaturated fatty acids; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

A.



B.



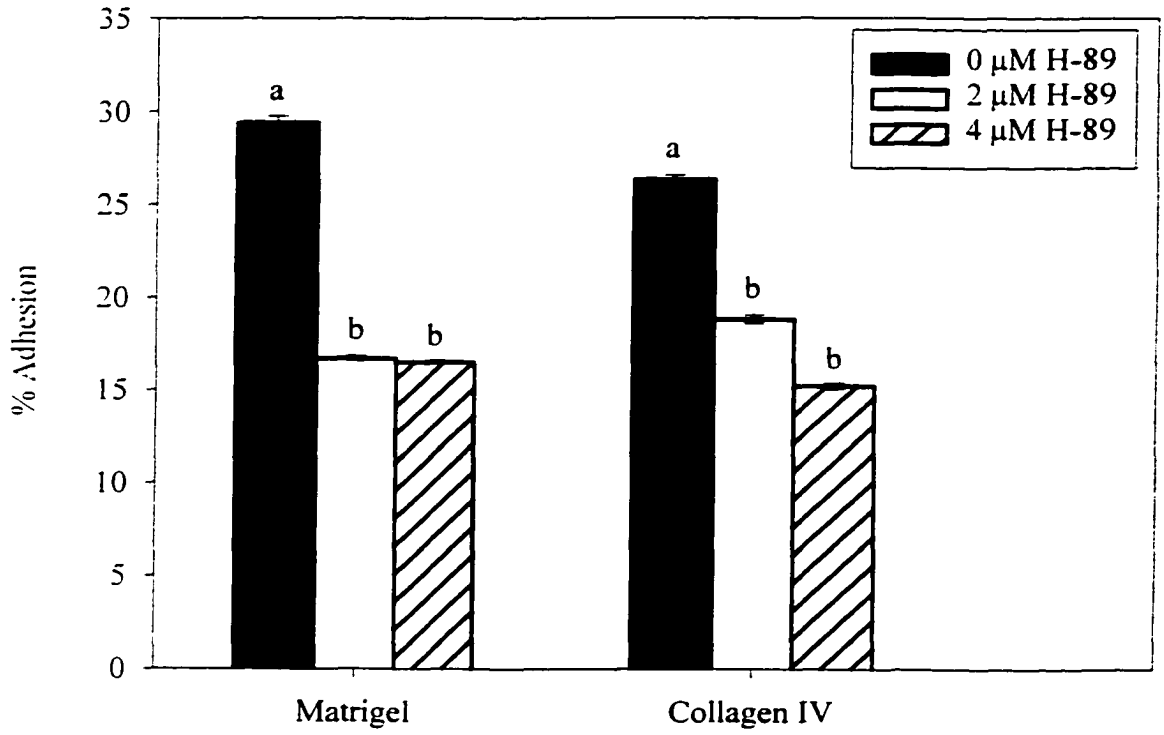


Figure 10. Effect of H-89 on adhesion of MDA-MB-231 cells to Matrigel and type IV collagen. Cells were treated in IMDM with H-89 at indicated concentrations for 60 min at 37 °C. Treated cells were washed (3X) with IMDM and plated onto precoated 96-well dishes for an adhesion assay. Values represent mean ± SEM ($n = 6$). For each substrate, treatments not sharing a common letter are different ($p < 0.05$). This figure represents one of two repeated experiments. H-89 = N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide•2 HCl; IMDM = Iscove's modified Dulbecco's medium.

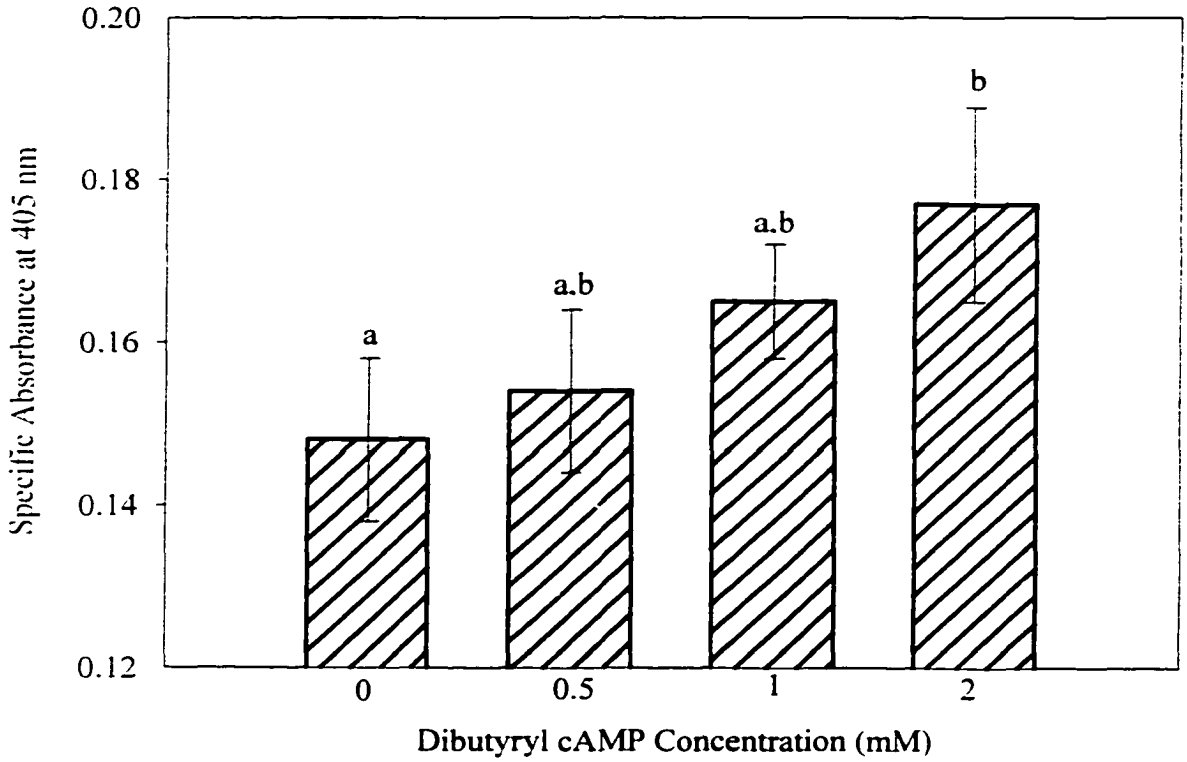


Figure 11. Effect of dibutyryl cAMP (dbcAMP) on the expression of the β1 integrin subunit. MDA-MB-231 cells grown in 96-well plates were cultured for 5 days without PUFAs followed by a 1-hr incubation with dbcAMP at the indicated concentrations. An ELISA was used to detect integrin expression. Values represent the mean ± SEM of the specific absorbance at 405 nm ($n = 6$). Values not sharing a common a letter are different ($p < 0.05$). This figure represents one of two repeated experiments. cAMP = cyclic adenosine monophosphate; PUFAs = polyunsaturated fatty acids; ELISA = enzyme-linked immunoassay.

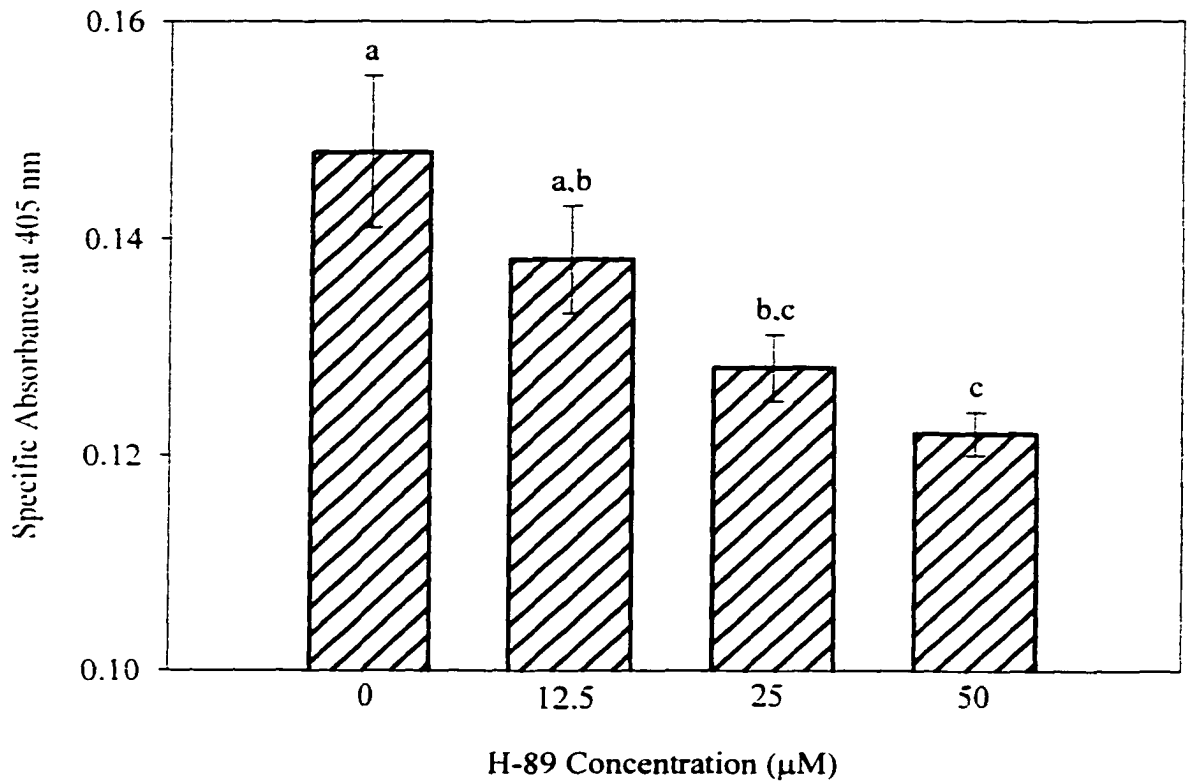


Figure 12. Effect of H-89 on the expression of the β1 integrin subunit. MDA-MB-231 cells grown in 96-well plates were cultured for 5 days without PUFAs followed by a 1-hr incubation with H-89 at the indicated concentrations. An ELISA, as described in Materials and Methods, was used to detect integrin expression. Values represent the mean ± SEM of the specific absorbance at 405 nm ($n = 6$). Treatments not sharing a common letter are different ($p < 0.05$). This figure represents one of two repeated experiments. H-89 = N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide•2 HCl; PUFAs = polyunsaturated fatty acids; ELISA = enzyme-linked immunoassay.

Figure 13. Effect of PUFAs on tyrosine phosphorylation of proteins isolated from MDA-MB-231 cells. A. Cells were grown for 5 days in IMDM containing 2.5 and 5 μg/ml EPA (lanes 3 and 4, respectively), 5 μg/ml DHA (lane 5), 5 μg/ml LA (lane 6), or no PUFA (lane 2). Cells were seeded at 4×10^6 cells/100-mm tissue culture dishes precoated with fibronectin (FN) or BSA (lane 1). After a 1-hr incubation at 37 °C, cells were lysed in lysis buffer, and equal aliquots (100 μg protein) of clarified lysates were electrophoresed onto a 7.5% SDS-PAGE gel. The gel was transferred to PVDF membrane and immunoblotted using an antiphosphotyrosine antibody (1:1,000 dilution). Visualization was performed by using the chemiluminescence detection system. Numbers at left indicate molecular weight markers in kilodaltons. B, graph of ratio of PUFA-treated cells to control calculated from densitometric scanning of the 122-, 73-, and 45-kDa protein bands in A. A represents one of three experiments, whereas B represents the average of the three experiments. Those not sharing letters are significantly different ($p < 0.05$). BSA = bovine serum albumin; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; IMDM = Iscove's modified Dulbecco's medium; LA = linoleic acid; PUFAs = polyunsaturated fatty acids; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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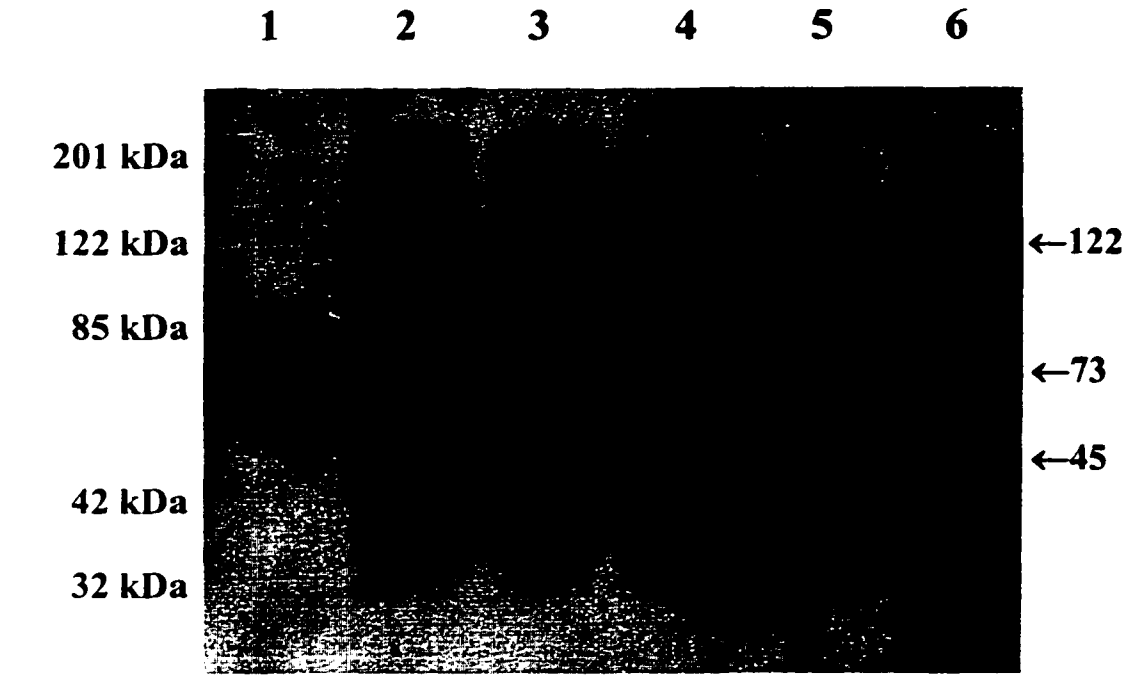

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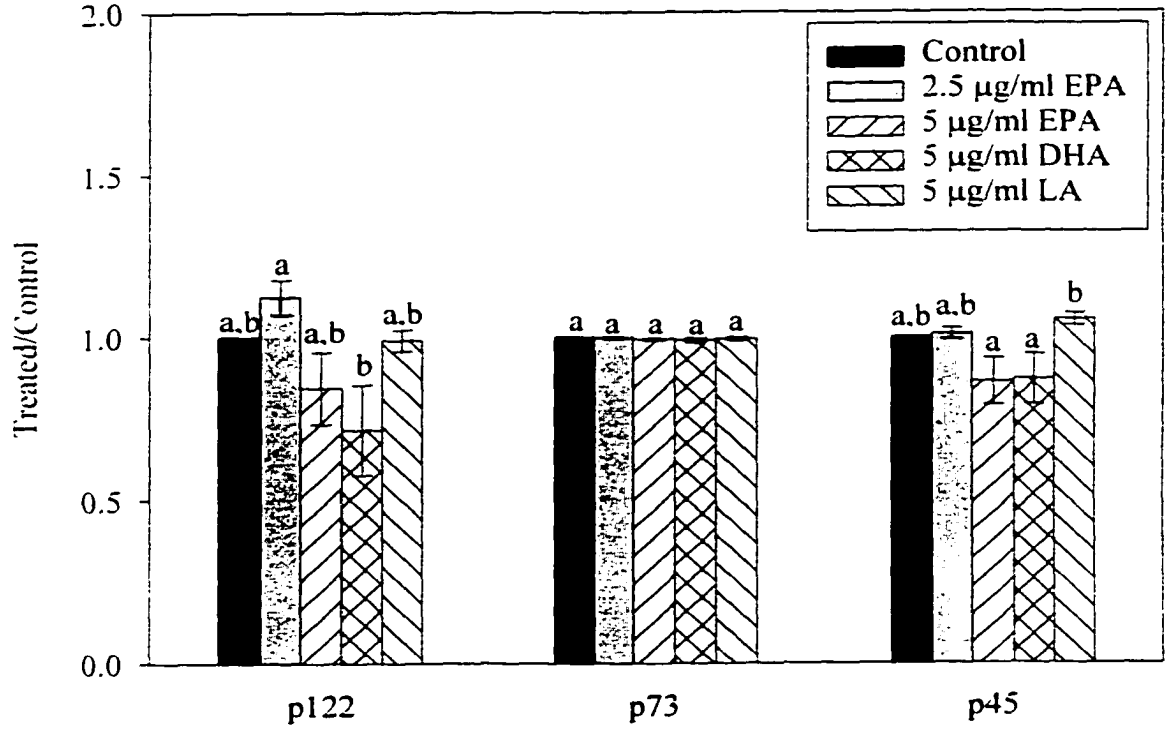

Figure 14. FAK tyrosine phosphorylation in MDA-MB-231 cells treated with PUFAs for 5 days. Cells were grown for 5 days in IMDM containing 2.5 and 5 μg/ml EPA (lanes 4 and 5, respectively), 5 μg/ml DHA (lane 6), 5 μg/ml LA (lane 7), or no PUFA (lane 3). Cells were seeded at 4×10^6 cells/100-mm tissue culture dishes precoated with FN or BSA (lane 2). After a 1-hr incubation at 37 °C, cells were lysed with lysis buffer, and equal aliquots (400 μg protein) of clarified lysate were immunoprecipitated with either 5 μg anti-FAK antibody or nonimmune mouse IgG (lane 1). Immunoprecipitates were separated on a 7.5 % SDS-PAGE gel and analyzed by immunoblotting using an anti-phosphotyrosine antibody. Numbers at left indicate molecular weight markers in kilodaltons. IgG heavy (H) and light (L) chains are indicated at the right. The asterisk (*) represents the region where FAK migrates. This figure represents one of three experiments. BSA = bovine serum albumin; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; FAK = focal adhesion kinase; FN = fibronectin; IgG = immunoglobulin G; IMDM = Iscove's modified Dulbecco's medium; LA = linoleic acid; PUFAs = polyunsaturated fatty acids; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

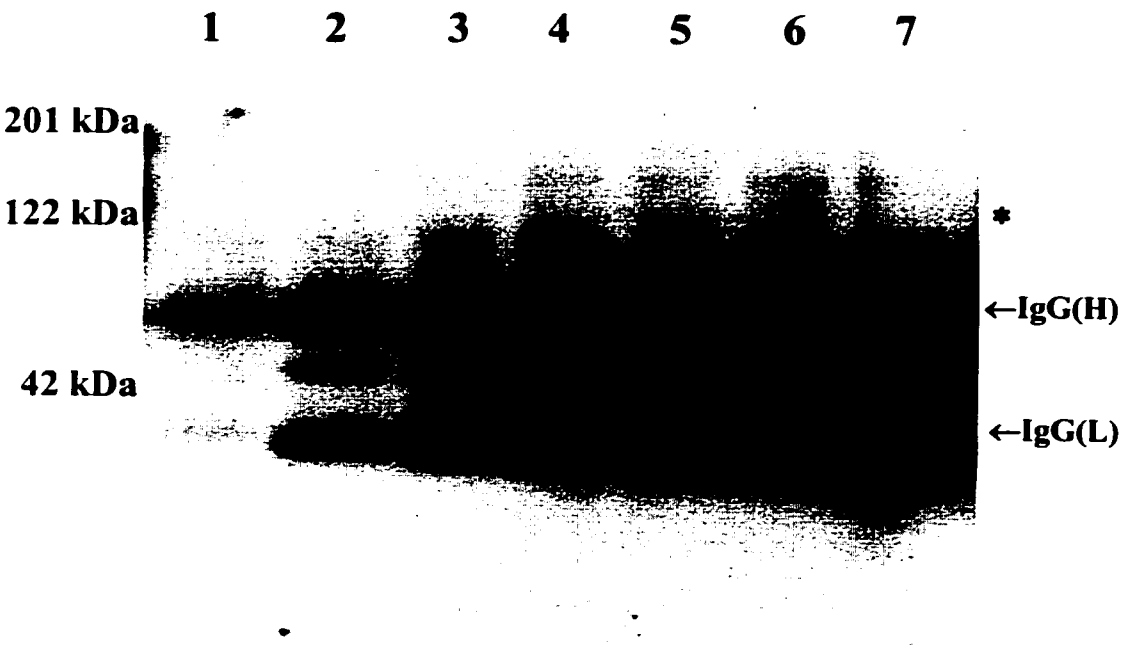

Figure 15. FAK tyrosine phosphorylation in MDA-MB-231 cells treated with PUFAs for 1 hr. Cells were treated with either 2.5 μg/ml EPA (lane 4), 5 μg/ml EPA (lane 5), 5 μg/ml DHA (lane 6), 5 μg/ml LA (lane 7), or no PUFA (lane 3) for 1 hr. Cells were seeded at 4×10^6 cells/100-mm tissue culture dishes precoated with FN or BSA (lane 2). Cells were lysed with lysis buffer, and equal aliquots (400 μg protein) of clarified lysate were immunoprecipitated with either 5 μg anti-FAK antibody or nonimmune mouse IgG (lane 1). Immunoprecipitates were separated on a 7.5 % SDS-PAGE gel and analyzed by immunoblotting using an antiphosphotyrosine antibody. Numbers at left indicate molecular weight markers in kilodaltons. IgG heavy and light chains are indicated at the right. The asterisk (*) represents the region where FAK migrates. This figure represents one of three experiments. BSA = bovine serum albumin; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; FAK = focal adhesion kinase; FN = fibronectin; IgG = immunoglobulin G; LA = linoleic acid; PUFAs = polyunsaturated fatty acids; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

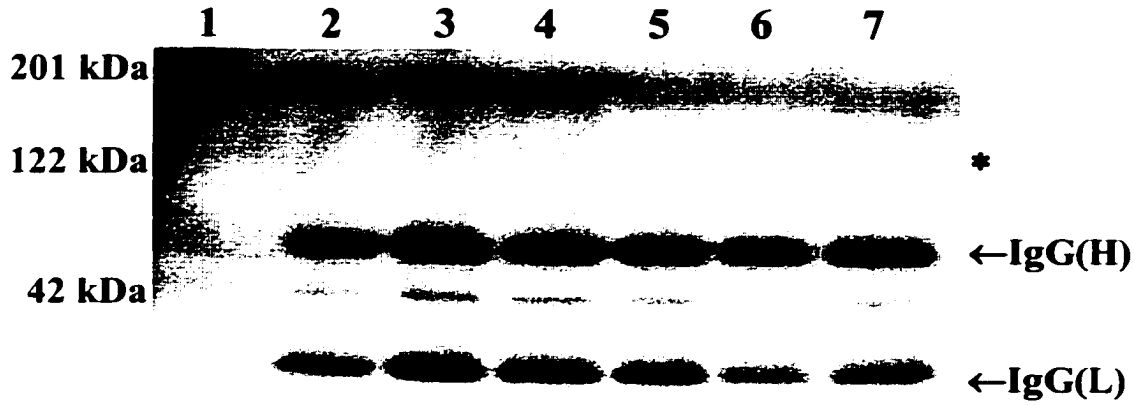

Figure 16. FAK expression in MDA-MB-231 cells treated with PUFAs for 5 days. A. Cells were grown for 5 days in IMDM containing 2.5 and 5 μg/ml EPA (lanes 3 and 4, respectively), 5 μg/ml DHA (lane 5), 5 μg/ml LA (lane 6), or no PUFA (lane 2). Cells were seeded at 4×10^6 cells/100-mm tissue culture dishes precoated with FN. After a 1-hr incubation at 37 °C, cells were lysed with lysis buffer, and equal aliquots (100 μg protein) of clarified lysate were electrophoresed onto a 7.5% SDS-PAGE gel. The gel was transferred to PVDF membrane and immunoblotted using an anti-FAK antibody (1:1,000 dilution). Visualization was performed by using the chemiluminescence detection system. B. graph of ratio of PUFA-treated cells to control calculated from densitometric scanning of A. A represents one of three experiments, whereas B represents the average of the three experiments. Those not sharing letters are significantly different ($p < 0.05$). DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; FAK = focal adhesion kinase; FN = fibronectin; IMDM = Iscove's modified Dulbecco's medium; LA = linoleic acid; PUFAs = polyunsaturated fatty acids; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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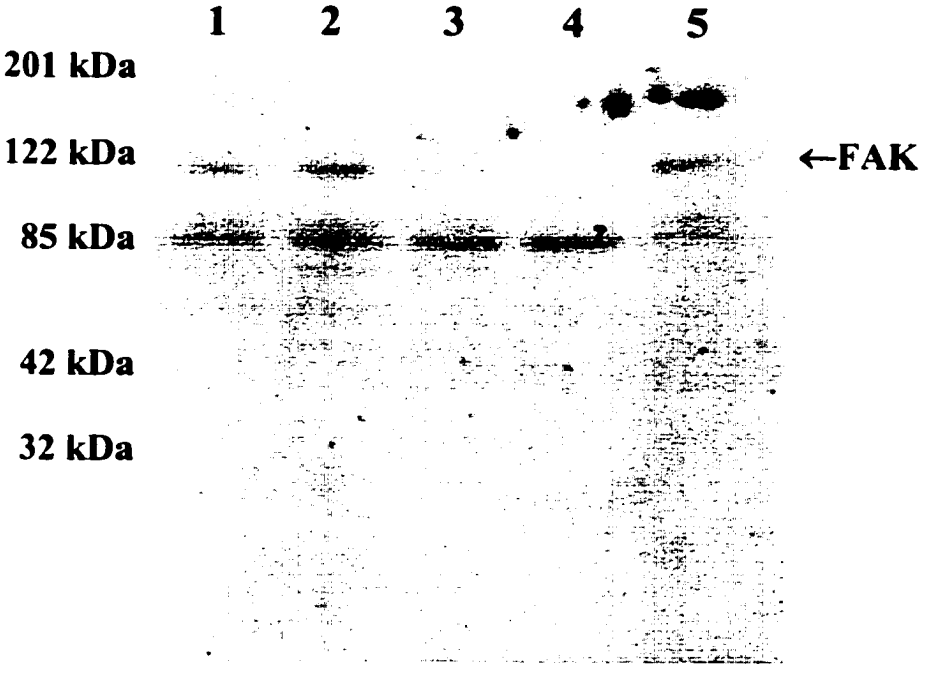

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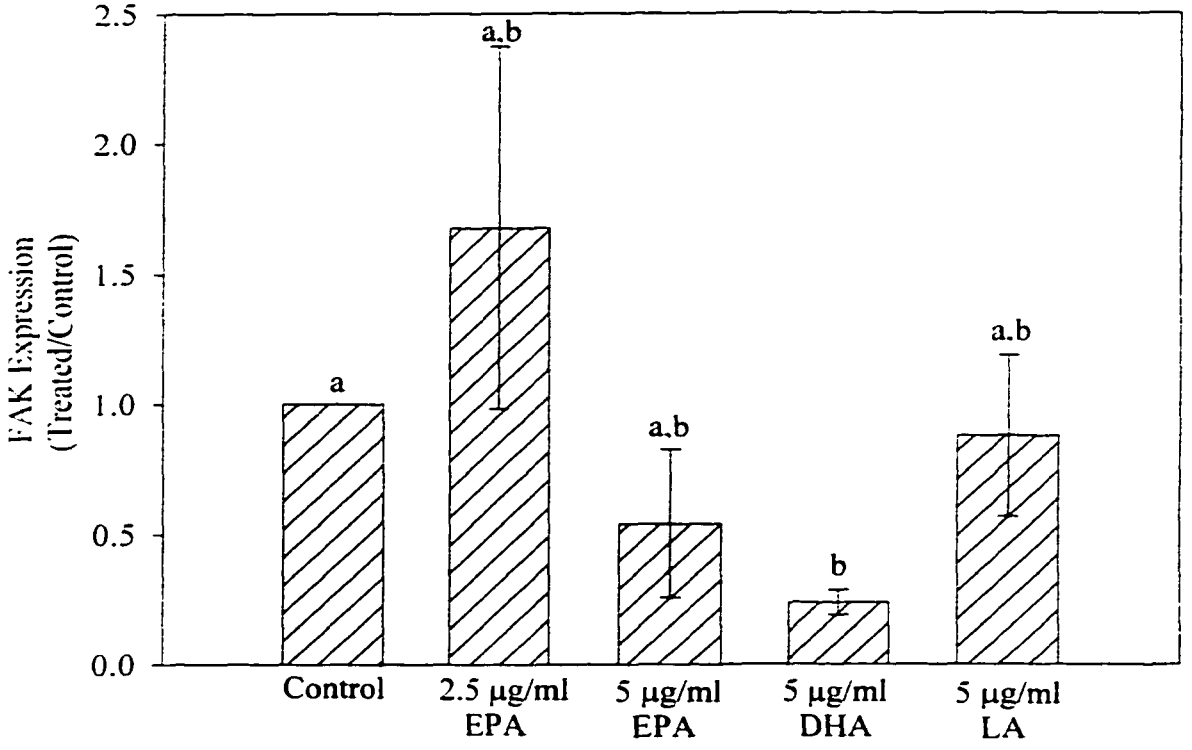
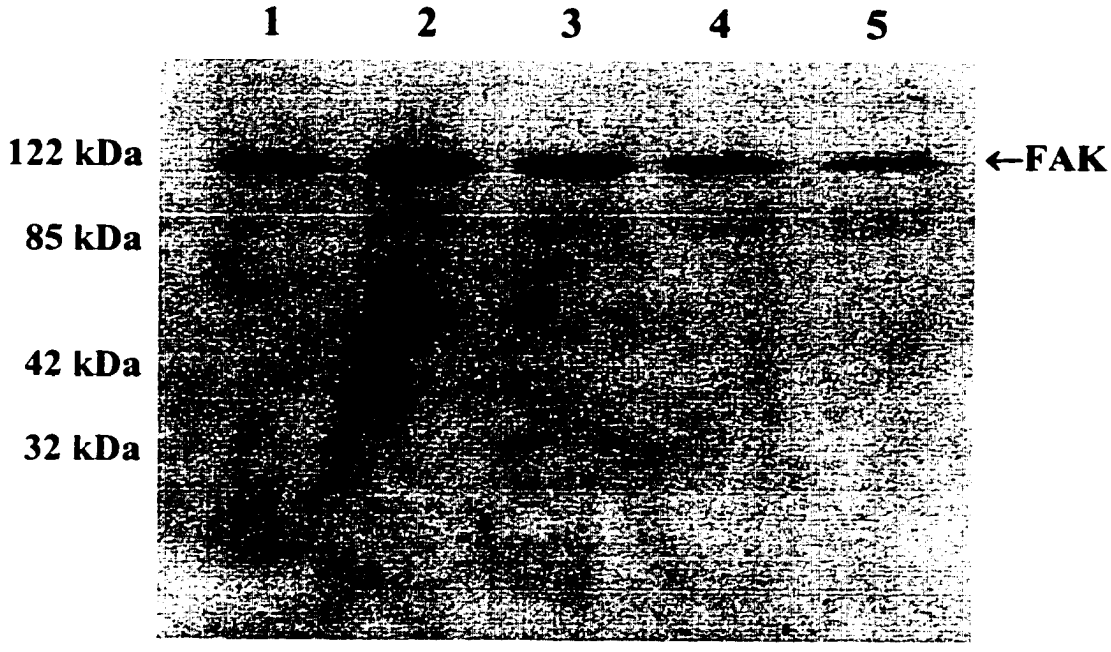


Figure 17. FAK expression in MDA-MB-231 cells treated with PUFAs for 1 hr. A. Cells were treated with either 2.5 μg/ml EPA (lane 3), 5 μg/ml EPA (lane 4), 5 μg/ml DHA (lane 5), or 5 μg/ml LA (lane 6) for 1 hr. Lane 2 represents cell lysate from cells treated with no PUFA Cells were seeded at 4×10^6 cells/100-mm tissue culture dishes precoated with FN. Cells were lysed with lysis buffer, and equal aliquots (100 μg protein) of clarified lysate were electrophoresed onto a 7.5% SDS-PAGE gel. The gel was transferred to PVDF membrane and immunoblotted using an anti-FAK antibody (1:1,000 dilution). Visualization was performed by using the chemiluminescence detection system. B, graph of ratio of PUFA-treated cells to control calculated from densitometric scanning of A. A represents one of three experiments, whereas B represents the average of the three experiments. Those not sharing letters are significantly different ($p < 0.05$). DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; FAK = focal adhesion kinase; FN = fibronectin; LA = linoleic acid; PUFAs = polyunsaturated fatty acids; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

A.



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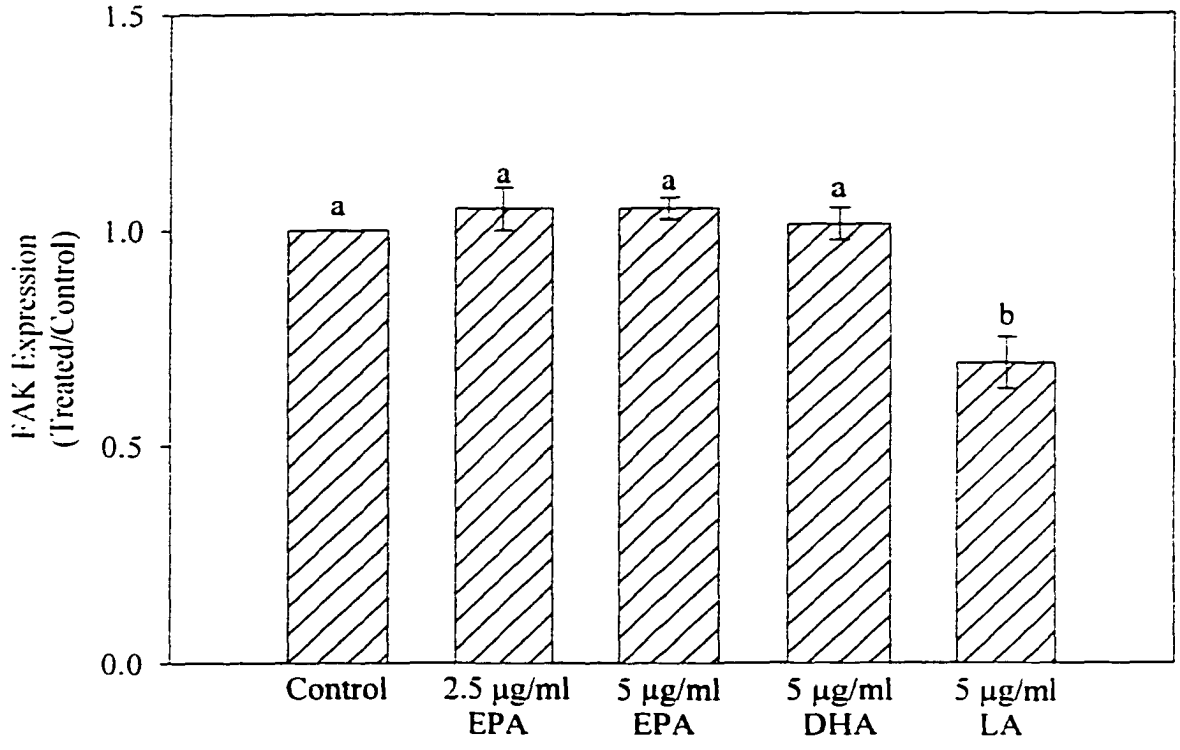

Figure 18. Effect of H-89 on FAK expression. A, Cells were treated with 0 (lane 2), 2 (lane 3), 4 (lane 4), or 8 (lane 5) μM H-89 for 1 hr. Cells were seeded at 4×10^6 cells/100-mm tissue culture dishes precoated with FN. Cells were lysed with lysis buffer, and equal aliquots (100 μg protein) of clarified lysate were electrophoresed onto a 7.5% SDS-PAGE gel. The gel was transferred to PVDF membrane and immunoblotted using an anti-FAK antibody (1:1,000 dilution). Visualization was performed by using the chemiluminescence detection system. B, graph of ratio of H-89-treated cells to control calculated from densitometric scanning of A. A represents one of three experiments, whereas B represents the average of the three experiments. Those not sharing letters are significantly different ($p < 0.05$). FAK = focal adhesion kinase; FN = fibronectin; H-89 = N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide•2HCl; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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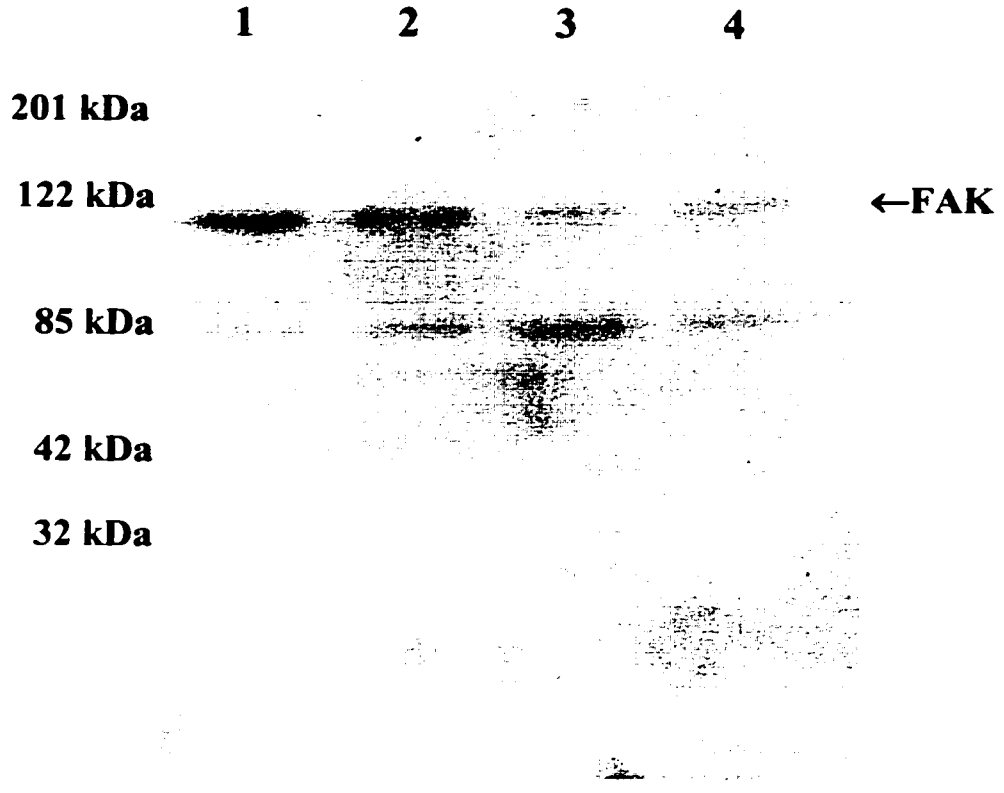

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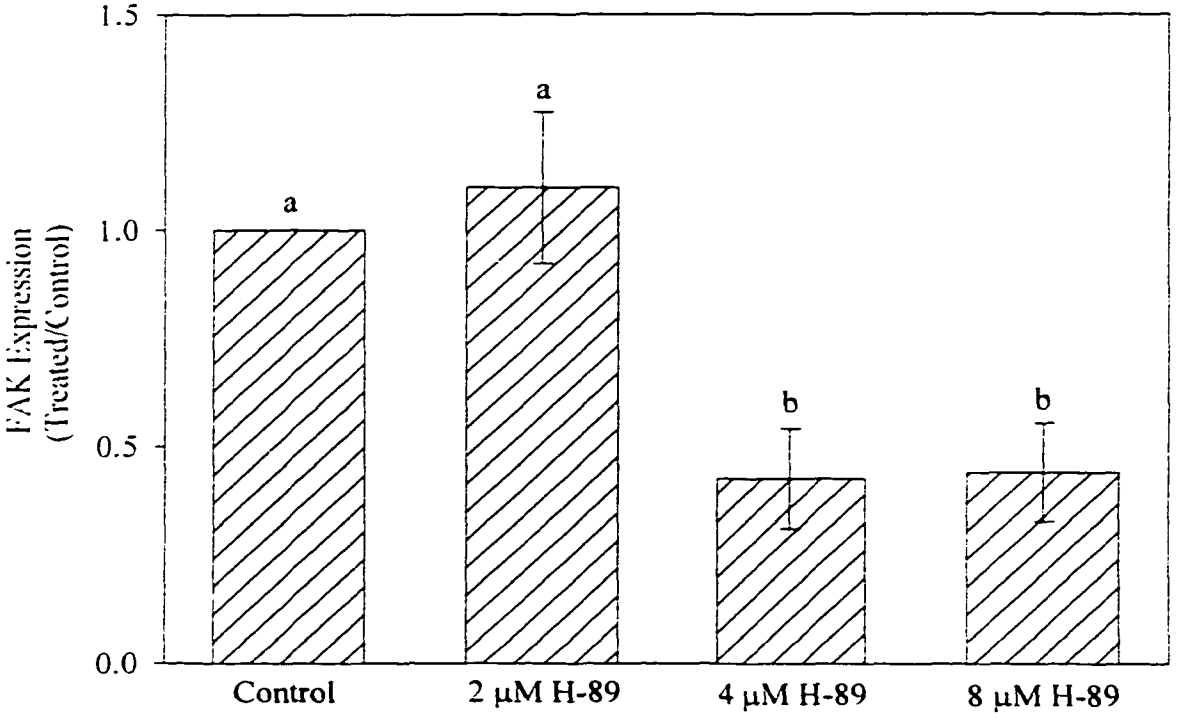

DISCUSSION

Environmental factors, including diet, play a role in the development of human cancer (Perera, 1997). Previous studies have demonstrated that ω-3 PUFAs found in fish oils can inhibit the growth and metastasis of human breast cancer cells in a nude mouse model (Rose & Connolly, 1993; Rose et al., 1995). The mechanisms by which ω-3 PUFAs suppress metastasis are not known with certainty, but they may act to inhibit enzymes involved in eicosanoid biosynthesis (Rose, Rayburn, et al., 1994) or directly suppress tumor cell growth in vitro (Falconer et al., 1994; Noguchi, Earashi, Minami, Kinoshita, & Miyazaki, 1995). Conversely, studies suggest that ω-6 PUFAs found in vegetable oils may act as promoters in the development and incidence of experimental mammary tumors (Rose et al., 1994b; Rose et al., 1993).

PUFAs may also suppress human breast cancer metastasis through effects on cell adhesion. Tumor cell adhesion is important in the complex process of tumor metastasis. Changes in adhesion of tumor cells to adjacent cells or to the nearby ECM are thought to be involved in many steps in the chain of events leading to metastasis formation (Albelda, 1993). Circulating cancer cells adhere to endothelial cells and the underlying subendothelial basement membrane as an initial step in the process of invading target organs during metastasis. Thus, alterations in tumor cell adhesion may result in increased or decreased cancer metastasis. Previous studies have demonstrated that ω-6 PUFAs stimulated and ω-3 PUFAs inhibited basement membrane invasiveness and adhesion of various cell lines (Connolly & Rose, 1993; Johanning & Lin, 1995; Palmantier et al., 1996; Reich

et al., 1989; Rose et al., 1994a). A mechanism to account for PUFA-mediated effects on breast cancer cell adhesion has not been proposed, but studies using other cell types suggest that ω-3 PUFAs inhibit PKC activity (Holian & Nelson, 1992; Tappia et al., 1995). PKC inhibition, in turn, has been associated with decreased cell adhesion to the endothelium (Liu, B., et al., 1992). Several mechanisms have been proposed to explain the stimulatory effect of ω-6 PUFAs on breast cancer cell adhesion. ω-6 PUFAs appear to increase adhesion of human breast cancer cells to type IV collagen via a lipoxygenase pathway, whereas PUFA-induced PKC activation leads to regulation of adhesion to both type IV collagen and vitronectin (Palmantier et al., 1996).

The influence of exogenous growth factors and nutrients on tumor cell adhesion to basement membrane ECM components has been recently investigated. EGF increased adhesion and in vitro invasiveness of high-metastatic RTC sarcoma cells to Matrigel and to the ECM components laminin and fibronectin (Yudoh et al., 1994). EGF did not, however, affect the invasive potential of the human breast cancer cell line MDA-MB-231 (Long & Rose, 1996). EGF decreased spreading on fibronectin and increased spreading on type I collagen by selectively upregulating expression of the α2β1 integrin collagen receptor in human cutaneous squamous carcinoma cells (Fujii et al., 1995).

The effect of EGF and PUFAs on adhesion of human breast cancer cells to ECM components has not been determined. In Experiment 1, we investigated the effect of EGF and the ω-3 PUFA EPA on adhesion of the human breast cancer cell line MDA-MB-231 to subendothelial matrix ECM components. MDA-MB-231 cells are estrogen-receptor-negative and express high EGFR numbers (3×10^4 EGF binding sites per cell; Mueller et al., 1994). Breast cancer cell lines as well as primary breast tumors that are EGFR-

positive and estrogen-receptor-negative are metastatically more aggressive (Sheikh, Garcia, Pujol, Fontana, & Rochefort, 1994-95). EGF decreased adhesion of MDA-MB-231 cells to Matrigel in a dose-dependent manner (Figure 4; German & Johanning, 1997). EGF also decreased adhesion to fibronectin and type IV collagen (Figure 5). Previous studies using the rat mammary adenocarcinoma cell line MTLn3, which express 5-6 × 10^4 EGFRs per cell (Lichtner et al., 1993), demonstrated that EGF increased adhesion of these cells to ECM components (Lichtner et al., 1993; Rohde-Schulz & Lichtner, 1995). Human epidermoid A431 cells, which also express high EGFR levels, were used as a control cell line in these studies. EGF decreased adhesion of A431 cells to fibronectin (Lichtner et al., 1993). The differential effect of EGF on adhesion in these two cell lines may have been due to increased phosphorylation in EGF-treated MTLn3 cells (but not in A431 cells) of a 240-kDa protein associated with microtubule formation (Lichtner et al., 1993). Treatment of NR6 fibroblasts with EGF resulted in decreased adhesion of these cells to Amgel, a human ECM (Xie et al., 1998). The latter study demonstrated the ability of EGF to directly induce focal adhesion disassembly. This disassembly of focal adhesions was concomitant with decreased cell adhesiveness to the substratum.

EPA also decreased adhesion of MDA-MB-231 cells to ECM substrates (Figure 5). EPA-mediated decreased adhesion of human breast cancer cells to the ECM may be one reason for the decreased metastasis of human breast cancer cells to the lungs of athymic nude mice consuming diets enriched in ω-3 PUFAs (Rose et al., 1994a).

Recent studies have demonstrated that fatty acids influence EGF-mediated cell signaling pathways in primary cultures of mammary epithelial cells (Bandyopadhyay, Imagawa, & Nandi, 1995; Sylvester, Birkenfeld, Hosick, & Briski, 1994). Mechanisms

involving PKC activation and activation of GTP-binding proteins have been proposed to explain fatty acid modulation of EGF effects. Sodium-arachidonate enhanced EGF-induced growth and PKC translocation into the membrane of mouse mammary epithelial cells (Sylvester et al., 1994). In contrast, sodium-stearate, a saturated fatty acid, decreased growth and PKC translocation in response to EGF. The changes in growth were accompanied by changes in cell membrane fatty acid composition and were attributed to altered PKC activation (Sylvester et al., 1994).

The effect of ω-3 PUFAs on EGF signaling in mammary- or breast-derived cells has not been well characterized. McKenzie, Bandyopadhyay, Imagawa, Sun, and Nandi (1994) showed that the growth of EGF/insulin-stimulated mammary epithelial cells was stimulated by low levels of ω-3 PUFAs and PGE_2, but the growth stimulation was lower than that obtained with ω-6 PUFAs. In addition, growth of EGF/insulin-stimulated cultures of mammary tumor cells was, in some cases, inhibited by ω-3 PUFAs. After becoming incorporated into the plasma membrane, EPA may thus alter the composition or physical properties of the breast cancer cell membrane in such a manner that EGF binding to its receptor is altered. Alternatively, second messengers downstream from the EGFR that are involved in cell adhesion may intersect with signaling events that are initiated by EPA incorporation into the cell membrane.

The effects of PUFAs on EGF-induced changes in cancer cell adhesion have not been previously reported. We found that the combined effect of EPA and EGF was not additive in decreasing adhesion of MDA-MB-231 cells to the three ECM components studied (Figure 5). These findings suggest that decreased adhesion to ECM substrates by

combined EPA and EGF treatment may be the result of a common postreceptor signaling pathway.

When cells in culture are supplemented with PUFA, the fatty acid is incorporated into both neutral lipids and phospholipids depending upon the fatty acid used and its concentration (DeAntueno et al., 1993). Likewise, addition of PUFA to cells in culture leads to a number of temporary changes in both the structural and functional aspects of the cells. Incorporation of PUFA into cell lipids provides substrate for both eicosanoid synthesis and lipid peroxidation. Lipid peroxidation is an oxidative deterioration of unsaturated fatty acids that give rise to a variety of oxidation products with the potential to interfere with cell replication, cell survival, or both. The presence of higher concentra-tions of long-chain ω-3 PUFAs (e.g., EPA and DHA) in certain dietary fats, such as fish oil, suggests that oxidation products of these fatty acids may play a role in the inhibitory or growth-rate-limiting action exhibited by these types of fats. Fish oil has been shown to suppress mammary gland tumorigenesis in rodent models (Karmali et al., 1984; Welsch, 1992) as well as inhibit the growth of human breast carcinomas maintained in immune-deficient animals (Gonzalez et al., 1991). Several studies have suggested that dietary fish oil-induced suppression of human breast carcinoma growth is a function, at least in part, of an accumulation of lipid peroxidation products in the tumor tissues (Gavino et al., 1981; Gonzalez et al., 1991, 1993).

In Experiment 2, we examined the hypothesis that an EPA-induced decrease in MDA-MB-231 cell adhesion to ECM components is due to increased production of lipid peroxidation products. Vitamin E is widely accepted to be the primary lipid-soluble antioxidant responsible for protection of unsaturated membrane components. Our results

show that addition of α-tocopherol (the most active and most abundant form of vitamin E) to EPA-treated cells substantially reduced the level of tumor lipid peroxidation products (Figure 6); however, there was no significant effect on cell adhesion to Matrigel or type IV collagen (Figure 7). Thus, it appears that lipid peroxidation products are not solely responsible for EPA-induced decreases in MDA-MB-231 cell adhesion to ECM components.

The signaling pathways that regulate tumor cell adherence to and invasion through ECM components are not very well defined. Studies suggest that PKA may have a role in increasing the metastatic potential of tumor cells (Lozano et al., 1996; Maier et al., 1996; Young et al., 1992). Young, Duffie, Lozano, Young, and Wright (1990) showed that metastatic clones of Lewis lung carcinoma tumors had more PKA activity than nonmetastatic clones. Likewise, Gordge et al. (1996) found an elevation of PKA and PKC activities in malignant human breast tissue. Our studies demonstrated that EPA, an ω-3 PUFA, as well as LA, an ω-6 PUFA, decreased the PKA activity in MDA-MB-231 human breast cancer cells (Table 2). Our results suggest a nonspecific inhibitory effect of PUFAs on PKA activity, an effect also seen in brush border membranes isolated from human placental vesicles (Doolan & Keenen, 1994). Tappia et al. (1995) also showed that EPA and DHA, at 100 μM fatty acid concentration, suppressed rat peritoneal macrophage PKA activity by 40% and 3%, respectively. In the presence of LA, PKA activity was enhanced by 34%. However, at 50 μM fatty acid concentration, both LA and EPA suppressed PKA activity by 21% and 42%, respectively, whereas DHA produced a marginal suppression of PKA activity (3%). Doolan and Keenan (1994) suggest that PKA localized to the brush border membrane of human placental vesicles is inhibited by fatty acids,

which may compete with cAMP for binding to the kinase regulatory subunit. Our studies did not indicate a significant difference in intracellular cAMP production in MDA-MB-231 cells treated with PUFAs. Other studies have shown that EPA inhibits the elevation of intracellular cAMP in intact adipocytes incubated with a tumor-derived lipid-mobilizing factor (LMF; Price & Tisdale, 1998; Tisdale & Beck, 1991). This inhibition arose from a direct inhibition of adenylate cyclase activity rather than indirectly through changes in cAMP phosphodiesterase. Because pertussis toxin eliminated the inhibition of lipolysis and the stimulation of adenylate cyclase by isoprenaline and LMF in the presence of EPA, the attenuation of adenylate cyclase stimulation by EPA may have been due, at least in part, to an inhibitory guanine nucleotide (G_i)-binding protein-mediated inhibition of adenylate cyclase activity.

The RIα subunit of PKA is generally overexpressed in human cancer cell lines and primary tumors (Cho-Chung & Clair, 1993; Cho-Chung et al., 1995; Gordge et al., 1996) and has been correlated with poor prognosis in breast cancer patients (Miller et al., 1993). Our studies showed that cells treated with ω-3 PUFAs exhibited a significant decrease in $PKA_{RI\alpha}$ expression, whereas LA-treated cells showed no significant effect on $PKA_{RI\alpha}$ expression (Figure 8). Further studies of the effect(s) of ω-3 PUFAs on other catalytic and regulatory subunits of PKA appear to be warranted, because the effect of these PUFAs was so strong on RIα expression.

In our study, the decreased PKA activity, in response to treatment with ω-3 PUFAs, was not as profound as the decrease in $PKA_{RI\alpha}$ subunit expression. We speculate that this may occur as a result of a compensatory mechanism in PKA holoenzyme formation, as seen in a previous study (Nesterova & Cho-Chung, 1995). The downregulation of

the RIα subunit by ω-3 PUFAs may result in an excess of free catalytic subunits that are then able to complex with all of the available RIIα and RIIβ subunits to form PKA-IIα and PKA-IIβ, respectively. Thus, a marked decrease in RIα subunit expression may not necessarily result in a marked decrease in PKA activity, as seen in our studies.

Our studies also show that treatment of MDA-MB-231 cells with an inhibitor of PKA, H-89, decreased adhesion of these cells to both Matrigel and type IV collagen (Figure 10). These results suggest that PKA signaling is important for modulating the tumor-ECM interaction.

Several studies suggest that aberrations in the PKC signal transduction pathway, specifically PKC-α, may be involved in the pathogenesis or progression of breast cancer and that PKC may be involved in the regulation of estrogen receptor expression, a parameter of prognostic and therapeutic importance (Fabbro, Regazzi, Costa, Borner, & Eppenberger, 1986; Kennedy, Presligiacoma, Tyler, May, & Davidson, 1992; Lee, S., Karaszkiewicz, & Anderson, 1992; O'Brian et al., 1989; Ways et al., 1995). In addition, an antisense oligonucleotide to PKC-α inhibited growth of a U-87 (glioblastoma), MDA-MB-231 (breast), and Calu-1 (lung) cell lines and decreased tumor size in ovarian cancer patients (Yuen et al., 1999).

Several studies have demonstrated the involvement of PKC in the modulation of cell attachment to ECM proteins (Dumont & Bitonti, 1994; Johanning & Lin, 1995; Liu, B., et al., 1991; Palmantier et al., 1996; Timar et al., 1992). Therefore, we examined the effect of PUFAs on expression of the α isoform of PKC. Results showed that, compared to control cell cultures, the higher level of EPA and DHA decreased expression of PKC-α (Figure 9). LA, an ω-6 PUFA, showed no significant effect on PKC-α expression (Figure

9). B. Liu et al. (1991) showed a role for 12(S)-hydroxyeicosatetraenoic acid (12[S]-HETE) and 13(S)-hydroxyoctadecadienoic acid (13[S]-HODE), lipoxygenase metabolites of AA and LA, respectively, in the modulation of adhesion of tumor cells via regulation of PKC. Tang, Tarrien, Dobrzynski, and Honn (1995) showed that 12(S)-HETE-promoted melanoma cell spreading on fibronectin involves tyrosine phosphorylation of FAK and PKC and tyrosine kinase-dependent focal adhesion formation. In the study by Tappia et al. (1995), 50- and 100-μM concentrations of EPA and DHA suppressed rat peritoneal macrophage PKC activity, whereas LA enhanced PKC activity. Collectively, these studies suggest that PKA and PKC signaling pathways are affected by fatty acids and these signaling pathways represent potential targets for therapeutic intervention in breast cancer.

CAMs play a fundamental role in tumor metastasis (Hart & Saini, 1992). Cell-cell and cell-ECM adhesive interactions are important in a wide range of phenomena involving normal and aberrant cells (Albelda, 1993). The integrins are the main receptors involved in interactions with the ECM (Hynes, 1992). The regulation of integrin expression and activation has been extensively studied in many cell types. The α3β1 integrin is a major integrin expressed in MDA-MB-231 cells (Coopman, Thomas, Gehlsen, & Mueller, 1996). This integrin recognizes a variety of ECM components, including collagen and laminin. Gui et al. (1995) reported that the invasive potential of MDA-MB-231 and Hs578T human breast cancer cells was inhibited by monoclonal antibodies directed against β1 and β5 integrins. This observation suggests that integrins play an important role in breast cancer cell metastasis.

The effect of PUFAs on integrin expression in human breast cancer cells has not been extensively studied; however, R. K. Singh et al. (1995) showed that stearate (a saturated fatty acid) inhibited human fibrosarcoma HT-1080 cell adhesion to human ECM via a mechanism involving a laminin integrin receptor. PUFAs have also been reported to regulate integrin receptor expression in neutrophils (Bates, Ferrante, Harvey, & Poulos, 1993; Capodici, Pillinger, Han, Philips, & Weissman, 1998). Several investigators have reported that PUFAs influence expression of other CAMs in a variety of cell types (Collie-Duguid & Wahle, 1996; DeCaterina & Libby, 1996; Jiang et al., 1995). These studies provide evidence that PUFAs are capable of modulating expression of CAMs that have important roles in the pathogenesis of chronic diseases such as cancer and atherosclerosis.

In our studies, EPA-treated MDA-MB-231 cells exhibited a significant decrease in β1 integrin expression, whereas LA showed a small, but nonsignificant increase (Table 3). The signaling pathways responsible for activation of protein kinases, leading to the downstream regulation of integrin function, are uncertain. Gimond, DeMelker, Aumailley, and Sonnenberg (1995) showed that the cytoplasmic domain of the α6A integrin subunit is an in vitro substrate for PKC. Rabinovitz, Toker, and Mercurio (1999) showed that the large β4 integrin subunit cytoplasmic domain (in A431 squamous carcinoma cells) contains multiple consensus motifs for PKC phosphorylation and that the presence of these motifs supports the possibility that PKC may phosphorylate α6β4 integrin directly. These studies indicate that integrins can be directly phosphorylated by protein kinases. Richardson et al. (1997) suggested a role for PKA before integrin binding to ECM. Treatment of fibroblasts with PKA inhibitors delayed serine phosphorylation of the C-

terminal noncatalytic domain of FAK, as well as cell spreading on fibronectin and tyrosine phosphorylation of FAK. This study provided evidence that PKA may play a role in phosphorylation of integrins or molecules involved in integrin signaling. Because our data shows that PUFAs modulate PKA and β1 integrin activity or expression, we evaluated the influence of PKA effectors on β1 integrin expression. Treatment of MDA-MB-231 cells with dbcAMP increased the expression of the β1 integrin subunit in a dose-dependent manner (Figure 11); however, this effect was only significant at 2 mM. Other studies have also demonstrated the ability of dbcAMP to alter integrin expression (Halvorson & Coligan, 1995; Jung et al., 1994) as well as cell adhesion (Halvorson & Coligan, 1995). Halvorson and Coligan (1995) examined the ability of cAMP to regulate VLA integrin receptor function in thymocytes. Results showed that VLA-dependent binding of thymocytes to fibronectin was enhanced by cAMP analogues (dbcAMP and 8-bromo-cAMP) and agents that increase intracellular cAMP, thus suggesting that cAMP acts as a secondary messenger to upregulate integrin function in thymocytes. In addition, cAMP-induced binding was blocked by monoclonal antibodies to the VLA integrin chains α4 and α5 and by the PKA inhibitor, (Rp)-cAMPs, indicating that activation of PKA enhances VLA-4 and VLA-5 receptor function. In contrast, Jung et al. (1994) showed that dbcAMP treatment decreased VLA-4 expression in a human leukemia cell line, Eol-1.Treatment with H-89 decreased expression of the β1 integrin subunit (Figure 12). However, the concentration range of H-89 employed in this study is highly non-specific, thus making the conclusion that β1 integrin expression and PKA activity are related speculative at best. In contrast, lower levels of H-89 were effective in decreasing MDA-MB-231 cell adhesion to ECM components (Figure 10). Because other CAMs con-

tribute to cell adhesion, it is possible that a strong effect of H-89 on any single CAM is not seen, but rather a modest effect is seen on several.

Increased tyrosine phosphorylation of several proteins that are associated with focal adhesions has been implicated in cell migration and invasion (Clarke & Brugge, 1995). Tyrosine phosphorylation of these proteins can be stimulated by several mechanisms. For example, growth factors, neuropeptides, cell adhesion to ECM ligands, and clustering of integrins can stimulate the tyrosine phosphorylation of a number of focal adhesion-associated proteins, including FAK and paxillin (Schaller & Parsons, 1994).

Buckley and Whorton (1995) demonstrated that AA as well as other unsaturated fatty acids (oleic acid, LNA, and GLA) stimulated protein tyrosine phosphorylation of proteins with masses of ~58, 93, and 120 kDa in cultured vascular endothelial cells. Our studies did not show a significant effect of PUFAs on protein tyrosine phosphorylation of some proteins in MDA-MB-231 cells (Figure 13). However, EPA, at 5 μg/ml, and DHA decreased tyrosine phosphorylation of the 185-, 148-, 122-, 98-, 45-, and 39-kDa proteins (Figure 13). Sanderson and Calder (1998) also reported that dietary fish oil and, to a lesser extent, olive oil caused a reduction in tyrosine phosphorylation of a range of proteins in lymphocytes isolated from rats fed a high fish oil or olive oil diet. This study suggested that feeding these diets led to an inhibition of a tyrosine kinase activity which is responsible for phosphorylating a number of proteins. A reduction in tyrosine kinase activity may account for the numerous inhibitory effects which accompany fish oil or olive oil feeding. To determine if the 122-kDa protein band in Figure 13 was FAK, anti-FAK immunoprecipitates were prepared from MDA-MB-231 cells that had been treated with PUFAs for either 5 days or 1 hr. Reports to date show that FAK is a single band of

~120-125 kDa on SDS-PAGE in a variety of cell types (Lipfert et al., 1992; Schaller et al., 1992). The absence of a protein band in the 120-125-kDa region suggests that FAK is not phosphorylated in response to PUFAs in this cell line (Figures 14 and 15). We cannot rule out the possibility that FAK phosphorylation did occur in MDA-MB-231 cells; it may have been below the limit of detection or it may not have been immunoprecipitated at all. Collectively, these results suggest that protein tyrosine phosphorylation is directly affected by unmetabolized unsaturated fatty acids.

Because there was no evidence of FAK phosphorylation in MDA-MB-231 cells treated with PUFAs, we examined the effects of PUFAs on FAK expression in this cell line. Treatment with 2.5 μg/ml EPA or 5 μg/ml LA for 5 days resulted in no significant change in FAK expression when compared to control cultures, although FAK expression was decreased in cells treated with 5 μg/ml EPA and DHA (Figure 16). Treatment with DHA resulted in a significant decrease in FAK expression.

The signaling events that are responsible for tumor cells traversing the ECM are incompletely understood but appear to be centered at focal adhesions, where signaling molecules such as integrins, FAK, and src are localized. Evidence suggests that FAK is involved in the pathogenesis of certain cancers. Studies show that FAK is consistently overexpressed in invasive and metastatic cancers of the breast, colon, thyroid, prostate, ovary, and oral cavity (Judson, He, Cance, & Le, 1999; Kornberg, 1998; Owens et al., 1995; Owens et al., 1996; Tremblay et al., 1996). Weiner et al. (1993) also showed that FAK mRNA overexpression occurs as human epithelial and mesenchymal tumors invade and metastasize. Agochiya et al. (1999) used in situ hybridization to confirm chromosome 8q as the genomic location of the human FAK gene and report that elevation of

FAK protein in cell lines derived from invasive squamous cell carcinomas is accompanied by gains in copy number of the FAK gene in all cases examined. These studies demonstrate that invasion and metastasis of transformed cells is accompanied by overexpression of FAK. In normal cells, FAK might be a sensor of cell adhesion, limiting growth in an anchorage-dependent manner, whereas, in transformed cells, overexpression of FAK may be part of the cascade of events required for transformed cells to overcome their normal anchorage-dependent growth and acquire an invasive phenotype (Liotta et al., 1991).

Similarly, the EGFR has an extremely high incidence of expression in human tumors of all origins (Fry, 1999). The EGFR has also been proposed to contribute to the invasiveness of human cancers (Jardines, Weiss, Fowble, & Greene, 1993). Brunton, Ozanne, Paraskeva, and Frame (1997) showed that both the EGFR and FAK protein levels were elevated in colon carcinoma but not in adenoma cells, thus suggesting a cooperation between the EGFR, c-Src, and possibly FAK to induce properties in the carcinoma cells, indicative of a more motile invasive phenotype.

Studies show that serine-threonine protein kinases may also affect FAK phosphorylation. Vuori and Ruoslahti (1993) reported that inhibition of PKC activity resulted in the lack of both cell spreading and FAK tyrosine phosphorylation, implying that these two phenomena could be connected to PKC activation. Likewise, Richardson et al. (1997) reported that cell spreading on fibronectin and tyrosine phosphorylation of FAK was delayed by treatment of chicken embryo cells with 10 μM H-89, an inhibitor of PKA. Because our studies showed an effect of PUFAs on FAK expression, but not tyrosine phosphorylation, we examined the effect of H-89 on FAK expression. The decrease

in FAK expression in those cells treated with either 4 or 8 μM H-89 (Figure 18) suggests that the FAK and PKA signaling pathways are somehow interrelated.

FAK may be a therapeutic target to interrupt the invasive and metastatic process (Owens et al., 1995). It is possible that attenuation of FAK expression or interruption of its signal transduction pathway in tumor cells may cause the loss of anchorage independent growth properties of a tumor. Thus, therapeutics against FAK may become useful in the treatment of invasive and metastatic cancer.

Summary

1. EPA and EGF each decreased MDA-MB-231 adhesion to the ECM components. The combined effect of EPA and EGF was not additive, however, which suggests that EPA and EGF may act via a common postreceptor signaling pathway.

2. Lipid peroxidation products are only partially responsible for EPA-induced decreased adhesion of MDA-MB-231 cells to ECM components.

3. Both ω-3 and ω-6 PUFAs decreased PKA activity in MDA-MB-231 cells. However, only ω-3 PUFAs inhibited the expression of the RIα subunit of PKA. The higher level of EPA (5 μg/ml) and DHA decreased expression of PKC-α, whereas, LA, an ω-6 PUFA, did not influence PKC-α expression.

4. PKA inhibition resulted in decreased MDA-MB-231 adhesion to ECM components.

5. EPA inhibited expression of the β1 integrin subunit in MDA-MB-231 cells.

6. FAK is probably not phosphorylated in response to PUFAs. However, the ω-3 PUFAs appear to decrease FAK expression during a 5-day incubation period.

7. Our studies suggest a possible link between the FAK and PKA signaling pathways.

With regard to the initial hypothesis, our studies showed a significant effect of EGF on cell adhesion; however, EGF interaction with the ω-3 PUFA, EPA, did not produce an additive effect on cell adhesion. This suggests a synergistic effect of EPA and EGF on cell adhesion and does not support our original hypothesis, which was that EPA (an ω-3 PUFA) would inhibit EGF-induced increased cell adhesion. PUFA treatment resulted in a nonspecific decrease in PKA activity; however, only the ω-3 PUFAs exhibited significant effects on $PKA_{RI\alpha}$ and PKC-α expression. The ω-6 PUFA, LA, had no effect on these molecules. Likewise, FAK and β1 integrin expression was affected only by the ω-3 PUFAs. Our original hypothesis was that the effects of PUFAs on breast cancer cell adhesion are mediated by the cell signaling molecules PKA, PKC, FAK, and β1 integrin. Our results support the original hypothesis for the ω-3 PUFAs; however, the results do not support the hypothesis for the ω-6 PUFA. These results suggest that ω-3 and ω-6 PUFAs may act through different signaling pathways to mediate their effects on cell adhesion.

In view of the results of the present study and other evidence discussed above, it can be said that the ω-3 PUFAs EPA and DHA decreased the expression of several key cell signaling molecules that are directly and indirectly associated with cell adhesion. These signaling molecules may, in turn, mediate PUFA-modulation of cell adhesion to ECM components. It is important to identify environmental factors that influence the metastatic cascade. Diet is a key environmental factor, and PUFAs are major dietary components. PUFAs are relatively safe components of the diet, and their levels in the diet

are easy to control. Thus, this study has the potential to explain how a common, safe, and inexpensive component of the diet may be useful in preventing the progression of breast cancer.

Future Studies

1. Determine the effect of ω-3 PUFAs on expression of other regulatory and catalytic subunits of PKA, as well as other isoforms of PKC.

2. Evaluate the influence of ω-3 and ω-6 PUFAs on the expression of other integrin subunits, as well as the effect of antiintegrin antibodies on cell adhesion to ECM components. These subunits might include α3, α5, α6, and β4, because most tumor cells demonstrate enhanced expression of these subunits. In addition, the ligands for these subunits are those ECM components utilized in our studies.

3. Evaluate the influence of PUFAs on β1 integrin subunit mRNA levels as well as protein expression. In addition, the relationship between FAK and β1 integrin in MDA-MB-231 cells needs to be examined, because studies show that FAK actually binds this integrin subunit.

4. Evaluate the influence of PUFAs on MDA-MB-231 cell invasion to correlate cell adhesion with a biological measure of metastasis.

5. Evaluate the influence of PUFAs on FAK distribution in MDA-MB-231 cells via immunofluorescence.

6. Evaluate the influence of PUFAs on the expression of paxillin, because studies show that FAK is associated with this protein. Paxillin, along with FAK, is involved in actin membrane attachment at focal adhesions and is phosphorylated with FAK.

REFERENCES

Abou-el-Ela, S. H., Prasse, K. W., Carroll, R., Wade, A. E., Dharwadkar, S., & Bunce, O. R. (1988). Eicosanoid synthesis in 7, 12-dimethylbenz (a) anthracene-induced mammary carcinomas in Sprague-Dawley rats fed primrose oil, menhaden oil or corn oil diet. Lipids, 23, 948-954.

Agochiya, M., Brunton, V. G., Owens, D. W., Parkinson, E. K., Paraskeva, C., Keith, W. N., & Frame, M. C. (1999). Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. Oncogene, 18, 5646-5653.

Akiyama, S. K., Nagata, K., & Yamada, K. M. (1990). Cell surface receptors for extracellular matrix components. Biochimica et Biophysica Acta, 1031, 91-110.

Albelda, S. M. (1993). Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Laboratory Investigation, 68, 4-17.

Albelda, S. M., & Buck, C. A. (1990). Integrins and other cell adhesion molecules. Federation of American Societies for Experimental Biology Journal, 4, 2868-2880.

Albelda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovich, L., Herlyn, M., & Buck, C. A. (1990). Integrin distribution in malignant melanoma: Association of the beta 3 subunit with tumor progression. Cancer Research, 50, 6757-6764.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., & Watson, J. D. (1994). Cell signaling. In Molecular Biology of the Cell (pp. 721-786). New York: Garland.

Arita, H., Nakano, T., & Hanasaki, K. (1989). Thromboxane A2: Its generation and role in platelet activation. Progress in Lipid Research, 28, 273-301.

Armstrong, B., & Doll, R. (1975). Environmental factors and cancer incidence and mortality in different countries with special reference to dietary practices. International Journal of Cancer, 15, 617-621.

Armstrong, D. K., Kaufmann, S. H., Ottaviano, Y. L., Furuya, Y., Buckley, J. A., Isaacs, J. T., & Davidson, N. E. (1994). Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells. Cancer Research, 54, 5280-5283.

Badawi, A. F., El-Sohemy, A., Stephen, L. L., Ghoshal, A. K., & Archer, M. C. (1998). The effect of dietary n-3 and n-6 polyunsaturated fatty acids on the expression of cyclooxygenase 1 and 2 and levels of $p21^{ras}$ in rat mammary glands. Carcinogenesis, 19, 903-91.

Bandyopadhyay, G. K., Hwang, S.-I., Imagawa, W., & Nandi, S. (1993). Role of polyunsaturated fatty acids as signal transducers: Amplification of signals from growth factor receptors by fatty acids in mammary epithelial cells. Prostaglandins, Leukotrienes and Essential Fatty Acids, 48, 71-78.

Bandyopadhyay, G. K., Imagawa, W., & Nandi, S. (1995). Role of GTP-binding proteins in the polyunsaturated fatty acid stimulated proliferation of mouse mammary epithelial cells. Prostaglandins Leukotrienes and Essential Fatty Acids, 52, 151-158.

Basu, A. (1993). The potential of protein kinase C as a target for anticancer treatment. Pharmacology and Therapeutics, 59, 257-280.

Bates, E. J., Ferrante, A., Harvey, D. P., & Poulos, A. (1993). Polyunsaturated fatty acids increase neutrophil adherence and integrin expression. Journal of Leukocyte Biology, 53, 420-426.

Bégin, M. E., Ells, G., & Horrobin, D. F. (1988). Polyunsaturated fatty acid-induced cytotoxicity against tumor cells and its relationship to lipid peroxidation. Journal of the National Cancer Institute, 80, 188-194.

Bennett, J. S., Hoxie, J. A., Leitman, S. F., Vilaire, G., & Cines, D. B. (1983). Inhibition of fibrinogen binding to stimulated human platelets by a monoclonal antibody. Proceedings of the National Academy of Sciences, USA, 80, 2417-2421.

Bernstein, L., & Ross, R. K. (1993). Endogenous hormones and breast cancer risk. Epidemiology Reviews, 15, 48-65.

Bézard, J., Blond, J. P., Bernard, A., & Clouet, P. (1994). The metabolism and availability of essential fatty acids in animal and human tissues. Reproduction, Nutrition, Development, 34, 539-568.

Birt, D. F. (1986). Dietary fat and experimental carcinogenesis: A summary of recent in vivo studies. Advances in Experimental Medicine and Biology, 206, 69-83.

Blobe, G. C., Khan, W. A., & Hannun, Y. A. (1995). Protein kinase C: Cellular target of the second messenger arachidonic acid? Prostaglandins, Leukotrienes and Essential Fatty Acids, 52, 129-135.

Blobe, G. C., Obeid, L. M., & Hannun, Y. A. (1994). Regulation of protein kinase C and role in cancer biology. Cancer and Metastasis Reviews, 13, 411-431.

Boissonneault, G. A., Elson, C. E., & Pariza, M. W. (1986). Net energy effects of dietary fat on chemically induced mammary carcinogenesis in F344 rats. Journal of the National Cancer Institute, 76, 335-338.

Borner, C., Filipuzzi, I., Wartmann, M., Eppenberger, U., & Fabbro, D. (1989). Biosynthesis and posttranslational modifications of protein kinase C in human breast cancer cells. The Journal of Biological Chemistry, 264, 13902-13909.

Bosman, F. T. (1994). The borderline: Basement membranes and the transition from premalignant to malignant neoplasia. Microscopy Research and Technique, 28, 216-225.

Boyd, N. F., Martin, L. J., Noffel, M., Lockwood, G. A., & Tritchler, D. L. (1993). A meta-analysis of studies of dietary fat and breast cancer risk. British Journal of Cancer, 68, 627-636.

Braden, L. M., & Carroll, K. K. (1986). Dietary polyunsaturated fat in relation to mammary carcinogenesis in rats. Lipids, 21, 285-288.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Bradlow, H. L., & Fishman, J. (1993). Diet and cancer. Nature, 361, 360.

Brenner, R. R. (1971). The desaturation step in the animal biosynthesis of polyunsaturated fatty acids. Lipids, 6, 567-575.

Brunton, V. G., Ozanne, B. W., Paraskeva, C., & Frame, M. C. (1997). A role for epidermal growth factor receptor, c-Src and focal adhesion kinase in an in vitro model for the progression of colon cancer. Oncogene, 14, 283-293.

Buckley, B. J., & Whorton, A. R. (1995). Arachidonic acid stimulates protein tyrosine phosphorylation in vascular cells. American Journal of Physiology, 269, C1489-C1495.

Buell, P. (1973). Changing incidence of breast cancer in Japanese-American women. Journal of the National Cancer Institute, 51, 1479-1483.

Burgering, B. M. (1993). cAMP antagonizes p21 ras-mediated activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide exchange factor. European Molecular Biology Organization Journal, 12, 4211-4220.

Burridge, K., Fath, K., Nuckolls, G., & Turner, C. (1988). Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. Annual Reviews in Cell Biology, 4, 487-525.

Burridge, K., Turner, C. E., & Romer, L. H. (1992). Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: A role in cytoskeletal assembly. Journal of Cell Biology, 119, 893-903.

Cantrill, R. C., Ells, G. W., DeMarco, A. C., & Horrobin, D. F. (1997). Mechanisms of the selective cytotoxic actions of certain essential fatty acids. Advances in Experimental Medicine and Biology, 400A, 539-544.

Capodici, C., Pillinger, M. H., Han, G., Philips, M. R., & Weissman, G. (1998). Integrin-dependent homotypic adhesion of neutrophils. Journal of Clinical Investigation, 102, 165-175.

Carroll, K. K. (1975). Experimental evidence of dietary factors and hormone-dependent cancers. Cancer Research, 35, 3374-3383.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., & Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. Journal of Biological Chemistry, 257, 7847-7851.

Cave, W. T., Jr. (1991). Dietary n-3 (ω-3) polyunsaturated fatty acid effects on animal tumorigenesis. Federation of American Societies for Experimental Biology Journal, 5, 2160-2166.

Cheresh, D. A., Smith, J. W., & Cooper, H. M. (1989). A novel vitronectin receptor integrin (αvβx) is responsible for distinct adhesive properties of carcinoma cells. Cell, 57, 59-69.

Chiang, H. S., Yang, R. S., & Huang, T. F. (1996). Thrombin enhances the adhesion and migration of human colon adenocarcinoma cells via increased β3 integrin expression on the tumour cell surface and their inhibition by the snake venom peptide rhodostomin. British Journal of Cancer, 73, 902-908.

Cho-Chung, Y. S. (1990). Role of cyclic AMP receptor proteins in growth, differentiation and suppression of malignancy: New approaches to therapy. Cancer Research, 50, 7093-7100.

Cho-Chung, Y. S. (1996). Protein kinase A-directed antisense restrains cancer growth: Sequence-specific inhibition of gene expression. Antisense Nucleic Acid Drug Development, 6, 237-244.

Cho-Chung, Y. S., & Clair, T. (1993). The regulatory subunit of cAMP-dependent protein kinase as a target for chemotherapy of cancer and other cellular dysfunctional-related diseases. Pharmacology & Therapeutics, 60, 265-288.

Cho-Chung, Y. S., Pepe, S., Clair, T., Budillon, A., & Nesterova, M. (1995). cAMP-dependent protein kinase: Role in normal and malignant growth. Critical Reviews in Oncology-Hematology, 21, 33-61.

Chow, S. C., Sisfontes, L., Björkhem, I., & Jondal, M. (1989). Suppression of growth in a leukemic T cell line by n-3 and n-6 polyunsaturated fatty acids. Lipids, 24, 700-704.

Christiansen, E. N., Lund, J. S., Rørtveit, T., & Rustan, A. C. (1991). Effect of dietary n-3 and n-6 fatty acids on fatty acid desaturation in rat liver. Biochimica et Biophysica Acta, 1082, 57-62.

Chrysogelos, S. A., & Dickson, R. B. (1994). EGF receptor expression, regulation, and function in breast cancer. Breast Cancer Research and Treatment, 29, 29-40.

Ciardiello, F., Pepe, S., Bianco, C., Baldassarre, G., Ruggiero, A., Bianco, C., Selvam, M. P., Bianco, A. R., & Tortora, G. (1993). Down-regulation of RI α subunit of cAMP-dependent protein kinase induces growth inhibition of human mammary epithelial cells transformed by c-Ha-ras and c-erbB-2 proto-oncogenes. International Journal of Cancer, 53, 438-443.

Clarke, E. A., & Brugge, J. S. (1995). Integrins and signal transduction pathways: The road taken. Science, 268, 233-239.

Cobb, B. S., Schaller, M. D., Leu, T.-H., & Parsons, J. T. (1994). Stable association of $pp60^{src}$ and $pp59f^{yn}$ with focal adhesion-associated protein tyrosine kinase, $pp125^{FAK}$. Molecular and Cellular Biology, 14, 147-155.

Collie-Duguid, E. S. R., & Wahle, K. W. J. (1996). Inhibitory effect of fish oil N-3 polyunsaturated fatty acids on the expression of endothelial cell adhesion molecules. Biochemical and Biophysical Research Communications, 220, 969-974.

Connolly, J. M., & Rose, D. P. (1993). Effects of fatty acids on invasion through reconstituted basement membrane (Matrigel) by a human breast cancer cell line. Cancer Letters, 75, 137-142.

Coopman, P. J., Thomas, D. M., Gehlsen, K. R., & Mueller, S. C. (1996). Integrin $\alpha 3\beta 1$ participates in the phagocytosis of extracellular matrix molecules by human breast cancer cells. Molecular Biology of the Cell, 7, 1789-1804.

Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., & McCarthy, D. (1978). Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3'5'-monophosphate-dependent protein kinase. Journal of Biological Chemistry, 253, 3997-4003.

Cuzick, J. (1992). Methodologic issues in the chemoprevention of breast cancer. Cancer Detection and Prevention, 16, 81-85.

Danker, K., Gabriel, B., Heidrich, C., & Reutter, W. (1998). Focal adhesion kinase pp125^{FAK} and the β1 integrin subunit are constitutively complexed in HaCaT cells. Experimental Cell Research, 239, 326-331.

Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., & Skriver, L. (1985). Plasminogen activators, tissue degradation, and cancer. Advances in Cancer Research, 44, 139-266.

Davidson, N. E., Gelmann, E. P., Lippman, M. E., & Dickson, R. B. (1987). Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Molecular Endocrinology, 1, 216-223.

DeAntueno, R. J., Cantrill, R. C., Ells, G. W., Elliot, M., Huang, Y.-S., & Horrobin, D. F. (1993). Metabolism of radiolabelled 18:2n-6 and 18:3n-6 by NIH-3T3 cells and the DT subclone. Anticancer Research, 13, 973-978.

DeCaterina, R., Cybulsky, M. A., Clinton, S. K., Gimbrone, M. A., Jr., & Libby, P. (1995). Omega-3 fatty acids and endothelial leukocyte adhesion molecules. Prostaglandins, Leukotrienes and Essential Fatty Acids, 52, 191-195.

DeCaterina, R., & Libby, P. (1996). Control of endothelial leukocyte adhesion molecules by fatty acids. Lipids, 31, S57-S63.

Dekker, L. V., & Parker, P. J. (1994). Protein kinase C-- a question of specificity. Trends in Biochemical Science, 19, 73-77.

Deryugina, E. I., Luo, G. X., Reisfeld, R. A., Bourdon, M. A., & Strongin, A. (1997). Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2. Anticancer Research, 17, 3201-3210.

Dole, V. P. (1956). A relation between non-esterified fatty acids in plasma and the metabolism of glucose. Journal of Clinical Investigation, 35, 150-154.

Dong, X.- F., Berthois, Y., & Martin, P. M. (1991). Effect of epidermal growth factor on the proliferation of human epithelial cancer cell lines: Correlation with the level of occupied EGF receptor. Anticancer Research, 11, 737-744.

Doolan, M., & Keenan, A. K. (1994). Inhibition by fatty acids of cyclic AMP-dependent protein kinase activity in brush border membranes isolated from human placental vesicles. Journal of Pharmacology, 111, 509-514.

Dumont, J. A., & Bitonti, A. J. (1994). Modulation of human melanoma cell metastasis and adhesion may involve integrin phosphorylation mediated through protein kinase C. Biochemical and Biophysical Research Communications, 204, 264-272.

Earp, H. S., Dawson, T. L., Li, X., & Yu, H. (1995). Heterodimerization and functional interaction between EGF receptor family members: A new signaling paradigm with implications for breast cancer research. Breast Cancer Research and Treatment, 35, 115-132.

Edelman, G. M., & Crossin, K. L. (1991). Cell adhesion molecules: Implications for a molecular histology. Annual Review of Biochemistry, 60, 155-190.

Elias, P. M., & Brown, B. E. (1978). Defective barrier function in essential fatty acid deficiency correlates with abnormal intercellular lipid deposition. Laboratory Investigation, 39, 574-583.

Elices, M. J., Osborn, L., & Takada, Y. (1990). VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell, 60, 577-584.

Eling, T. E., & Glasgow, W. C. (1994). Cellular proliferation and lipid metabolism: Importance of lipoxygenases in modulating epidermal growth factor-dependent mitogenesis. Cancer and Metastasis Reviews, 13, 397-410.

Epand, R. M. (1994). In vitro assays of protein kinase C activity. Analytical Biochemistry, 218, 241-247.

Eynard, A. R., Manzur, T., Mogano, A., Quiroga, P. L., Muñoz, S. E., & Silva, R. A. (1997). Dietary deficiency or enrichment on essential fatty acids modulates tumorigenesis on the whole body cobalt 60-irradiated mice. Prostaglandins, Leukotrienes and Essential Fatty Acids, 56, 239-244.

Fabbro, D., Regazzi, R., Costa, S., Borner, C., & Eppenberger, U. (1986). Protein kinase C desensitization by phorbol esters and its impact on growth of human breast cancer cells. Biochemical and Biophysical Research Communications, 135, 65-73.

Falconer, J. S., Ross, J. A., Fearon, K. C., Hawkins, R. A., O'Riordain, M. G., & Carter, D. C. (1994). Effect of eicosapentaenoic acid and other fatty acids on the growth in vitro of human pancreatic cancer cell lines. British Journal of Cancer, 69, 826-832.

Faull, R. J., & Ginsberg, M. H. (1996). Inside-out signaling through integrins. Journal of the American Society of Nephrology, 7, 1091-1097.

Feldman, L. E., Shin, K. C., Natale, R. B., & Todd, R. F., III. (1991). β1 integrin expression on human small cell lung cancer cells. Cancer Research, 51, 1065-1070.

Ferretti, A., & Flanagan, V. P. (1990). Modification of prostaglandin metabolism in vivo by long-chain omega-3 polyunsaturates. Biochimica et Biophysica Acta, 1045, 299-301.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Medicine, 1, 27-31.

Ford-Hutchinson, A. W., Bray, M. A., Doig, M. V., Shipley, M. E., & Smith, M. J. H. (1980). Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature, 286, 264-265.

Fox, S. B., Turner, G. D., Gatter, K. C., & Harris, A. L. (1995). The increased expression of adhesion molecules ICAM-3, E- and P-selectins on breast cancer endothelium. Journal of Pathology, 177, 369-376.

Frandsen, T. L., Boysen, B. E., Jirus, S., Zwiebel, J., Spang-Thomsen, M., Thompson, E. W., & Brünner, N. (1992). Assays for the study of human cancer cell invasion and metastasis. Fibrinolysis, 6, 71-76.

Frayn, K. N. (1998). Non-esterified fatty acid metabolism and postprandial lipaemia. Atherosclerosis, 141 (Suppl. 1), S41-S46.

Frederickson, D. S., & Gordon, R. S., Jr.(1958). The metabolism of albumin-bound ^{14}C-labeled unesterified fatty acids in normal human subjects. Journal of Clinical Investigation, 37, 1504-1515.

Freedman, L. S., Clifford, C., & Messina, M. (1990). Analysis of dietary fat, calories, body weight, and the development of mammary tumors in rat and mice: A review. Cancer Research, 50, 5710-5719.

Frixen, U. H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D., & Birchmeier, W. (1991). E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. Journal of Cell Biology, 113, 173-185.

Fry, D. W. (1999). Inhibition of the epidermal growth factor receptor family of tyrosine kinases as an approach to cancer chemotherapy: Progression from reversible to irreversible inhibitors. Pharmacology & Therapeutics, 82, 207-218.

Fujii, K., Nakajima, N. D., & Imamura, S. (1995). Epidermal growth factor enhancement of HSC-1 human cutaneous squamous carcinoma cell adhesion and migration on type I collagen involves selective up-regulation of $\alpha 2\beta 1$ integrin expression. Experimental Cell Research, 216, 261-272.

Gavino, V. C., Miller, J. S., Ikharebha, S. O., Milo, G. E., & Cornwell, D. G. (1981). Effect of polyunsaturated fatty acids and antioxidants on lipid peroxidation in tissue cultures. Journal of Lipid Research, 22, 763-769.

German, N. S., & Johanning, G. L. (1997). Eicosapentaenoic acid and epidermal growth factor modulation of human breast cancer cell adhesion. Cancer Letters, 118, 95-100.

Gimond, C., DeMelker, A., Aumailley, M., & Sonnenberg, A. (1995). The cytoplasmic domain of α6A integrin subunit is an in vitro substrate for protein kinase C. Experimental Cell Research, 216, 232-235.

Ginsberg, M. H., Du, X., & Plow, E. F. (1992). Inside-out integrin signalling. Current Opinion in Cell Biology, 4, 766-771.

Godley, P. A. (1995). Essential fatty acid consumption and risk of breast cancer. Breast Cancer Research and Treatment, 35, 91-95.

Gonzalez, M. J., Schemmel, R. A., Dugan, L., Jr., Gray, J. I., & Welsch, C. W. (1993). Dietary fish oil inhibits human breast carcinoma growth: A function of increased lipid peroxidation. Lipids, 28, 827-832.

Gonzalez, M. J., Schemmel, R. A., Gray, J. I., Dugan, L., Jr., Sheffield, L. G., & Welsch, C. W. (1991). Effect of dietary fat on growth of MCF-7 and MDA-MB-231 human breast carcinomas in athymic nude mice: Relationship between carcinoma growth and lipid peroxidation product levels. Carcinogenesis, 12, 1231-1235.

Goodman, D. S. (1958). The interaction of human serum albumin with long-chain fatty acid anions. Journal of the American Chemical Society, 80, 3892-3898.

Gopalakrishna, R., & Barsky, S. H. (1988). Tumor promoter-induced membrane-bound protein kinase C regulates hematogenous metastasis. Proceedings of the National Academy of Sciences, 85, 612-616.

Gordge, P. C., Hulme, M. J., Clegg, R. A., & Miller, W. R. (1996). Elevation of protein kinase A and protein kinase C activities in malignant as compared with normal human breast tissue. European Journal of Cancer, 32A, 2120-2126.

Gordon, R. S., Jr., & Cherkes, A. (1956). Unesterified fatty acid in human blood plasma. Journal of Clinical Investigation, 35, 206-212.

Gordon, R. S., Jr., & Cherkes, A. (1958). Production of unesterified fatty acids from isolated rat adipose tissue incubated in vitro. Proceedings of the Society for Experimental Biology and Medicine, 97, 150-151.

Grammatikos, S. I., Subbaiah, P. V., Victor, T. A., & Miller, W. M. (1994). n-3 and n-6 fatty acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial cell lines. British Journal of Cancer, 70, 219-227.

Gui, G. P., Puddefoot, J. R., Vinson, G. P., Wells, C. A., & Carpenter, R. (1995). In vitro regulation of human breast cancer cell adhesion and invasion via integrin receptors to the extracellular matrix. British Journal of Surgery, 82, 1192-1196.

Guriec, N., Marcellin, L., Gairard, B., Calderoli, H., Wilk, A., Renaud, R., Bergerat, J. P., & Oberling, F. (1996). E-cadherin mRNA expression in breast carcinomas correlates with overall and disease-free survival. Invasion and Metastasis, 16, 19-26.

Halvorson, M. J., & Coligan, J. E. (1995). Enhancement of VLA integrin receptor function on thymocytes by cAMP is dependent on the maturation stage of thymocytes. Journal of Immunology, 155, 4567-4574.

Hamada, J.-I., Nagayasu, H., Takayama, M., Kawano, T., Hosokawa, M., & Takeichi, N. (1995). Enhanced effect of epidermal growth factor on pulmonary metastasis and in vitro invasion of rat mammary carcinoma cells. Cancer Letters, 89, 161-167.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1986). Protein kinase C activation in mixed micelles: Mechanistic implications of phospholipid, diacylglycerol, and calcium interdependencies. Journal of Biological Chemistry, 261, 7184-7190.

Hart, I. R., & Saini, A. (1992). Biology of tumor metastasis. Lancet, 339, 1453-1457.

Hatta, K., Nose, A., Nagafuchi, A., & Takeichi, M. (1988). Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: Its identity in the cadherin gene family. The Journal of Cell Biology, 106, 873-881.

Hellberg, C. D., Eierman, D., Sjolander, A., & Anderson, T. (1995). The Ca^{2+} signaling capacity of the beta 2-integrin on HL60-granulocytic cells is abrogated following phosphorylation of its CD18-chain: Relation to impaired protein tyrosine phosphorylation. Experimental Cell Research, 217, 140-148.

Henderson, J. F., & LePage, G. A. (1959). The nutrition of tumors: A review. Cancer Research, 19, 887-902.

Herbert, J. M. (1993). Protein kinase C: A key factor in the regulation of tumor cell adhesion to the endothelium. Biochemical Pharmacology, 45, 527-537.

Hildebrand, J. D., Taylor, J. M., & Parsons, J. T. (1996). An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. Molecular and Cellular Biology, 16, 3169-3178.

Hirasawa, M., Shijubo, N., Uede, T., & Abe, S. (1994). Integrin expression and ability to adhere to extracellular matrix proteins and endothelial cells in human lung cancer lines. British Journal of Cancer, 70, 466-473.

Holian, O., & Nelson, R. (1992). Action of long-chain fatty acids on protein kinase C activity: Comparison of omega-6 and omega-3 fatty acids. Anticancer Research, 12, 975-980.

Holley, R. W., Baldwin, J. H., & Kiernan, J. A. (1974). Control of growth of a tumor cell by linoleic acid. Proceedings of the National Academy of Sciences, USA, 71, 3976-3978.

Holman, R. T. (1960). The ratio of trienoic:tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. Journal of Nutrition, 70, 405-410.

Holmes, M. D., Hunter, D. J., Colditz, G. A., Stampfer, M. J., Hankinson, S. E., Speizer, F. E., Rosner, B., & Willett, W. C. (1999). Association of dietary intake of fat and fatty acids with risk of breast cancer. Journal of the American Medical Association, 281, 914-920.

Howe, G. R., Hirohata, T., Hislop, T. G., Isovich, J. M., Yuan, J.-M., Katsouyanni, K., Lubin, F., Marubini, E., Modan, B., Rohan, T., Toniolo, P., & Shunzhang, Y. (1990). Dietary factors and risk of breast cancer: Combined analysis of 12 case-control studies. The Journal of the National Cancer Institute, 82, 561-569.

Hubbard, N. E., Chapkin, R. S., & Erickson, K. L. (1988). Inhibition of growth and linoleate-enhanced metastasis of a transplantable mouse mammary tumor by indomethacin. Cancer Letters, 43, 111-120.

Hubbard, N. E., & Erickson, K. L. (1987). Enhancement of metastasis from a transplantable mouse mammary tumor by dietary linoleic acid. Cancer Research, 47, 6171-6175.

Hubbard, N. E., & Erickson, K. L. (1989). Effect of dietary linoleic acid level on lodgement, proliferation and survival of mammary tumor metastases. Cancer Letters, 44, 117-125.

Hunkapiller, T., & Hood, L. (1989). Diversity of the immunoglobulin gene superfamily. Advances in Immunology, 44, 1-63.

Hunter, D. J., Spiegelman, D., Adami, H.-O., Beeson, L., Van Den Brandt, P. A., Folsom, A. R., Fraser, G. E., Goldbohm, R. A., Graham, S., Howe, G. R., Kushi, L. H., Marshall, J. R., McDermott, A., Miller, A. B., Speizer, F. E., Wolk, A., Yaun, S.-S., & Willett, W. (1996). Cohort studies of fat intake and the risk of breast cancer--A pooled analysis. The New England Journal of Medicine, 334, 356-361.

Hunter, D. J., & Willett, W. C. (1996). Nutrition and breast cancer. Cancer Causes and Control, 7, 56-68.

Hursting, S. D., Thomquist, M., & Henderson, M. M. (1990). Types of dietary fat and the incidence of cancer at five sites. Preventive Medicine, 19, 242-253.

Hussey, H. J., & Tisdale, M. J., (1994). Effect of polyunsaturated fatty acids on the growth of murine colon adenocarcinomas in vitro and in vivo. British Journal of Cancer, 70, 6-10.

Hynes, R. O. (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. Cell, 69, 11-25.

Ip, C. (1993). Controversial issues of dietary fat and experimental mammary carcinogenesis. Prevention and Medicine, 22, 728-737.

Ip, C., Singh, M., Thompson, H. J., & Scimeca, J. A. (1994). Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Research, 54, 1212-1215.

Jardines, L., Weiss, M., Fowble, B., & Greene, M. (1993). neu (c-erbB-2/HER2) and the epidermal growth factor receptor (EGFR) in breast cancer. Pathobiology, 61, 268-282.

Jiang, W. G., Hiscox, S., Hallett, M. B., Horrobin, D. F., Mansel, R. E., & Puntis, M. C. A. (1995). Regulation of the expression of E-cadherin on human cancer cells by γ-linolenic acid (GLA). Cancer Research, 55, 5043-5048.

Johanning, G. L., & Lin T.-Y. (1995). Unsaturated fatty acid effects on human breast cancer cell adhesion. Nutrition and Cancer, 24, 57-66.

Jones, J. L., Royall, J. E., & Walker, R. A. (1996). E-cadherin relates to EGFR expression and lymph node metastasis in primary breast carcinoma. British Journal of Cancer, 74, 1237-1241.

Judson, P. L., He, X., Cance, W. G., & Le, L. V. (1999). Overexpression of focal adhesion kinase, a protein tyrosine kinase, in ovarian carcinoma. Cancer, 86, 1551-1556.

Jung, E.-Y., Ohshima, Y., Shintaku, N., Sumimoto, S.-I., Heike, T., Katamura, K., & Mayumi, M. (1994). Effects of cyclic AMP on expression of LFA-1, Mac-1, and VLA-4 and eosinophilic differentiation of a human leukemia cell line, EoL-1. European Journal of Haematology, 53, 156-162.

Kaizer, L., Boyd, N. F., Kriukov, V., & Tritchler, D. (1989). Fish consumption and breast cancer risk: An ecological study. Nutrition and Cancer, 12, 61-68.

Kanner, S. B., Grosmaire, L. S., Ledbetter, J. A., & Damle, N. K. (1993). Beta 2-integrin LFA-1 signaling through phospholipase C-gamma 1 activation. Proceedings of the National Academy of Sciences, USA, 90, 7099-7103.

Karmali, R. A., Adams, L., & Trout, J. R. (1993). Plant and marine n-3 fatty acids inhibit experimental metastasis of rat mammary adenocarcinoma cells. Prostaglandins, Leukotrienes and Essential Fatty Acids, 48, 309-314.

Karmali, R. A., Marsh, J., & Fuchs, C. (1984). Effect of omega-3 fatty acids on growth of a rat mammary tumor. Journal of the National Cancer Institute, 73, 457-461.

Katz, E. B., & Boylan, E. S. (1987). Stimulatory effect of a high polyunsaturated fat diet on lung metastasis from the 13762 mammary adenocarcinoma in female retired breeder rats. Journal of the National Cancer Institute, 79, 351-358.

Kennedy, M., Presligiacoma, L., Tyler, G., May, W., & Davidson, N. (1992). Differentiation effects of bryostatin 1 and phorbol ester on human breast cancer cell lines. Cancer Research, 52, 1278-1283.

Khan, W. A., Blobe, G. C., & Hannun, Y. A. (1992). Activation of protein kinase C by oleic acid. Determination and analysis of inhibition by detergent micelles and physiologic membranes: Requirement for free oleate. Journal of Biological Chemistry, 267, 3605-3612.

Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., & Juliano, R. L. (1992). Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. Journal of Biological Chemistry, 267, 23439-23442.

Kornberg, L. J. (1998). Focal adhesion kinase expression in oral cancers. Head and Neck, 20, 634-639.

Koukoulis, G. K., Virtanen, I., Korhonen, M., Laitinen, L., Quaranta, V., & Gould, V. E. (1991). Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. Correlations with their functions as receptors and cell adhesion molecules. American Journal of Pathology, 139, 787-799.

Kuhajda, F. P., Jenner, K., Wood, F. D., Randolph, A. H., Jacobs, L. B., Dick, J. D., & Pasternack, G. R. (1994). Fatty acid synthesis: A potential selective target for antineoplastic therapy. Journal of Biological Chemistry, 91, 6379-6383.

Lauri, D., Needham, L., Martin-Padura, I., & Dejana, E. (1991). Tumor cell adhesion to endothelial cells: Endothelial leukocyte adhesion molecule-1 as an inducible adhesive receptor for colon carcinoma cells. Journal of the National Cancer Institute, 83, 1321-1324.

Lee, C. S. L., deFazio, A., Ormandy, C. J., & Sutherland, R. L. (1996). Inverse regulation of oestrogen receptor and epidermal growth factor receptor gene expression in MCF-7 breast cancer cells treated with phorbol ester. Journal of Steroid Biochemistry and Molecular Biology, 58, 267-275.

Lee, S., Karaszkiewicz, J., & Anderson, W. (1992). Elevated level of nuclear protein kinase C in multidrug-resistant MCF-7 human breast carcinoma cells. Cancer Research, 52, 3750-3759.

Lichtner, R. B., Wiedemuth, M., Noeske-Jungblut, C., & Schirrmacher, V. (1993). Rapid effects of EGF on cytoskeletal structures and adhesive properties of highly metastatic rat mammary adenocarcinoma cells. Clinical and Experimental Metastasis, 11, 113-125.

Lin, C.-S., Zhang, K., & Kramer, R. (1993). α6 integrin is up-regulated in step increments accompanying neoplastic transformation and tumorigenic conversion of human fibroblasts. Cancer Research, 53, 2950-2953.

Linscheer, W. G., & Vergroesen, A. J. (1994). Lipids. In M. E. Shils, J. A. Olson, & M. Shike (Eds.), Modern nutrition in health and disease (pp. 47-88). Philadelphia: Lea & Febiger.

Liotta, L. A. (1992). Cancer cell invasion and metastasis. Scientific American, 266, 54-63.

Liotta, L. A., Steeg, P. S., & Stetler-Stevenson, W. G. (1991). Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell, 64, 327-336.

Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., & Brugge, J. B. (1992). Integrin-dependent phosphorylation and activation of the protein kinase pp125^{FAK} in platelets. Journal of Cell Biology, 119, 905-912.

Liu, B., Renaud, C., Nelson, K. K., Chen, Y. Q., Bazaz, R., Kowynia, J., Timar, J., Diglio, C A., & Honn, K. V. (1992). Protein kinase C inhibitor calphostin C reduces B16 amelanotic cell adhesion to endothelium and lung colonization. International Journal of Cancer, 52, 147-152.

Liu, B., Timar, J., Howlett, J., Diglio, C. A., & Honn, K. V. (1991). Lipoxygenase metabolites of arachidonic and linoleic acids modulate the adhesion of tumor cells to endothelium via regulation of protein kinase C. Cell Regulation, 2, 1045-1055.

Liu, J., Yeo, H. C., Doniger, S. J., & Ames, B. N. (1997). Assay of aldehydes from lipid peroxidation: Gas chromatography-mass spectrometry compared to thiobarbituric acid. Analytical Biochemistry, 245, 161-166.

Livingstone, M. B. E., Prentice, A. M., Strain, J. J., Coward, W. A., Black, A. E., Barker, M. E., McKenna, P. G., & Whitehead, R. G. (1990). Accuracy of weighed dietary records in studies of diet and health. British Medical Journal, 300, 708-712.

Long, B. J., & Rose, D. P. (1996). Invasive capacity and regulation of urokinase-type plasminogen activator in estrogen receptor (ER)-negative MDA-MB-231 human breast cancer cells, and a transfectant (S30) stably expressing ER. Cancer Letters, 99, 209-215.

Louw, L., Engelbrecht, A. M., & Cloete, F. (1998). Comparison of the fatty acid compositions in intraepithelial and infiltrating lesions of the cervix: Part I. Total fatty acid profiles. Prostaglandins, Leukotrienes and Essential Fatty Acids, 59, 247-251.

Lozano, Y., Taitz, A., Petruzzelli, G. J., Djordjevic, A., & Young, M. R. I. (1996). Prostaglandin E_2-protein kinase A signaling and protein phosphatases-1 and -2A regulate human head and neck squamous cell carcinoma motility, adherence, and cytoskeletal organization. Prostaglandins, 51, 35-48.

Lund, E., & Bonaa, K. H. (1993). Reduced breast cancer mortality among fisherman's wives. Cancer Causes and Control, 4, 283-287.

Maier, G. D., Vellody, K., Meisinger, J., Djordjevic, A., Lozano, Y., & Young, M. R. I. (1996). Protein kinase A regulates Lewis lung carcinoma adherence to extracellular matrix components and spontaneous metastasis. Clinical and Experimental Metastasis, 14, 314-322.

Marcel, Y. L., Christiansen, K., & Holman, R. T. (1968). The preferred metabolic pathway from linoleic acid to arachidonic acid in vitro. Biochimica et Biophysica Acta, 164, 25-34.

Marguerie, G. A., Plow, E. F., & Edgington, T. S. (1979). Human platelets possess an inducible and saturable receptor specific for fibrinogen. Journal of Biological Chemistry, 254, 5357-5363.

Martinez-Lacaci, I., & Dickson, R. B. (1996). Dual regulation of the epidermal growth factor family of growth factors in breast cancer by sex steroids and protein kinase C. Journal of Steroid Biochemistry and Molecular Biology, 57, 1-11.

McCarty, M. F. (1996). Fish oil may impede tumour angiogenesis and invasiveness by down-regulating protein kinase C and modulating eicosanoid production. Medical Hypotheses, 46, 107-115.

McEver, R. P. (1997). Selectin-carbohydrate interactions during inflammation and metastasis. Glycoconjugate Journal, 14, 585-591.

McGregor, B. C., McGregor, J. L., Weiss, L. M., Wood, G. S., Hu, C.-H., Boukerche, H., & Warnke, R. A. (1989). Presence of cytoadhesins (IIb- IIa- like glycoproteins) on human metastatic melanomas but not on benign melanocytes. American Journal of Clinical Pathology, 92, 495-499.

McKenzie, K. E., Bandyopadhyay, G. K., Imagawa, W., Sun, K., & Nandi, S. (1994). ω-3 and ω-6 fatty acids and PGE_2 stimulate the growth of normal but not tumor mouse mammary epithelial cells: Evidence for alterations in the signaling pathways in tumor cells. Prostaglandins, Leukotrienes and Essential Fatty Acids, 51, 437-443.

McPhail, L. C., Clayton, C. C., & Snyderman, R. (1984). A potential second messenger role for unsaturated fatty acids: Activation of Ca^{2+}-dependent protein kinase. Science, 224, 622-625.

Mechtersheimer, G., Barth, T., Quentmeier, A., & Moller, P. (1994). Differential expression of β1, β3, and β4 integrin subunits in nonneoplastic neural cells of the peripheral and autonomic nervous system and in tumors derived from these cells. Laboratory Investigation, 70, 740-752.

Miller, W. R., Watson, D. M. A., Jack, W., Chetty, U., & Elton, R. A. (1993). Tumor cyclic AMP binding proteins: An independent prognostic factor for disease recurrence and survival in breast cancer. Breast Cancer Research and Treatment, 26, 89-94.

Miyamoto-Tiaven, M. J., Hillyard, L. A., & Abraham, S. (1981). Influence of dietary fat on the growth of mammary ducts in BALB/c mice. Journal of the National Cancer Institute, 67, 179-188.

Moncada, S., & Vane, J. R. (1978). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. Pharmacological Reviews, 30, 293-331.

Mueller, H., Loop, P., Liu, R., Wosikowski, K., Kueng, W., & Eppenberger, U. (1994). Differential signal transduction of epidermal-growth-factor receptors in hormone-dependent and hormone-independent human breast cancer cells. European Journal of Biochemistry, 221, 631-637.

Murakami, K., Chan, S. Y., & Routtenberg, A. (1986). Protein kinase C activation by cis-fatty acid in the absence of Ca^{2+} and phospholipid. Journal of Biological Chemistry, 261, 15424-15429.

Murphy, M. G. (1990). Dietary factors and membrane protein function. Journal of Nutrition and Biochemistry, 1, 68-79.

Natarajan, R., Esworthy, R., Bai, W., Gu, J.-L., Wilczynski, S., & Nadler, J. (1997). Increased 12-lipoxygenase expression in breast cancer tissues and cells. Regulation by epidermal growth factor. Journal of Clinical Endocrinology and Metabolism, 82, 1790-1798.

Nesterova, M., & Cho-Chung, Y. S. (1995). A single-injection protein kinase A-directed antisense treatment to inhibit tumour growth. Nature Medicine, 1, 528-533.

Neuringer, M., Anderson, G. J., & Connor, W. E. (1988). The essentiality of n-3 fatty acids for the development and function of the retina and brain. Annual Review of Nutrition, 8, 517-541.

Nguyen, M., Corless, C. L., Kraling, B. M., Tran, C., Atha, T., Bischoff, J., & Barsky, S. H. (1997). Vascular expression of E-selectin is increased in estrogen-receptor-negative breast cancer: A role for tumor-cell-secreted interleukin-1α. American Journal of Pathology, 150, 1307-1314.

Nicolson, G. L. (1987). Tumor cell instability, diversification, and progression to the metastatic phenotype: From oncogene to oncofetal expression. Cancer Research, 47, 1473-1487.

Noguchi, M., Earashi, M., Minami, M., Kinoshita, K., & Miyazaki, I. (1995). Effects of eicosapentaenoic and docosahexaenoic acid on cell growth and prostaglandin E and leukotriene B production by a human breast cancer cell line (MDA-MB-231). Oncology, 52, 458-464.

Noguchi, M., Rose, D. P., Earashi, M., & Miyazaki, I. (1995). The role of fatty acids and eicosanoid synthesis inhibitors in breast carcinoma. Oncology, 52, 265-271.

Nose, A., Tsuji, K., & Takeichi, M. (1990). Localization of specificity determining sites in cadherin cell adhesion molecules. Cell, 61, 147-155.

O'Brian, C. A., Vogel, V. G., Singletary, S. E., & Ward, N. E. (1989). Elevated protein kinase C expression in human breast tumour biopsies relative to normal breast tissue. Cancer Research, 49, 3215-3217.

Owens, L. V., Xu, L., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T., & Cance, W. G. (1995). Overexpression of the focal adhesion kinase ($p125^{FAK}$) in invasive human tumors. Cancer Research, 55, 2752-2755.

Owens, L. V., Xu, L., Dent, G. A., Yang, X., Sturge, G. C., Craven, R. J., & Cance, W. G. (1996). Focal adhesion kinase as a marker of invasive potential in differentiated human thyroid cancer. Annals of Surgical Oncology, 3, 100-105.

Ozawa, M., Baribault, H., & Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structur-

ally related in different species. European Molecular Biology Organization Journal, 8, 1711-1717.

Padma, M., & Das, V. N. (1999). Effects of cis-unsaturated fatty acids on the activity of protein kinases and protein phosphorylation in macrophage tumor (AK-5) cells in vitro. Prostaglandins, Leukotrienes and Essential Fatty Acids, 60, 55-63.

Palmantier, R., Roberts, J. D., Glasgow, W. C., Eling, T. E., & Olden, K. (1996). Regulation of the adhesion of a human breast carcinoma cell line to type IV collagen and vitronectin: Roles for lipoxygenase and protein kinase C. Cancer Research, 56, 2206-2212.

Parsons, J. T., Schaller, M. D., Hildebrand, J., Leu, T. H., Richardson, A., & Otey, C. (1994). Focal adhesion kinase: Structure and signalling. Journal of Cell Science, 18, 109-113.

Perera, F. P. (1997). Environment and cancer: Who are susceptible? Science, 278, 1068-1073.

Pignatelli, M., Hanby, A. M., & Stamp, G. W. H. (1991). Low expression of β1, α2 and α3 subunits of VLA integrins in malignant mammary tumors. Journal of Pathology, 165, 25-32.

Pike, M. C., Spicer, D. V., Dahmoush, L., & Press, M. F. (1993). Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiology Reviews, 15, 17-35.

Piomelli, D. (1996). Biosynthesis, storage and mobilization of arachidonic acid. In Arachidonic acid in cell signaling (pp. 15-20). Austin, TX: R. G. Landes.

Prentice, R. L., Kakar, F., Hursting, S., Sheppard, L., Klein, R., & Kushi, L. H. (1988). Aspects of the rationale for the women's health trial. Journal of the National Cancer Institute, 80, 802-814.

Prentice, R. L., & Sheppard, L. (1990). Dietary fat and cancer: Consistency, of the epidemiologic data, and disease prevention that may follow from a practical reduction in fat consumption. Cancer Causes and Control, 1, 81-97.

Price, J. E., Polyzos, A., Zhang, R. D., & Daniels, L. M. (1990). Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Research, 50, 717-721.

Price, S. A., & Tisdale, M. J. (1998). Mechanism of inhibition of a tumor lipid-mobilizing factor by eicosapentaenoic acid. Cancer Research, 58, 4827-4831.

Rabinovitz, I., Toker, A., & Mercurio, A. M. (1999). Protein kinase C-dependent mobilization of the α6β4 integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. The Journal of Cell Biology, 146, 1147-1159.

Reich, R., Royce, L., & Martin, G. R. (1989). Eicosapentaenoic acid reduces the invasive and metastatic activities of malignant tumor cells. Biochemical and Biophysical Research Communications, 160, 559-564.

Reiss, U., & Tappel, A. L. (1973). Fluorescent product formation and changes in structure of DNA reacted with peroxidizing arachidonic acid. Lipids, 8, 199-202.

Richardson, A., Shannon, J. D., Adams, R. B., Schaller, M. D., & Parsons, J. T. (1997). Identification of integrin-stimulated sites of serine phosphorylation in FRNK, the separately expressed C-terminal domain of focal adhesion kinase: A potential role for protein kinase A. Biochemical Journal, 324, 141-149.

Roberts, J. D., Klein, J.-L., Palmantier, R., Dhume, S. T., George, M. D., & Olden K. (1998). The role of protein glycosylation inhibitors in the prevention of metastasis and therapy of cancer. Cancer Detection and Prevention, 22, 455-462.

Rohde-Schulz, B., & Lichtner, R. B. (1995). EGF enhances attachment of metastatic rat mammary adenocarcinoma cell clone MTLn3 to fibronectin and collagen. Invasion and Metastasis, 15(1-2), 1-10.

Rose, D. P. (1997). Dietary fatty acids and prevention of hormone-responsive cancer. Proceedings of the Society for Experimental Biology and Medicine, 216, 224-233.

Rose, D. P., & Connolly, J. M. (1989). Stimulation of growth of human breast cancer cell lines in culture by linoleic acid. Biochemical and Biophysical Research Communications, 165, 277-283.

Rose, D. P., & Connolly, J. M. (1990). Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture. Cancer Research, 50, 7139-7144.

Rose, D. P., & Connolly, J. M. (1993). Effects of dietary omega-3 fatty acids on human breast cancer growth and metastases in nude mice. Journal of the National Cancer Institute, 85, 1743-1747.

Rose, D. P., Connolly, J. M., & Liu, X.-H. (1994a). Dietary fatty acids and human breast cancer cell growth, invasion, and metastasis. In Diet and Breast Cancer (pp. 83-91). New York: Plenum Press.

Rose, D. P., Connolly, J. M., & Liu, X.-H. (1994b). Effects of linoleic acid on the growth and metastasis of two human breast cancer cell lines in nude mice and the invasive capacity of these cell lines in vitro. Cancer Research, 54, 6557-6562.

Rose, D. P., Connolly, J. M., Rayburn, J., & Coleman, M. (1995). Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. Journal of the National Cancer Institute, 87, 587-592.

Rose, D. P., & Hatala, M. A. (1994). Dietary fatty acids and breast cancer invasion and metastasis. Nutrition and Cancer, 21, 103-111.

Rose, D. P., Hatala, M. A., Connolly, J. M., & Rayburn, J. (1993). Effect of diets containing different levels of linoleic acid on human breast cancer growth and lung metastasis in nude mice. Cancer Research, 53, 4686-4690.

Rose, D. P., Rayburn, J., Hatala, M. A., & Connolly, J. M. (1994). Effects of dietary fish oil on fatty acids and eicosanoids in metastasizing human breast cancer cells. Nutrition and Cancer, 22, 131-141.

Rosen, S. D., & Bertozzi, C. R. (1994). The selectins and their ligands. Current Opinion in Cell Biology, 6, 663-673.

Roubal, W. T., & Tappel, A. L. (1966). Damage to proteins, enzymes, and amino acids by peroxidizing lipids. Archives of Biochemistry and Biophysics, 113, 5-8.

Ruoslahti, E., & Pierschbacher, M. D. (1987). New perspectives in cell adhesion: RGD and integrins. Science, 238, 491-497.

Samuelsson, B. (1981). Prostaglandins, thromboxanes, and leukotrienes: Formation and biological roles. Harvey Lectures, 75, 1-40.

Sanderson, P., & Calder, P. C. (1998). Dietary fish oil appears to prevent the activation of phospholipase Cγ in lymphocytes. Biochimica et Biophysica Acta, 1392, 300-308.

Sardesai, V. M. (1992). The essential fatty acids. Nutrition in Clinical Practice, 7, 179-186.

Sasaki, S., Horacsek, M., & Kesteloot, H. (1993). An ecological study of the relationship between dietary fat intake and breast cancer mortality. Preventive Medicine, 22, 187-202.

Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., & Parsons, J. T. (1992). pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proceedings of the National Academy of Sciences, USA, 89, 5192-5196.

Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, F. W., Vines, R. R., & Parsons, J. T. (1994). Autophosphorylation of the focal adhesion kinase, $pp125^{FAK}$, directs Sh2-dependent binding of $pp60^{src}$. Molecular and Cellular Biology, 14, 1680-1688.

Schaller, M. D., Otey, C. A., Hildebrand, J. D., & Parsons, J. T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. Journal of Cell Biology, 130, 1181-1187.

Schaller, M. D., & Parsons, J. T. (1994). Focal adhesion kinase and associated proteins. Current Opinions in Cell Biology, 6, 705-710.

Schlaepfer, D. D., Hanks, S. K., Hunter, T., & van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. Nature, 372, 786-791.

Schwartz, G. K., Redwood, S. M., Ohnuma, T., Holland, J. F., Droller, M. J., & Liu, B. C. (1990). Inhibition of invasion of invasive human bladder carcinoma cells by protein kinase C inhibitor staurosporine. Journal of the National Cancer Institute, 82, 1753-1756.

Schwartz, M. A., & Ingber, D. E. (1994). Integrating with integrins. Molecular Biology of the Cell, 5, 389-393.

Schwartz, M. A., Schaller, M. D., & Ginsberg, M. H. (1995). Integrins: Emerging paradigms of signal transduction. Annual Reviews in Cellular and Developmental Biology, 11, 549-599.

Seifert, R., Schachtele, C., Rosenthal, W., & Schultz, G. (1988). Activation of protein kinase C by cis- and trans-fatty acids and its potentiation by diacylglycerol. Biochemical and Biophysical Research Communications, 154, 20-26.

Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, U., & Nishizuka, Y. (1987). Three distinct forms of rat brain protein kinase C: Differential response to unsaturated fatty acids. Biochemical and Biophysical Research Communications, 145, 797-802.

Sheikh, M. S., Garcia, M., Pujol, P., Fontana, J. A., & Rochefort, H. (1994-1995). Why are estrogen-receptor-negative breast cancers more aggressive than the estrogen-receptor-positive breast cancers? Invasion and Metastasis, 14, 329-336.

Sheikh, M. S., Shao, Z.-M., Chen, J.-C., Li, X.-S., Hussain, A., & Fontana, J. A. (1994). Expression of estrogen receptors in estrogen receptor-negative human breast carcinoma cells: Modulation of epidermal growth factor-receptor (EGF-R) and transforming growth factor α (TGFα) gene expression. Journal of Cellular Biochemistry, 54, 289-298.

Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M., Shimosato, Y., Takeichi, M., & Abe, O. (1989). Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. Cancer Research, 49, 2128-2133.

Siitonen, S. M., Kononen, J. T., Helin, H. J., Rantala, I. S., Holli, K. A., & Isola, J. J. (1996). Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. American Journal of Clinical Pathology, 105, 394-402.

Sinclair, H. M. (1984). Essential fatty acids in perspective. Human Nutrition: Clinical Nutrition, 38C, 245-260.

Singh, J., Hamid, R., & Reddy, B. S. (1997). Dietary fat and colon cancer: Modulation of cyclooxygenase-2 by types and amount of dietary fat during the postinitiation stage of colon carcinogenesis. Cancer Research, 57, 3465-3470.

Singh, R. K., Hardy, R. W., Wang, M.-H., Williford, J., Gladson, C. L., McDonald, J. M., & Siegal, G. P. (1995). Stearate inhibits human tumor cell invasion. Invasion Metastasis, 15, 144-155.

Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., & Press, M. F. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science, 244, 707-712.

Spector, A. A. (1967). The importance of free fatty acid in tumor nutrition. Cancer Research, 27, 1580-1586.

Spector, A. A., & Burns, C. P. (1987). Biological and therapeutic potential of membrane lipid modification in tumors. Cancer Research, 47, 4529-4537.

Spector, A. A., & Steinberg, D. (1965). The utilization of unesterified palmitate by Ehrlich ascites tumor cells. Journal of Biological Chemistry, 240, 3747-3753.

Spector, A. A., & Steinberg, D. (1967). The effect of fatty acid structure on utilization by Ehrlich ascites tumor cells. Cancer Research, 27, 1587-1594.

Spector, A. A., Steinberg, D., & Tanaka, A. (1965). Uptake of free fatty acids by Ehrlich ascites tumor cells. Journal of Biological Chemistry, 240, 1032-1041.

Speizer, L. A., Watson, M. J., & Brunton, L. L. (1991). Differential effects of omega-3 fish oils on protein kinase activities in vitro. American Journal of Physiology, 261, E109-E114.

Sprecher, H. (1989). Interactions between the metabolism of n-3 and n-6 fatty acids. Journal of Internal Medicine, 225, 5-9.

Springer, T. A. (1990). Adhesion receptors of the immune system. Nature, 346, 425-434.

Srivastava, R. K., Srivastava, A. R., Park, Y. G., Agrawal, S., & Cho-Chung, Y. S. (1998). Antisense depletion of RIα subunit of protein kinase A induces apoptosis and growth arrest in human breast cancer cells. Breast Cancer Research and Treatment, 49, 97-107.

Srivastava, R. K., Srivastava, A. R., Seth, P., Agrawal, S., & Cho-Chung, Y. S. (1999). Growth arrest and induction of apoptosis in breast cancer cells by antisense depletion of protein kinase A- RIα subunit: p53-independent mechanism of action. Molecular and Cellular Biochemistry, 195, 25-36.

Starzec, A. B., Spanakis, E., Nehme, A., Salle, V., Veber, N., Mainguene, C., Planchon, P., Valette, A., Prevost, G., & Israel, L. (1994). Proliferative responses of epithelial cells to 8-bromo-cyclic AMP and to a phorbol ester change during breast pathogenesis. Journal of Cellular Physiology, 161, 31-38.

Stetler-Stevenson, W. G., Liotta, L. A., & Kleiner, D. E., Jr. (1993). Extracellular matrix 6: Role of matrix metalloproteinases in tumor invasion and metastasis. Federation of American Societies for Experimental Biology Journal, 7, 1434-1441.

Stracke, M. L., & Liotta, L. A. (1992). Multi-step cascade of tumor cell metastasis. In Vivo, 6, 309-316.

Strobl, J. S., Wonderlin, W. F., & Flynn, D. C. (1995). Mitogenic signal transduction in human breast cancer cells. General Pharmacology, 26, 1643-1649.

Stromblad, S., & Cheresh, D. A. (1996). Cell adhesion and angiogenesis. Trends in Cell Biology, 6, 462-467.

Sylvester, P. W., Birkenfeld, H. P., Hosick, H. L., & Briski, K. P. (1994). Fatty acid modulation of epidermal growth factor-induced mouse mammary epithelial cell proliferation in vitro. Experimental Cell Research, 214, 145-153.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science, 251, 1451-1455.

Tang, D. G., Grossi, I. M., Tang, K. Q., Diglio, C. A., & Honn, K. V. (1995). Inhibition of TPA and 12 (S)-HETE-stimulated tumor cell adhesion by prostacyclin and its stable analogs: Rationale for their antimetastatic effects. International Journal of Cancer, 60, 418-425.

Tang, D. G., & Honn, K. V. (1994-1995). Adhesion molecules and tumor metastasis: An update. Invasion Metastasis, 14, 109-122.

Tang, D. G., Tarrien, M., Dobrzynski, P., & Honn, K. V. (1995). Melanoma cell spreading on fibronectin induced by 12(S)-HETE involves both protein kinase C-and protein tyrosine kinase-dependent focal adhesion formation and tyrosine phosphorylation of focal adhesion kinase ($pp125^{FAK}$). Journal of Cellular Physiology, 165, 291-306.

Tappia, P. S., Man, W. J., & Grimble, R. F. (1995). Influence of unsaturated fatty acids on the production of tumour necrosis factor and interleukin-6 by rat peritoneal macrophages. Molecular and Cellular Biochemistry, 143, 89-98.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990). cAMP-dependent protein kinase: Framework for a diverse family of regulatory enzymes. Annual Reviews in Biochemistry, 59, 971-1005.

Thaler-Dao, H., Crastes de Paulet, A., & Paoletti, R. (1983). Icosanoids and tumor metastasis. In H. Thaler-Dao, A. Crastes de Paulet, & R. Paoletti (Eds.). Icosanoids and cancer (pp. 205-223). New York: Raven Press.

Timar, J., Chen, Y. Q., Liu, B., Bazaz, R., Taylor, J. D., & Honn, K. V. (1992). The lipoxygenase metabolite 12(S)-HETE promotes $\alpha IIb\beta 3$ integrin-mediated tumor cell spreading on fibronectin. International Journal of Cancer, 52, 594-603.

Tisdale, M. J., & Beck, S. A. (1991). Inhibition of tumour-induced lipolysis in vitro and cachexia and tumour growth in vivo by eicosapentaenoic acid. Biochemistry and Pharmacology, 41, 103-107.

Tortora, G., Damiano, V., Bianco, C., Baldassarre, G., & Bianco, A. R. (1997). The $RI\alpha$ subunit of protein kinase A (PKA) binds to Grb2 and allows PKA interaction with the activated EGF-receptor. Oncogene, 14, 923-928.

Tremblay, L., Hauck, W., Aprikian, A. G., Begin, L. R., Chapdelaine, A., & Chevalier, S. (1996). Focal adhesion kinase (pp125FAK) expression, activation and association with paxillin and p50csk in human metastatic prostate carcinoma. International Journal of Cancer, 68, 164-171.

Umbas, R., Isaacs, W. B., Bringuier, P. P., Schaafsma, H. E., Karthaus, H. F., Oosterhof, G. O., Debruyrie, F. M., & Schalken, J. A. (1994). Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Research, 54, 3929-3933.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature-- New Biology, 231, 232-235.

Van Noorden, C. J. F., Meade-Tollin, L. C., & Bosman, F. T. (1998). Metastasis. American Scientist, 86, 130-141.

Varki, A. (1994). Selectin ligands. Proceedings of the National Academy of Sciences, USA, 91, 7390-7397.

Veber, N., Prévost, G., Planchon, P., & Starzec, A. (1994). Evidence for a growth effect of epidermal growth factor on MDA-MB-231 breast cancer cells. European Journal of Cancer, 30A, 1352-1359.

Verkest, V., McArthur, M., & Hamilton, S. (1988). Fatty acid activation of protein kinase C: Dependence on diacylglycerol. Biochemical and Biophysical Research Communications, 152, 825-829.

Virtanen, I., Korhonen, M., Kariniemi, A. L., Gould, V. E., Laitinen, L., & Ylanne, J. (1990). Integrins in human cells and tumors. Cell Differentiation and Development, 32, 215-227.

Volk, T., & Geiger, B. (1986). A-CAM: A 135-kD receptor of intercellular adherens junctions. I. Immunoelectron microscopic localization and biochemical studies. The Journal of Cell Biology, 103, 1441-1450.

Vukicevic, S. (1992). Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. Experimental Cell Research, 202, 1-8.

Vuori, K., & Ruoslahti, E. (1993). Activation of protein kinase C precedes α5β1 integrin-mediated cell spreading on fibronectin. The Journal of Biological Chemistry, 268, 21459-21462.

Walcheck, B., Kahn, J., Fisher, J. M., Wang, B. B., Fisk, R. S., Payan, D. G., Feehan, C., Betageri, R., & Darlak, K. (1996). Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. Nature, 380, 720-723.

Wang, F., Nohara, K., Olivera, A., Thompson, E. W., & Spiegel, S. (1999). Involvement of focal adhesion kinase in inhibition of motility of human breast cancer cells by sphingosine 1-phophate. Experimental Cell Research, 247, 17-28.

Ways, D. K., Kukoly, C. A., deVente, J., Hooker, J. L., Bryant, W. O., Posekany, K. J., Fletcher, D. J., Cook, P. P., & Parker, P. J. (1995). MCF-7 breast cancer cells transfected with protein kinase C-α exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. Journal of Clinical Investigation, 95, 1906-1915.

Weber, W., & Hilz, H. (1986). cAMP-dependent protein kinases I and II: Divergent turnover of subunits. Biochemistry, 25, 5661-5667.

Weidner, N. (1995). Intratumor microvessel density as a prognostic factor in cancer. American Journal of Pathology, 147, 9-19.

Weiner, T. M., Liu, E. T., Craven, R. J., & Cance, W. G. (1993). Expression of focal adhesion kinase gene and invasive cancer. Lancet, 342, 1024-1025.

Weiss, L. (1994-1995). Cell adhesion molecules: A critical examination of their roles in metastasis. Invasion Metastasis, 14, 192-197.

Welsch, C. W. (1992). Relationship between dietary fat and experimental mammary tumorigenesis: A review and critique. Cancer Research, 52, (Suppl. 7), 2040S-2048S.

Welsch, C. W. (1994). Interrelationship between dietary lipids and calories and experimental mammary gland tumorigenesis. Cancer Supplement, 74, 1055-1062.

Welsch, C. W. (1995). Review of the effects of dietary fat on experimental mammary gland tumorigenesis: Role of lipid peroxidation. Free Radical Biology and Medicine, 18, 757-773.

Welsch, C. W., Oakley, C. S., Chang, C.-C., & Welsch, M. A. (1993). Suppression of growth by dietary fish oil of human breast carcinomas maintained in three different strains of immune-deficient mice. Nutrition and Cancer, 20, 119-127.

Wertz, P. W., Swartzendruber, D. C., Abraham, W., Madison, K. C., & Downing, D. T. (1987). Essential fatty acids and epidermal integrity. Archives in Dermatology, 123, 1381-1384.

Wickramasinghe, N. S., Jo, H., McDonald, J. M., & Hardy, R. W. (1996). Stearate inhibition of breast cancer cell proliferation. A mechanism involving epidermal growth factor receptor and G-proteins. American Journal of Pathology, 148, 987-995.

Willett, W. C., & Stampfer, M. J. (1990). Dietary fat and cancer: Another view. Cancer Causes and Control, 1, 103-109.

Williams, A. F., & Barclay, A. N. (1988). The immunoglobulin superfamily-domains for cell surface recognition. Annual Review of Immunology, 6, 381-405.

World Cancer Research Fund. (1997). Breast. In G. Cannon (Ed.), Food, nutrition and the prevention of cancer: A global perspective (pp. 252-287). Washington, DC: The American Institute for Cancer Research.

World Health Organization. (1997). Nutrition and prevention of chronic diseases. (Technical Report 797). Geneva:WHO.

Wu, A. H., Pike, M. C., & Stram, D. O. (1999). Meta-analysis: Dietary fat intake, serum estrogen levels, and the risk of breast cancer. Journal of the National Cancer Institute, 91, 529-534.

Wynder, E. L., Cohen, L. A., Muscat, J. E., Winters, B., Dwyer, J. T., & Blackburn, G. (1997). Breast cancer: Weighing the evidence for a promoting role of dietary fat. Journal of the National Cancer Institute, 89, 766-775.

Xie, H., Pallero, M. A., Gupta, K., Chang, P., Ware, M. F., Witke, W., Kwiatkowski, D. J., Lauffenburger, D. A., Murphy-Ullrich, J. E., & Wells, A. (1998). EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCγ signaling pathway. Journal of Cell Science, 111, 615-624.

Xing, Z., Chen, H.-C., Nowlen, J. K., Taylor, S. J., Shalloway, D., & Guan, J. L. (1994). Direct interaction of v-Src with the focal adhesion kinase mediated by the Src Sh2 domain. Molecular Biology of the Cell, 5, 413-421.

Young, M. R. I., Duffie, G. P., Lozano, Y., Young M. E., & Wright, M. A. (1990). Association of a functional prostaglandin E_2-protein kinase A coupling with responsiveness of metastatic Lewis lung carcinoma variants to prostaglandin E_2 and to prostaglandin E_2-producing nonmetastatic Lewis lung carcinoma variants. Cancer Research, 50, 2973-2978.

Young, M. R. I., Young, M. E., Lozano, Y., & Bagash, J. M. (1992). Activation of protein kinase A increases the in vitro invasion, migration, and adherence to reconstituted basement membrane by Lewis lung carcinoma tumor cells. Invasion and Metastasis, 12, 253-263.

Yudoh, K., Matsui, H., Kanamori, M., Maeda, A., Ohmori, K., & Tsuji, H. (1994). Effects of epidermal growth factor on invasiveness through the extracellular matrix in high- and low-metastatic clones of RCT sarcoma in vitro. Japanese Journal of Cancer Research, 85, 63-71.

Yuen, A. R., Halsey, J., Fisher, G. A., Holmlund, J. T., Geary, R. S., Kwoh, T. J., Dorr, A., & Sikic, B. I. (1999). Phase I study of an antisense oligonucleotide to protein kinase C-α (ISIS 3521/CGP 64128A) in patients with cancer. Clinical Cancer Research, 5, 3357-3363.

Zhang, M., Singh, R. K., Wang, M. H., Wells, A., & Siegal, G. P. (1996). Epidermal growth factor modulates cell attachment to hyaluronic acid by the cell surface glycoprotein CD44. Clinical and Experimental Metastasis, 14, 268-276.

Ziegler, R. G., Hoover, R. N., Pike, M. C., Hildesheim, A., Nomura, A. M. Y., West, D. W., Wu-Williams, A. H., Kolonel, L. N., Horn-Ross, P. L., Rosenthal, J. F., & Hyer, M. B. (1993). Migration patterns and breast cancer risk in Asian-American women. Journal of the National Cancer Institute, 85, 1819-1827.

Zutter, M. M., Mazoujian, G., & Santoro, S. A. (1990). Decreased expression of integrin adhesive protein receptors in adenocarcinoma of the breast. American Journal of Pathology, 137, 863-870.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

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