

# Cell Cycle Arrest and Apoptosis of Melanoma Cells by Docosahexaenoic Acid: Association with Decreased pRb Phosphorylation<sup>1</sup>

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## ABSTRACT

The incidence of cutaneous malignant melanoma is undergoing a dramatic increase in persons with light-color skin in all parts of the world. The prognosis for individuals with advanced disease is dismal due to the lack of effective treatment options. Thus, there is a need for new approaches to control tumor progression. Epidemiological, experimental, and mechanistic data implicate  $\omega$ -6 polyunsaturated fatty acids (PUFAs) as stimulators and long-chain  $\omega$ -3 PUFAs as inhibitors of development and progression of a range of human cancers, including melanoma. The aim of this study was to assess the mechanisms by which docosahexaenoic acid (DHA), an  $\omega$ -3 PUFA, affects human melanoma cells.

Exponentially growing melanoma cell lines were exposed *in vitro* to DHA and then assessed for (a) inhibition of cell growth; (b) expression of cyclins and cyclin-dependent kinase inhibitors in individual cells by flow cytometry and immunocytochemistry using specific monoclonal antibodies to cyclin D1, cyclin E, p21<sup>WAF1/CIP1</sup>, or p27<sup>KIP1</sup>; and (c) expression of total pRb<sup>T</sup> independent of phosphorylation state and hypophosphorylated pRb<sup>P-</sup> in fixed cells by flow cytometry and immunocytochemistry using specific monoclonal antibodies to pRb<sup>T</sup> or pRb<sup>P-</sup>, respectively. After treatment with increasing concentrations of DHA, cell growth in a majority of melanoma cell lines (7 of 12) was inhibited, whereas in 5 of 12 cell lines, cell growth was minimally affected. Two melanoma cell lines were examined in detail, one resistant (SK-Mel-29) and one sensitive (SK-Mel-110) to the inhibitory activity of DHA. SK-Mel-29 cells were unaffected by treatment with up to 2  $\mu$ g/ml DHA whether grown in the absence or presence of 1% fetal bovine serum (FBS). No appreciable change was observed in cell growth, cell cycle distribution, the status of pRb phosphorylation, cyclin D1 expression, or the levels of the cyclin-dependent kinase inhibitors p21 and p27. In contrast, SK-Mel-110 cell growth was inhibited by DHA with the cells accumulating either in G<sub>1</sub> or S phase: 0% in SK-Mel-29 versus 13.3 or 41.2% in SK-Mel-110 in the absence or presence of FBS, respectively. In the absence of serum, considerable death occurred by apoptosis. In addition, DHA treatment resulted in increasing numbers of SK-Mel-110 cells (from 12 to >40%) expressing hypophosphorylated pRb, whereas the levels of cyclin D1 and p21 changed little. Expression of p27 in these cells increased >2.5 times when grown in the absence of FBS but not in the presence of 1% FBS.

Thus, we show for the first time that DHA inhibits the growth of cultured metastatic melanoma cells. Furthermore, growth inhibition correlates with a quantitative increase in hypophosphorylated pRb in the representative sensitive melanoma cell line SK-Mel-110. Although multiple factors influence pRb phosphorylation, it appears that both cyclin D1 and p21 expression do not change in the presence of DHA, although p27 was strikingly increased in SK-Mel-110 cells in the absence of FBS. The fact that pRb became hypophosphorylated after exposure to DHA suggests a cross-talk mechanism between fatty acid metabolism and the pRb pathway. Determining the mechanism by which PUFAs can inhibit melanoma growth will be an important first step in the rational use of PUFAs as antitumor agents.

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## INTRODUCTION

Epidemiological studies predict that, given the present rate of increase, 1 in 75 Americans born today will develop cutaneous malignant melanoma sometime in their lifetime (1). Moreover, although early stage melanoma that is confined to the epidermis or superficial dermis is curable, the prognosis for individuals with deep invasion of the dermis and metastases is dismal, with a 5-year survival rate of only ~10%. This poor outcome is due to the lack of effective treatment options (2–4).

One potential and innovative adjuvant therapeutic modality is a dietary intervention designed to modify the relative consumption of the essential PUFAs<sup>3</sup> (3). There is a growing body of data indicating that dietary fat influences the development and progression of many cancers (5–8), including malignant melanoma (9–14), with the polyunsaturated  $\omega$ -6 fatty acids (n-6 PUFAs) exerting stimulatory effects (9, 10) and  $\omega$ -3 fatty acids (n-3 PUFAs) having suppressive activity (11). These epidemiological observations are supported by experimental studies, which demonstrated enhancing effects of high-fat diets on UV-induced skin carcinogenesis (15–18) and inhibition by feeding dietary n-3 PUFA (17).

Previously published studies have shown that n-6 PUFA stimulates the growth of human breast (19, 20) and prostate (21) cancer cell lines *in vitro*, whereas DHA and EPA, two long-chain n-3 PUFAs that are present at high concentrations in some fish oils, inhibit growth (19, 21–23). We report here that DHA inhibited the growth of >50% of human melanoma cell lines studied by us *in vitro*. Furthermore, in a representative sensitive melanoma cell line, this inhibition was accompanied by alterations in the phosphorylation status of the pRb gene product, the primary role of which is to control the commitment of cells to enter S phase (24–26). At present, little is known about the effects of fatty acids on cell cycle control. Thus, the present study provides information about potential mechanistic interactions of fatty acids with specific components of the cell cycle machinery.

## MATERIALS AND METHODS

**Melanoma Cell Lines.** Twelve human metastatic melanoma (SK-Mel) cultures were established as described previously (27, 28) and routinely passaged in DMEM (BioWhittaker, Walkersville, MD) containing 7.5% FBS (Gemini Bioproducts, Calabasas, CA), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). All cell lines were confirmed to be free of *Mycoplasma* before use.

**Calculation of Cell Doubling Times.** Cells were plated in DMEM containing 7.5% FBS at a density of 2000 cells per well of a 12-well tissue culture plate and incubated at 37°C. After 24 h, the cells were washed twice with PBS and refed with DMEM in the presence or absence of 1.0% FBS. Every 2 days thereafter, the cells were harvested by trypsinization and cell number was determined using a Coulter Counter (Coulter Electronics, Hialeah, FL). The cultures were refed every 3 days of the experiment. After 8 days of growth, the

<sup>3</sup> The abbreviations used are: PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; Rb, retinoblastoma; CKI, cyclin dependent kinase inhibitor; mAb, monoclonal antibody; EPA, eicosapentaenoic acid; CDK, cyclin dependent kinase; FBS, fetal bovine serum; DAPI, 4',6-diamidino-2-phenylindole.

total number of cell doublings and the doubling time of the cell populations were determined.

**DHA Treatment.** Cells were plated in 7.5% FBS-containing DMEM at a density of 2,000–10,000 cells per well of a 12-well plastic plate and incubated at 37°C. After 24 h, the cells were washed twice with PBS and treated with DHA (Sigma, St. Louis, MO) at concentrations of 0.5, 0.75, 1, 1.5, 3, and 5  $\mu\text{g/ml}$ . The DHA was dissolved in 100% ethanol, with a final concentration of 0.1% in each well; wells containing 0.1% ethanol but no DHA were included as solvent controls. These growth experiments were performed in DMEM containing DHA plus delipidized BSA (Collaborative Research, Lexington, MA), 1 mg/ml, and in the presence or absence of 1% FBS. At the designated time points (e.g., as shown in legends to Fig. 1 and Table 1), the cells were harvested by trypsinization, and cell number was determined using a Coulter Counter. The cultures were refed on day 3 of the experiment. Melanoma cell lines were assessed for growth inhibition and cell cycle perturbations in at least two independent experiments. Although maintenance of melanoma cells in medium supplemented with FBS, all experiments were performed using medium that was either FBS-free or supplemented with 1% FBS for reasons discussed in the text. No preadaptation of cells was necessary for sustained growth in either of these serum conditions, predominantly due to the fact that most melanoma cells in culture produce a variety of autocrine growth factors. Melanoma cell growth, in terms of doubling times, does diminish in the absence of all serum as explained in "Results," but the cells can be maintained indefinitely in culture under these conditions.

**Statistics.** The data are presented as the mean values  $\pm$  SE. Comparisons were performed using a one-way ANOVA test, followed by a *t* test for statistical significance for which  $P < 0.05$  was accepted.

**Immunocytochemical Detection of Cyclins and CKIs.** Control cells and cells treated with DHA (at concentrations of 0, 0.5, and 2  $\mu\text{g/ml}$ ) as described above were harvested after 6 days of growth in 75-cm<sup>2</sup> flasks by trypsinization and fixed in ice-cold 80% ethanol for up to 24 h. They were then washed twice in PBS, suspended in 1 ml of 0.25% Triton X-100 in PBS, kept on ice for 5 min, and centrifuged, and the pellet was suspended in 100  $\mu\text{l}$  of PBS containing 0.5  $\mu\text{g}$  of one of the following mAbs (all from PharMingen, San Diego, CA), anti-cyclin D1 (clone G124-326), anti-cyclin E (clone HE12), anti-p21<sup>WAF1/CIP1</sup> (clone SX118), or anti-p27<sup>KIP1</sup> (clone G173-524), and 1% BSA (Sigma) and incubated for 2 h at room temperature. The cells were then rinsed with PBS containing 1% BSA and incubated with FITC-conjugated goat antimouse IgG antibody (Molecular Probes, Eugene, OR) diluted 1:30 in 1% BSA-containing PBS for 30 min at room temperature in the dark. The cells were washed again, resuspended in 5  $\mu\text{g/ml}$  propidium iodide (Molecular Probes) and 0.1% RNase A (Sigma) in PBS, and incubated at room temperature for 20 min before measurement. Control cells were treated identically, except that instead of using the anti-cyclin or anti-CKI antibody, they were incubated with the appropriate isotypic antibody (IgG1, clone MOPC-21; PharMingen) at the same titer. Details of the detection of intracellular proteins by flow cytometry are presented elsewhere (29, 30).

**Immunocytochemical Detection of pRb<sup>T</sup> and pRb<sup>P-</sup>.** The procedure has been described in detail elsewhere (31). Briefly, after harvesting by trypsinization, the cells were fixed by suspension in 1% formaldehyde in PBS for 15 min on ice, washed with PBS, and resuspended in ice-cold 80% ethanol for up to 24 h. After fixation, the cells were washed twice with PBS and then suspended in 1 ml of 0.25% Triton X-100 in PBS on ice for 5 min. After centrifugation, the cell pellet was suspended in 100  $\mu\text{l}$  of PBS containing 1% BSA and 0.5  $\mu\text{g}$  of the anti-pRb<sup>T</sup> mAb (PharMingen; clone G3-245) conjugated with Cy-Chrome and/or with 0.5  $\mu\text{g}$  of anti-pRb<sup>P-</sup> mAb (PharMingen; clone G99-549) conjugated with FITC and incubated for 2 h at room temperature. The cells were then rinsed with PBS containing 1% BSA and counterstained with DAPI (Molecular Probes), and their fluorescence was measured by flow cytometry. The anti-pRb<sup>P-</sup> mAb used is a specific marker of hypophosphorylated pRb, whereas anti-pRb<sup>T</sup> mAb detects this protein independent of its state of phosphorylation (31–34). The interactions of pRb *in situ* with anti-pRb<sup>P-</sup> and anti-pRb<sup>T</sup> mAbs are not mutually exclusive; i.e., the cells that are stained with anti-pRb<sup>P-</sup> mAb also are reactive with anti-pRb<sup>T</sup> mAb. The respective epitopes (between amino acids 514 and 610 of human pRb for anti-pRb<sup>P-</sup> and 300 and 380 for anti-pRb<sup>T</sup>) do not spatially overlap; therefore, there is no steric hindrance to mAb binding (31). The epitope recognized by anti-pRb<sup>P-</sup> is located within the "A box" of the large T antigen binding pocket domain of human pRb. This antibody does not recognize the phosphorylation status of the

phosphorylation consensus *per se* but senses a specific structural conformation of hypophosphorylated pRb, at a site distant from the phosphorylation consensus (31). To demonstrate the specificity of the mAbs with respect to pRb phosphorylation, before the incubation with the mAbs the cells were preincubated with 4 units of alkaline phosphatase (type VII from bovine intestinal mucosa; Sigma) in 100  $\mu\text{l}$  of Tris buffer (Sigma) at pH 9.4 for 30 min. Binding of the control fluorochrome tagged isotypic mAb (IgG1, clone MOPC-21; PharMingen) was also determined as described (31, 35). The cells defined as reactive with anti-pRb<sup>P-</sup> mAb were those with fluorescence intensity that exceeded that of 99% of cells stained with an isotype control mAb.

**Analysis of Cellular Fluorescence.** Cellular fluorescence was determined with the Coulter ELITE ESP flow cytometer and cell sorter using either the argon ion laser (emission at 488 nm) or the argon ion laser combined with the helium-cadmium laser, emitting UV light. For analysis of DNA content *versus* expression of cyclins D1 and E, and CKIs p21<sup>WAF1</sup> and p27<sup>KIP1</sup>, fluorescence signals were collected using the standard configuration of the flow cytometer (green fluorescence for FITC and red fluorescence for propidium iodide). To determine the phosphorylation status of pRb, DNA content was analyzed based on DAPI fluorescence (blue emission) excited by UV light, whereas the anti-pRb<sup>P-</sup> mAb (FITC) and anti-pRb<sup>T</sup> (Cy-Chrome)-related emission was excited with blue laser light. Additional details of the multicolor measurement of cellular fluorescence and multivariate cell analysis are presented elsewhere (29–31, 35). It is noted that the type of multiparameter analysis used in this study allows a direct determination of the relative expression of various proteins as a function of cell cycle compartment (DNA content) and, therefore, precludes the need to synchronize cells, which has been shown to invariably lead to unbalanced growth and subsequent protein levels in excess of that present in unperturbed cultures (29).

## RESULTS

**Effect of DHA on Melanoma Cell Growth.** Twelve melanoma cell lines were exposed to DHA over a 0.5–5.0- $\mu\text{g/ml}$  concentration range and assessed for cell growth. The concentrations of DHA selected were those that, in a previous study of a human breast cancer cell line, were found to inhibit growth without inducing nonspecific cytotoxic effects due to the production of peroxidation products (19). Table 1 summarizes these data and shows that in 7 of 12 cases (SK-Mel-13, -17, -28, -110, -133, and -245 and MeWo) there was moderate to high inhibition of cell growth, which was dose dependent over the range of DHA levels. In five cell lines (SK-Mel-29, -93, -94, -119, and -127) there was minimal inhibition of cell growth. Fig. 1 shows the impact of increasing amounts of DHA on the growth of two representative melanoma cell lines: SK-Mel-29 cells, which were minimally inhibited, and SK-Mel-110 cells, which were highly inhibited over the entire range of DHA concentrations tested.

The SK-Mel-110 and SK-Mel-29 lines, as representative examples of cells that were either sensitive or refractory to the inhibitory effects of DHA, respectively, were chosen for further study. Both of these lines grew *in vitro* at two ploidy levels, although proportionally more

Table 1 Inhibitory effects of DHA on cell growth<sup>a</sup>  
Cells were grown for 7 days in presence of DHA and counted.

Cell line	1.5 $\mu\text{g/ml}$	3 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$
SK-Mel-13	26	37	48
SK-Mel-17	18	35	73
SK-Mel-28	21	53	70
SK-Mel-110	74	89	94
SK-Mel-133	9	40	67
SK-Mel-245	87	91	90
MeWo	18	59	81
SK-Mel-29	<1	<1	17
SK-Mel-93	17	7	14
SK-Mel-94	<1	<1	6
SK-Mel-119	14	2	7
SK-Mel-127	<1	<1	<1

<sup>a</sup> Values represent the percentage of growth inhibition compared with control untreated cells.

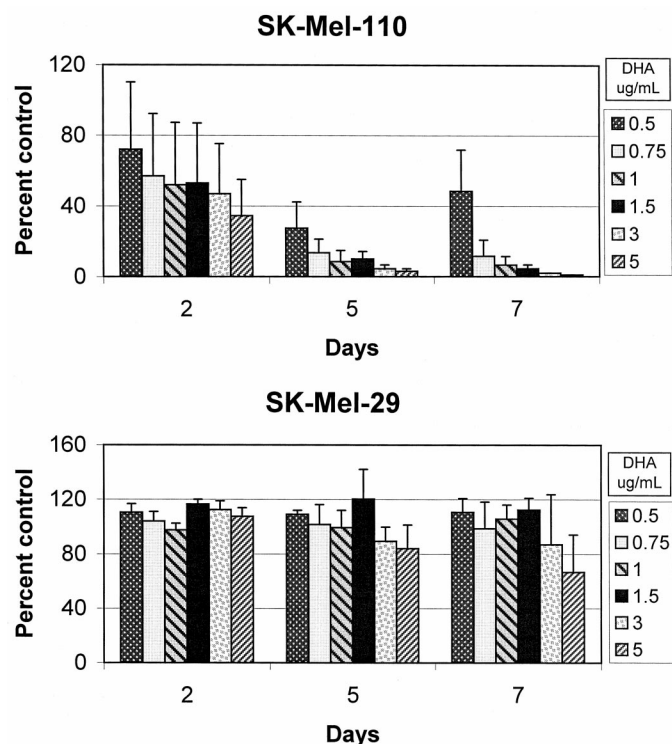


Fig. 1. Growth of SK-Mel-29 and SK-Mel-110 cells in DMEM supplemented with 1% FBS and DHA at the concentrations denoted. In each case, values represent percent inhibition of cell growth compared with untreated control cells. Multiple independent experiments were performed for SK-Mel-110 and SK-Mel-29. Bars, interexperimental SDs.

SK-Mel-29 cells were at the higher DNA ploidy compared with SK-Mel-110 cells.

**DHA Effects on Cell Growth in the Presence of FBS.** The cell cycle progression of SK-Mel-29 cells grown in the presence of 1% FBS was unaffected by DHA up to 2.0  $\mu\text{g/ml}$ ; neither DNA ploidy nor the cell cycle distribution was altered in this cell line. In contrast, SK-Mel-110 cells when treated with as little as 0.5  $\mu\text{g/ml}$  DHA showed a shift in growth pattern from higher to lower DNA ploidy (Table 2). In addition, the proportion of S phase cells increased >2-fold (from 16 to 36%) after treatment with 2.0  $\mu\text{g/ml}$  DHA. The loss of high DNA ploidy cells thus was compensated for by the increase in proportion of S phase cells and to a lesser degree by an increase in  $G_1$  cells of the lower ploidy.

In addition to the increase in S phase cells, the presence of apoptotic cells in SK-Mel-110 cultures became apparent after exposure to 2.0  $\mu\text{g/ml}$  DHA. Apoptotic cells had a fractional DNA content and were represented on the DNA content histograms as a distinct “sub- $G_1$ ” peak characteristic of apoptotic cells (36). However, the frequency of

apoptotic cells was low, and even at a DHA concentration of 2.0  $\mu\text{g/ml}$ , they failed to exceed 5% of the total cell population (Table 2). Confirmation of the induction of apoptosis under these culture conditions was based on cell morphology, which showed that the cells had condensed and structure-less chromatin, hyperchromatic DNA, diminished size, and blebbing of the plasma membrane but not classical nuclear fragmentation (Fig. 2). Although nuclear fragmentation is often observed during apoptosis, it is not unusual to find apoptotic cells that have hyperchromatic DNA but do not show typical apoptotic body formation (36, 37).

**DHA Effects on Cell Growth in Serum-free Medium.** Experiments in serum-free medium were carried out to eliminate the potentially confounding effect of other fatty acids and polypeptide growth factors that may be present in FBS. A growth rate analysis showed that the doubling time of SK-Mel-110 cells was extended from 21.9 h in the presence of 1% FBS to 50.2 h when grown in its absence. There was no evidence of cell death in these cultures, which can be grown in serum-free media for extended periods. The lengthening of the replicative cycle of SK-Mel-110 cells was accompanied by changes in the cell cycle distribution, manifested as an increase in the proportion of  $G_1$  cells and a corresponding decrease in the percentage of S phase cells at both DNA ploidy levels (Table 2).

Similar to growth in the presence of FBS, SK-Mel-29 cells were refractory to growth inhibition by DHA in FBS-free cultures; *i.e.*, neither significant changes in DNA ploidy nor in cell cycle distribu-

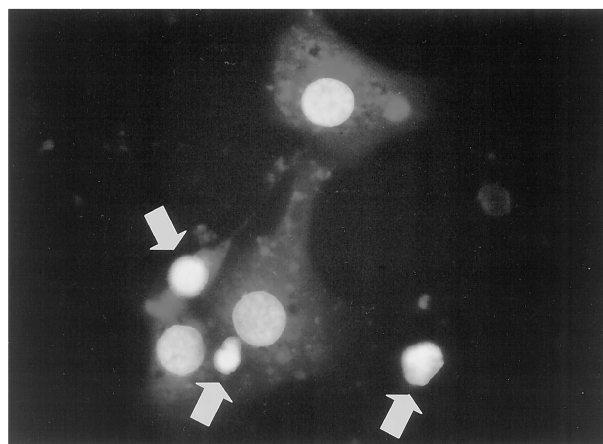


Fig. 2. Photomicrograph of SK-Mel-110 cells treated with 2.0  $\mu\text{g/ml}$  DHA for 5 days in the absence of FBS. The cells growing on microscope slides were fixed, and their DNA and protein were stained with DAPI and sulforhodamine 101, respectively, as described (36). When viewed under a fluorescence microscope, apoptotic cells were characterized by shrinkage, condensation of chromatin, and DNA hyperchromicity, all features characteristic of apoptosis (36). These cells were also in the process of detaching from the slide, often slightly out of focus under conditions in which contiguous nonapoptotic cells were in focus. However, the often classic morphological observation of chromatin fragmentation was not apparent.

Table 2 Cell cycle effects of DHA

Cells	Serum (%)	DHA	$G_1$	S	$G_2\text{-M}/G_{1T}^a$	$S_T$	$G_2\text{-M}_T$	Apoptosis
SK-Mel-29	1%	0	11.3 $\pm$ 0.6	6.3 $\pm$ 0.6	60.1 $\pm$ 8.7	16.3 $\pm$ 5.1	6.0 $\pm$ 4.5	0
		0.5	10.3 $\pm$ 1.5	5.3 $\pm$ 1.5	64.0 $\pm$ 9.5	14.7 $\pm$ 3.8	5.7 $\pm$ 2.9	0
		2.0	11.3 $\pm$ 3.2	5.3 $\pm$ 1.5	62.7 $\pm$ 9.9	14.4 $\pm$ 2.5	6.3 $\pm$ 3.2	0
SK-Mel-110	1%	0	43.5 $\pm$ 1.5	17.3 $\pm$ 2.3	32.2 $\pm$ 2.5	5.0 $\pm$ 1.7	2.0 $\pm$ 1.0	0
		0.5	47.3 $\pm$ 2.5	25.0 $\pm$ 1.0	22.3 $\pm$ 1.5	4.0 $\pm$ 1.7	1.3 $\pm$ 0.6	0
		2.0	52.3 $\pm$ 1.5	36.0 $\pm$ 1.0	11.7 $\pm$ 1.2	0	0	7.3 $\pm$ 3.0
	0	0	52.7 $\pm$ 0.6	5.7 $\pm$ 1.5	37.3 $\pm$ 0.6	2.7 $\pm$ 0.5	1.7 $\pm$ 0.6	0
		0.5	71.7 $\pm$ 1.5	8.3 $\pm$ 0.6	20.0 $\pm$ 1.7	0	0	2.0 $\pm$ 2.6
		2.0	<sup>b</sup>	<sup>b</sup>	<sup>b</sup>	<sup>b</sup>	<sup>b</sup>	43 $\pm$ 15

<sup>a</sup> It is not possible from DNA distributions alone to discriminate between  $G_2$  cells of the lower ploidy and  $G_1$  cells of the higher ploidy ( $G_{1T}$ ), which stain identically with the DNA-specific fluorochrome propidium iodide.

<sup>b</sup> The large percentage of apoptotic cells precluded determination of the cell cycle distribution.

tion were apparent in these cultures when treated with DHA up to 2.0  $\mu\text{g/ml}$  (data not shown). In contrast, the growth of SK-Mel-110 cells was quite dramatically altered (Table 2). At a concentration of 0.5  $\mu\text{g/ml}$  DHA, growth of SK-Mel-110 cells was almost entirely shifted to the lower DNA ploidy. Furthermore, cell arrest in  $G_1$  became apparent as the proportion of  $G_1$  cells increased to 72% compared with 53% in the untreated cultures. When exposed to 2.0  $\mu\text{g/ml}$  DHA, a large proportion of SK-Mel-110 cells underwent apoptosis, and  $\sim 33\%$  of the total cell population had a fractional DNA content that was represented on the DNA frequency histograms as a distinct sub- $G_1$  peak. The apoptotic mode of death in these cultures was confirmed by analysis of cell morphology as described above.

**Effects of DHA on pRb Phosphorylation.** The use of mAbs that specifically react either with hypophosphorylated pRb ( $\text{pRb}^{\text{P}^-}$ ) or with total pRb regardless of its phosphorylation state ( $\text{pRb}^{\text{T}}$ ; Ref. 31) allowed us to immunocytochemically probe the status of pRb phosphorylation in individual cells that were untreated or treated with DHA (Fig. 3). As is evident in Fig. 3, treatment of SK-Mel-110 with 2.0  $\mu\text{g/ml}$  DHA in the presence of FBS led to the increase in proportion of cells reacting with the  $\text{pRb}^{\text{P}^-}$  mAb. The increase was observed across all phases of the cell cycle, with no evidence of cell cycle phase specificity. No similar increase was apparent in SK-Mel-29 cultures.

Fig. 4 presents the proportions of SK-Mel-29 and SK-Mel-110 cells reactive with the  $\text{pRb}^{\text{P}^-}$  mAb in cultures maintained with or without

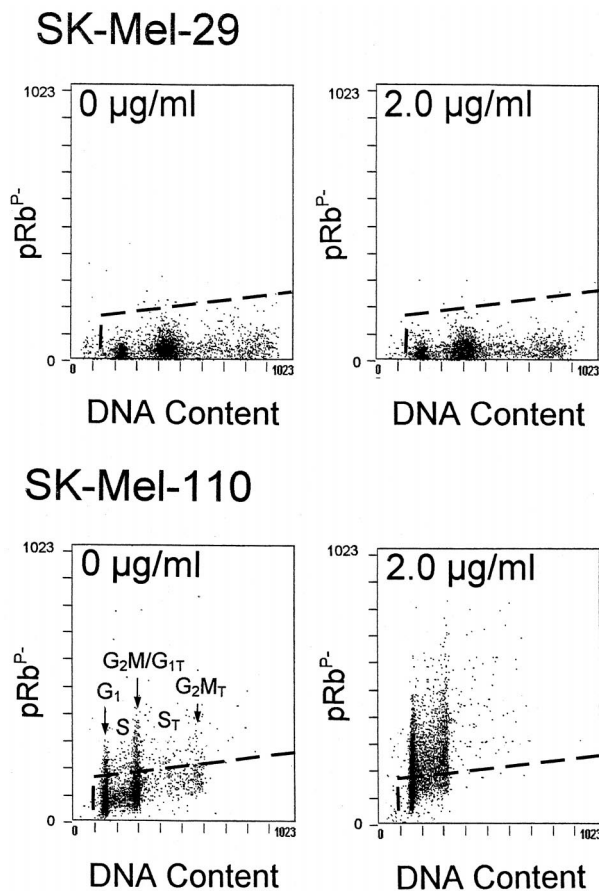


Fig. 3. Bivariate DNA versus  $\text{pRb}^{\text{P}^-}$  distributions of SK-Mel-29 and SK-Mel-110 cells growing in the presence of 1% FBS and in the absence (0  $\mu\text{g/ml}$ ) or presence of DHA (2.0  $\mu\text{g/ml}$ ) for 6 days. The reactivity of SK-Mel-110 cells with the mAb specific to hypophosphorylated pRb ( $\text{pRb}^{\text{P}^-}$ ) is higher than that of SK-Mel-29 cells and is further increased after treatment with DHA. The dashed line represents the threshold level of  $\text{pRb}^{\text{P}^-}$  above which the cells are considered to have hypophosphorylated pRb (see "Materials and Methods").

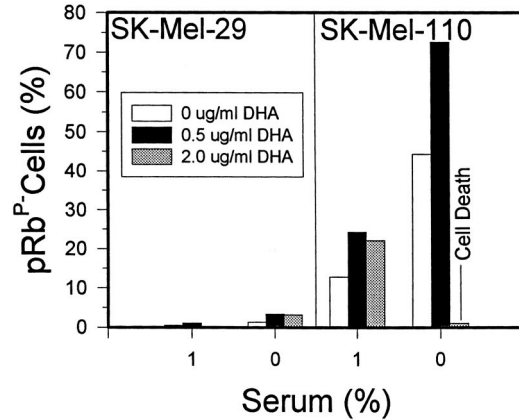


Fig. 4. Percentage of SK-Mel-29 and SK-Mel-110 cells reacting with anti- $\text{pRb}^{\text{P}^-}$  mAb after growth in different concentrations of DHA in the presence and absence of 1% FBS.

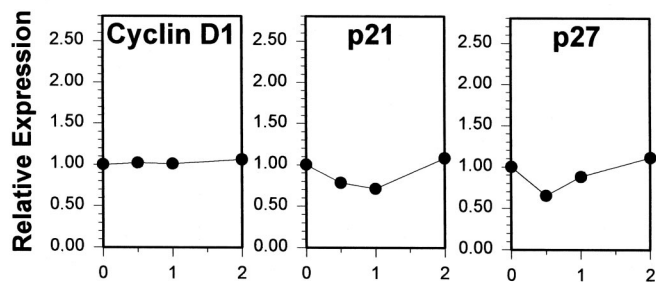
FBS and treated with 0.5 or 2.0  $\mu\text{g/ml}$  DHA. In SK-Mel-29 cultures that were grown either in the presence or absence of FBS, very few cells ( $<4\%$ ) reacted with the  $\text{pRb}^{\text{P}^-}$  mAb. The percentage of cells that was reactive with the  $\text{pRb}^{\text{P}^-}$  mAb (*i.e.*,  $<4\%$ ) remained virtually unaltered throughout the range of DHA used (*i.e.*, 0.5–2.0  $\mu\text{g/ml}$ ). In contrast, 12% of the untreated SK-Mel-110 cells grown in 1% FBS were positive for  $\text{pRb}^{\text{P}^-}$  (Fig. 4). The removal of FBS increased the proportion of untreated SK-Mel-110 cells with  $\text{pRb}^{\text{P}^-}$  to  $>40\%$ , whereas treatment of the cells with 0.5  $\mu\text{g/ml}$  DHA increased the number of cells expressing  $\text{pRb}^{\text{P}^-}$  to  $>70\%$ . As mentioned above, considerable apoptosis was observed after treatment with 2.0  $\mu\text{g/ml}$  DHA in the absence of FBS, precluding determination of the pRb status in these cells.

**Effect of DHA on Expression of Cyclins D1 and E and Inhibitors of Cyclin Kinase Inhibitors  $\text{p21}^{\text{WAF1}}$  and  $\text{p27}^{\text{KIP1}}$ .** To reveal mechanisms that may be responsible for the changes in cell proliferation (cell cycle distribution) and apoptosis, the upstream events known to play a role in pRb phosphorylation were investigated. Thus, we measured the effect of DHA on expression of cyclins D1 and E and the CKIs  $\text{p21}^{\text{WAF1}}$  and  $\text{p27}^{\text{KIP1}}$ . These proteins were detected immunocytochemically, and their expression was measured as the mean immunofluorescence of the  $G_1$  cell subpopulations minus the nonspecific fluorescence determined from the isotype control. Such cells were selected on the basis of their DNA content by gating analysis of the bivariate DNA content versus immunofluorescence distributions (scatterplots). To monitor changes in expression of cyclins or CKI inhibitors in response to DHA and for comparison of cell lines with each other, the mean immunofluorescence of  $G_1$  cells from the treated cultures was normalized to that of the  $G_1$  cells from the untreated control cultures (1.0).

As is evident from Fig. 5, the level of cyclin D1 in SK-Mel-29 cells was invariable over the entire range of DHA concentrations tested (0.5–2.0  $\mu\text{g/ml}$ ). A modest rise ( $\sim 40\%$ ) in cyclin D1 was observed in SK-Mel-110 cultures at 0.5  $\mu\text{g/ml}$  DHA, but no additional increase in cyclin D1 was found with increasing concentrations of DHA (to 2.0  $\mu\text{g/ml}$ ). Expression of cyclin E was comparable in the two cell lines and was unaffected by growth in the presence of DHA at any concentration tested (data not shown). Similarly, DHA had no marked effect on expression of  $\text{p21}^{\text{WAF1}}$  in either the SK-Mel-29 or SK-Mel-110 cell lines, because the level of this inhibitor was only slightly lower after treatment of these cell cultures with DHA in the range of 0.5–2.0  $\mu\text{g/ml}$ .

Finally, no marked changes in expression of  $\text{p27}^{\text{KIP1}}$  were apparent in either SK-Mel-29 or SK-Mel-110 cells treated with DHA (0.5–2.0  $\mu\text{g/ml}$ ) in the presence of 1% FBS. However, because DHA triggered

## SK-Mel-29



## SK-Mel-110

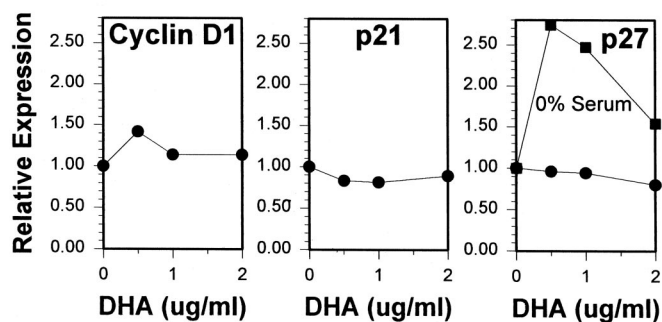


Fig. 5. Reactivity of anti-cyclin D1, anti-p21<sup>WAF1</sup>, and anti-p27<sup>KIP1</sup> mAb in SK-Mel-29 and SK-Mel-110 cells grown in the presence of 0–2.0 µg/ml DHA. The mean mAb fluorescence of the G<sub>1</sub> population selected based on DNA content (propidium iodide staining) for each untreated culture was determined and considered to be 100%. Expression of each of the proteins was then calculated as the mean mAb fluorescence of the G<sub>1</sub> population, normalized to the untreated control for each cell type. In each case, the *solid circles* represent data based on cells grown in the presence of 1% FBS, whereas for p27, SK-Mel-110 cells were exposed to 0–2.0 µg/ml DHA in the absence of FBS, as indicated by *solid squares*.

a high frequency of apoptosis in cultures of SK-Mel-110 cells in serum-free medium, we also explored the effect of DHA on p27<sup>KIP1</sup> expression when these cells were grown in the absence of FBS. As shown in Fig. 5, there was an ~3-fold increase in the expression of p27<sup>KIP1</sup> with treatment of these cells with 0.5 µg/ml DHA. There were no additional increases in p27<sup>KIP1</sup> expression when these cells were treated with up to 2.0 µg/ml DHA.

## DISCUSSION

We have shown, for the first time, that a long-chain n-3 PUFA inhibits the proliferation of a majority of cell lines derived from human metastatic melanomas and that inhibition involves the pRb pathway. Determination of the mechanism by which PUFAs inhibit melanoma cell growth will be an important first step in the rational use of PUFAs as antitumor agents. The molecular events by which DHA suppresses the growth of melanoma cells were examined in detail in two melanoma cell lines: one refractory (SK-Mel-29) and the other sensitive (SK-Mel-110) to the inhibitory effects of DHA. At the concentrations tested (*i.e.*, 0.5–2 µg/ml), DHA had little or no effect on SK-Mel-29 cells in terms of proliferation or distribution of cells in the various phases of the cell cycle. The observed dose-dependent suppression of proliferation of SK-Mel-110 cells by DHA, however, seemed to be due to cell cycle perturbation.

Analysis of the cell cycle phase distributions of the SK-Mel-29 and SK-Mel-110 cell lines revealed that both cell lines grew at two ploidies, one at twice the DNA content of the other. At the lowest concentration of DHA used (0.5 µg/ml), SK-Mel-110 cells displayed an increase in the proportion of lower ploidy S phase cells. There was no evidence of cell death under these conditions, suggesting that the

impaired progression through S was primarily responsible for the decrease in growth rate. At the higher DHA concentration (2 µg/ml), the cell cycle distribution of SK-Mel-110 cells was affected in two ways: (a) the higher ploidy cells, which consisted of approximately one-third of the total cells in the untreated parental SK-Mel-110 culture, virtually disappeared (although it was not possible to rule out that some of the cells with a G<sub>2</sub>-M DNA content did not represent G<sub>1</sub> cells of the higher ploidy, G<sub>1T</sub>); and (b) the cell cycle distribution of the lower ploidy cells indicated that cells continued to accumulate in S and to a lesser extent G<sub>1</sub> phases (Table 2). Because the disappearance of high DNA ploidy cells was not accompanied by any significant increase in the frequency of apoptotic cells (4%), it is unlikely that the loss of these cells was a result of their selective death induced by DHA. It appears more likely that progression through the cell cycle at the higher ploidy was inhibited to a greater extent than that of the low DNA ploidy cells, which led to overgrowth of the latter in terms of relative cell number.

Despite the fact that melanoma cells, like many tumor types, synthesize a range of mitogenic growth factors and develop the potential for autocrine stimulation of cell growth *in vitro*, these cells usually grow with different kinetics in the absence or presence of exogenously supplied growth factors present in FBS (38, 39). Thus, the doubling time of logarithmically growing SK-Mel-110 cells was prolonged from 21.9 h in medium supplemented with 1% FBS to 50.2 h in FBS-free medium. We examined the effects of DHA on SK-Mel-110 cells in the absence of FBS. The slowdown in growth of SK-Mel-110 cells after removal of FBS was mirrored in the cell cycle distribution, which, as might be predicted, was characterized by more cells in G<sub>1</sub> phase and fewer cells in S phase, although the relative percentage of cells growing at the higher ploidy did not change appreciably (see Table 2). Interestingly, in the absence of FBS, the higher ploidy cells present in SK-Mel-110 cultures disappeared at a DHA concentration of 0.5 µg/ml, whereas the cells accumulated in the G<sub>1</sub> phase (Table 2 and Fig. 3). More striking, however, was the induction of cell death by apoptosis in SK-Mel-110 cultures exposed to 2 µg/ml DHA. A significant proportion (>33%) of the cells appeared to be dying by apoptosis, which precluded analysis of the cell cycle distribution, because apoptotic cells tended to lose DNA as a result of endonuclease activity, resulting in a shift to lower DNA values of cells in each cell cycle phase. Such massive cell death of melanoma cells contrasts dramatically with the limited amount of apoptosis observed in the same cells treated with DHA in the presence of 1% FBS (Table 2).

The inhibitory effects of DHA on cell cycle progression of SK-Mel-110 cells in the presence of 1% FBS were directly paralleled by an increase in the proportion of cells with hypophosphorylated pRb. Thus, at 0.5 µg/ml DHA, the percentage of S phase cells increased, whereas the percentage of pRb<sup>P-</sup> S phase cells rose substantially (data not shown). At a DHA concentration of 2.0 µg/ml, the percentage of S phase cells more than doubled, whereas the percentage of pRb<sup>P-</sup> S phase cells reached 80%. Clearly, the perturbation of cell progression through S phase was accompanied by maintenance of pRb in its hypophosphorylated state. Although the frequency of G<sub>1</sub> cells with pRb<sup>P-</sup> also increased after treatment with DHA, there was no evidence of cell arrest in G<sub>1</sub>. This can be explained by the fact that the cell arrest in, or slowdown in progression through, S phase precluded cell entrance to G<sub>2</sub>-M and, subsequently, reentrance to G<sub>1</sub> such that the percentage of G<sub>1</sub> cells remained essentially unchanged. In the absence of FBS, by contrast, cell arrest in G<sub>1</sub> was paralleled by a dramatic increase in the proportion of pRb<sup>P-</sup> G<sub>1</sub> cells (Fig. 3). These data suggest that the suppression of cell progression either through S (as in the presence of FBS) or through G<sub>1</sub> (in its absence) induced by

DHA was mediated via the maintenance of pRb in its hypophosphorylated state.

pRb is the master switch regulating cell cycle progression, and its continuing phosphorylation parallels cell transit through G<sub>1</sub> and S (24, 40). However, although the overwhelming majority of invasive and metastatic melanoma specimens and cell lines (including those examined in the present study<sup>4</sup>) expresses normal RB protein (41–44), virtually 100% of these tissues and cell lines have defects in one or more of regulators of the pRb regulatory circuit, *i.e.*, the cyclin-dependent kinase inhibitor 2A (CDKN2A) or *p16<sup>INK4a</sup>* gene, the *CDKN2B* or *p15<sup>INK4b</sup>* gene, and the D-type cyclins or their functional partners Cdk4 and Cdk6 (42, 44–49). For example, homozygous deletions encompassing the p16 gene have been detected in SK-Mel-110 cells; however, the SK-Mel-29 cells possess a wild-type *p16* gene but a mutation of the *CDK4* gene, which abrogates its ability to bind to p16 (45).<sup>4</sup> Thus, in both these cell lines phosphorylation of pRb (and subsequent promotion of cell cycle progression) by the CDK4-cyclin D complex cannot be attenuated by p16.

The loss of normal pRb control through phosphorylation would result in its being either constitutively in the inactive position (*i.e.*, when it is hyperphosphorylated and not bound to growth-promoting transcription factors) or erratic in response to mitogenic or inhibitory signals and would be manifested as a low percentage of G<sub>1</sub> cells with hypophosphorylated (active) pRb. Indeed, our data indicate that only 13% of SK-Mel-110 and <1% of SK-Mel-29 G<sub>1</sub> cells had hypophosphorylated pRb in untreated cultures. Even total depletion of growth factors by FBS removal failed to revert pRb to the hypophosphorylated state in >50% of G<sub>1</sub> cells in SK-Mel-110 and in >98% of SK-Mel-29 cells. However, exposure of SK-Mel-110 cells to DHA restored, to a large degree, the active mode of pRb, as evidenced by the increased proportion of cells with hypophosphorylated pRb and the decrease in overall cell proliferation. We have confirmed that a second sensitive cell line (SK-Mel-28) also responds to DHA by increased levels of hypophosphorylated pRb, whereas a second resistant cell line (SK-Mel-186) predictably did not show increased hypophosphorylated pRb (data not shown).

In the presence of 1% FBS, we did not observe any significant effect of DHA on several important cell cycle regulators, manifested either as down-regulation of Cdk activators (*e.g.*, cyclin D1 and cyclin E) or as up-regulation of p27<sup>WAF1</sup> and p27<sup>KIP1</sup>, two direct inhibitors of Cdk activity. Given the lack of change in any of these proteins, it is possible that other components of the cell cycle regulatory machinery known to control pRb phosphorylation status were affected by DHA. Studies are presently under way to assess the status of these components. In contrast to the effects when FBS is present, SK-Mel-110 cells treated with DHA in the absence of FBS exhibited a striking increase in the expression levels of p27<sup>KIP1</sup> protein. Interestingly, only in these cells did we observe both cell cycle arrest and the induction of apoptosis. Considering that cell arrest alone, and not apoptosis, was observed in DHA-treated SK-Mel-110 cells in the presence of FBS, *i.e.*, when p27<sup>KIP1</sup> did not increase, it is tempting to speculate that the up-regulation of p27<sup>KIP1</sup> may contribute more to inducing apoptosis than cell cycle arrest in these cells. Supporting evidence for this conjecture comes from several studies showing that overexpression of p27<sup>KIP1</sup> triggers apoptosis (50, 51), and increased levels of this inhibitor precedes, or is associated with, apoptosis (52). Thus, further studies are needed to resolve whether it is DHA that sensitizes melanoma cells to respond to growth factor withdrawal occurring in the absence of serum by apoptosis, or alternatively, the absence of

stimulatory signals from growth factor receptors preconditions the cells to respond to DHA by apoptosis.

Dietary PUFAs (both n-3 and n-6) may play important roles in the evolution and/or progression of a broad range of cancers, including melanoma (for reviews, see Refs. 53–57). For example, murine melanoma cells cultured in medium supplemented with EPA showed a dose-dependent decrease in invasiveness, collagenase IV production, and ability to metastasize to the lung after *i.v.* injection (58). In another study, mice fed an n-3-rich fish oil diet then challenged with B-16 melanoma cells showed a >50% decrease in lung metastases compared with mice fed an n-6-rich corn oil diet (59). Thus, n-3 PUFAs have the potential to reduce melanoma metastasis. Although the biochemical mechanism(s) by which PUFAs influence tumor cell growth and metastasis is unclear, altered eicosanoid biosynthesis is likely to play a role (19–21, 23, 60). Feeding a diet supplemented with DHA or the closely related EPA inhibits the cyclooxygenase- and lipoxygenase-catalyzed formation of prostaglandins and hydroxyeicosatetraenoic acids by breast cancer cells (23), and the pharmacological inhibition of cyclooxygenase-2 (61) or lipoxygenases (62) induces apoptosis in some malignant cell lines. An inhibitory effect of DHA was observed on the growth of the highly invasive and metastatic MDA-MB-231 human breast cancer cell line *in vitro* (19); more recently, dietary DHA supplementation was shown to inhibit MDA-MB-231 cell solid tumor growth in nude mice, with both suppression of cell proliferation and induction of apoptosis.<sup>5</sup> These findings are in agreement with other reports that n-3 PUFAs induce apoptosis in cultured pancreatic cells *in vitro* (63) and in a transplantable Morris hepatocarcinoma growing *in vivo* (64). However, a mechanistic link among DHA, eicosanoid biosynthesis, and apoptosis is not, as yet, clear. Data presented in this report provide the first evidence that one such link may be manipulation of pRb phosphorylation status in DHA-treated cells.

Experiments are currently under way to extend these observations and to dissect the specific role played by pRb as well as other important regulators of both cell cycle progression and apoptosis. If DHA is capable of suppressing cell and tumor growth and metastatic potential in *in vivo* models of melanoma, a clinical trial of DHA would be warranted as an adjuvant to current surgical and chemotherapeutic interventions. Theoretically, reconstitution of a functional pRb pathway in melanoma cells by the induction of pRb hypophosphorylation via supplementation with PUFAs could result in the suppression of cell proliferation and provide the basis for a novel antitumor strategy.

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<sup>4</sup> A. P. Albino, unpublished data.

<sup>5</sup> J. M. Connolly, E. M. Gilhooly, and D. P. Rose, unpublished data.

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