Cell Cycle Arrest and Apoptosis of Melanoma Cells by Docosahexaenoic Acid: Association with Decreased pRb Phosphorylation

Anthony P. Albino, Gloria Juan, Frank Traganos, Lisa Reinhardt, Jeanne Connolly, David P. Rose, and Zbigniew Darzynkiewicz


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 ω-6 polyunsaturated fatty acids (PUFAs) as stimulators and long-chain ω-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 ω-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 \textit{WAF1/CIP1}, or p27 \textit{KIP1}; and (c) expression of total pRb \textsuperscript{+} independent of phosphorylation state and hypophosphorylated pRb \textsuperscript{−} in fixed cells by flow cytometry and immunocytochemistry using specific monoclonal antibodies to pRb \textsuperscript{+} or pRb \textsuperscript{−}, 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 μg/ml DHA whereas 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 G1 or S phase: 0% expression of p21 and p27, with increased levels of the cyclin-dependent kinase inhibitors p21 and p27. 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.
were performed using a one-way ANOVA test, followed by a t-test. The antibodies were washed again, resuspended in 5 μg/ml antimouse IgG antibody (Molecular Probes, Eugene, OR) diluted 1:30 in 1% BSA. For intracellular proteins, the cells were fixed in ice-cold 80% ethanol for up to 24 h. After fixation, the cells were washed twice with PBS and then suspended in ice-cold 80% ethanol for up to 24 h. They were then rinsed with PBS containing 1% BSA and incubated with FITC-conjugated goat antirabbit 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 μg/ml propidium iodide (Sigma) and incubated for 20 min before measurement. Control cells were treated identically, except that instead of using the anti-rabbit 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 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-μg/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, -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.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>1.5 μg/ml</th>
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<tr>
<td>SK-Mel-13</td>
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<td>SK-Mel-17</td>
<td>18</td>
<td>35</td>
<td>73</td>
</tr>
<tr>
<td>SK-Mel-28</td>
<td>21</td>
<td>53</td>
<td>70</td>
</tr>
<tr>
<td>SK-Mel-110</td>
<td>74</td>
<td>89</td>
<td>94</td>
</tr>
<tr>
<td>SK-Mel-133</td>
<td>9</td>
<td>40</td>
<td>67</td>
</tr>
<tr>
<td>SK-Mel-245</td>
<td>87</td>
<td>91</td>
<td>90</td>
</tr>
<tr>
<td>MeWo</td>
<td>18</td>
<td>59</td>
<td>81</td>
</tr>
<tr>
<td>SK-Mel-29</td>
<td>&lt;1</td>
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<td>&lt;1</td>
</tr>
<tr>
<td>SK-Mel-93</td>
<td>17</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>SK-Mel-94</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>SK-Mel-119</td>
<td>14</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>SK-Mel-127</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Values represent the percentage of growth inhibition compared with control untreated cells.

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...
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 μg/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 μg/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 μg/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 G1 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 μg/ml DHA. Apoptotic cells had a fractional DNA content and were represented on the DNA content histograms as a distinct “sub-G1” peak characteristic of apoptotic cells (36). However, the frequency of apoptotic cells was low, and even at a DHA concentration of 2.0 μg/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 G2 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 distrib-

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![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.](image1)

![Fig. 2. Photomicrograph of SK-Mel-110 cells treated with 2.0 μg/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.](image2)

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**Table 2**  
**Cell cycle effects of DHA**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Serum (%)</th>
<th>DHA</th>
<th>G2</th>
<th>S</th>
<th>G2-M/G1 res</th>
<th>Sf</th>
<th>G2-Mf</th>
<th>Apoptosis</th>
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</thead>
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<tr>
<td>SK-Mel-29</td>
<td>1%</td>
<td>0.5</td>
<td>10.3 ± 1.5</td>
<td>6.3 ± 1.5</td>
<td>64.0 ± 6.5</td>
<td>16.3 ± 3.5</td>
<td>4.7 ± 2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>11.3 ± 1.5</td>
<td>5.3 ± 1.5</td>
<td>62.7 ± 5.9</td>
<td>14.4 ± 2.5</td>
<td>6.3 ± 3.2</td>
<td>0</td>
</tr>
<tr>
<td>SK-Mel-110</td>
<td>1%</td>
<td>0.5</td>
<td>43.5 ± 1.5</td>
<td>17.3 ± 2.3</td>
<td>32.2 ± 5.6</td>
<td>5.0 ± 1.7</td>
<td>2.0 ± 1.0</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>47.3 ± 1.5</td>
<td>25.0 ± 1.0</td>
<td>22.3 ± 1.5</td>
<td>4.4 ± 1.0</td>
<td>1.3 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>52.5 ± 1.5</td>
<td>36.0 ± 1.5</td>
<td>11.7 ± 1.2</td>
<td>0</td>
<td>0</td>
<td>7.3 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>58.7 ± 1.5</td>
<td>57.1 ± 1.5</td>
<td>37.3 ± 6.5</td>
<td>2.7 ± 0.5</td>
<td>1.7 ± 0.6</td>
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<tr>
<td></td>
<td></td>
<td>0.6</td>
<td>71.7 ± 1.5</td>
<td>8.3 ± 0.6</td>
<td>20.0 ± 1.7</td>
<td>0</td>
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<td>2.0 ± 2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>73.7 ± 1.5</td>
<td>8.3 ± 0.6</td>
<td>19.0 ± 1.7</td>
<td>0</td>
<td>0</td>
<td>43 ± 15</td>
</tr>
</tbody>
</table>

*It is not possible from DNA distributions alone to discriminate between G2 cells of the lower ploidy and G1 cells of the higher ploidy (G1T), which stain identically with the DNA-specific fluorochrome propidium iodide.

*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 μg/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 μg/ml DHA, growth of SK-Mel-110 cells was almost entirely shifted to the lower DNA ploidy. Furthermore, cell arrest in G1 became apparent as the proportion of G1 cells increased to 72% compared with 53% in the untreated cultures. When exposed to 2.0 μg/ml DHA, a large proportion of SK-Mel-110 cells underwent apoptosis, and ~33% of the total cell population had a fractional DNA content that was represented on the DNA frequency histograms as a distinct sub-G1 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 (pRb\(^{P^0}\)) or with total pRb regardless of its phosphorylation state (pRb\(^T\); 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 μg/ml DHA in the presence of FBS led to the increase in proportion of cells reacting with the pRb\(^{P^0}\) 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 pRb\(^{P^0}\) mAb in cultures maintained with or without FBS and treated with 0.5 or 2.0 μg/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 pRb\(^{P^0}\) mAb. The percentage of cells that was reactive with the pRb\(^{P^0}\) mAb (i.e., <4%) remained virtually unaltered throughout the range of DHA used (i.e., 0.5–2.0 μg/ml). In contrast, 12% of the untreated SK-Mel-110 cells grown in 1% FBS were positive for pRb\(^{P^0}\) (Fig. 4). The removal of FBS increased the proportion of untreated SK-Mel-110 cells with pRb\(^{P^0}\) to >40%, whereas treatment of the cells with 0.5 μg/ml DHA increased the number of cells expressing pRb\(^{P^0}\) to >70%. As mentioned above, considerable apoptosis was observed after treatment with 2.0 μg/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 p21\(^{WAF1}\) and p27\(^{KIP1}\). 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 p21\(^{WAF1}\) and p27\(^{KIP1}\). These proteins were detected immunocytochemically, and their expression was measured as the mean immunofluorescence of the G1 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 G1 cells from the treated cultures was normalized to that of the G1 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 μg/ml). A modest rise (~40%) in cyclin D1 was observed in SK-Mel-110 cultures at 0.5 μg/ml DHA, but no additional increase in cyclin D1 was found with increasing concentrations of DHA (to 2.0 μg/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 p21\(^{WAF1}\) 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 μg/ml.

Finally, no marked changes in expression of p27\(^{KIP1}\) were apparent in either SK-Mel-29 or SK-Mel-110 cells treated with DHA (0.5–2.0 μg/ml) in the presence of 1% FBS. However, because DHA triggered
a high frequency of apoptosis in cultures of SK-Mel-110 cells in serum-free medium, we also explored the effect of DHA on p27KIP1 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 p27KIP1 with treatment of these cells with 0.5 μg/ml DHA. There were no additional increases in p27KIP1 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 G2-M DNA content did not represent G1 cells of the higher ploidy, G1T); and (b) the cell cycle distribution of the lower ploidy cells indicated that cells continued to accumulate in S and to a lesser extent G1 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 G1 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 G1 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−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−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 G1 cells with pRb− also increased after treatment with DHA, there was no evidence of cell arrest in G1. This can be explained by the fact that the cell arrest in, or slowdown in progression through, S phase precluded entrance to G2-M and, subsequently, reentrance to G1 such that the percentage of G1 cells remained essentially unchanged. In the absence of FBS, by contrast, cell arrest in G1 was paralleled by a dramatic increase in the proportion of pRb− G1 cells (Fig. 3). These data suggest that the suppression of cell progression either through S (as in the presence of FBS) or through G1 (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 G1 and S (24, 40). However, although the overwhelming majority of invasive and metastatic melanoma specimens and cell lines (including those examined in the present study5) 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 p16INK4a gene, the CDKN2B or p15INK4B 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).4 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 G1 cells with hypophosphorylated (active) pRb. Indeed, our data indicate that only 13% of SK-Mel-110 and <1% of SK-Mel-29 G1 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 G1 cells, SK-Mel-110 and in >98% of SK-Mel-29 cells. However, exposure of SK-Mel-110 cells to DHA restored, to a 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 p21WAF1 and p27KIP1, 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 p27KIP1 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 p27KIP1 did not increase, it is tempting to speculate that the up-regulation of p27KIP1 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 p27KIP1 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 PUFA (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 PUFA have the potential to reduce melanoma metastasis. Although the biochemical mechanism(s) by which PUFA 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 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.5 These findings are in agreement with other reports that n-3 PUFA 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 vitro 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 PUFA could result in the suppression of cell proliferation and provide the basis for a novel antitumor strategy.

REFERENCES


4 A. P. Albino, unpublished data.

5 J. M. Connolly, E. M. Gilhooly, and D. P. Rose, unpublished data.
CELL CYCLE ARREST OF MELANOMA CELLS BY DOCOSAHEXAENOIC ACID


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