Effect of Cellular Fatty Acid Alteration on Adriamycin Sensitivity in Cultured L1210 Murine Leukemia Cells

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ABSTRACT

We have investigated the effect of cellular fatty acid alteration on Adriamycin cytotoxicity using the L1210 lymphoblastic leukemia cell line. Cells growing in Roswell Park Memorial Institute Medium 1640 with 5% fetal bovine serum were modified with respect to fatty acid composition by supplementing their growth medium with 32 \( \mu \)M docosahexaenoic acid (22:6) or oleic acid (18:1). A soft agar clonogenic assay was then used to assess survival following incubation with Adriamycin. When exposed to the drug at a concentration of 0.4 \( \mu \)M, cells grown in the 22:6-supplemented medium were more sensitive (min of drug treatment required to reduce survival by 63% on the exponential portion of the survival curve, 64.9 ± 4.2 min) to the cytotoxic effects of Adriamycin than cells grown in unsupplemented medium (min of drug treatment required to reduce survival by 63% on the exponential part of the survival curve, 106 ± 9.7 min) (p < 0.005). Cytotoxicity of L1210 cells grown in 18:1-supplemented medium was similar to that of cells grown in unsupplemented medium (min of drug treatment required to reduce survival by 63% on the exponential part of the survival curve, 126.6 ± 9.1 min). The heightened sensitivity to Adriamycin of cells whose medium contained 22:6 increased as the concentration of fatty acid used to supplement the growth medium was increased. The cytotoxicity was also a function of the concentration of Adriamycin from 0.1 to 1.6 \( \mu \)M. When compared to cells grown in unsupplemented medium, those grown in 22:6-supplemented medium contained 3- to 4-fold more polyunsaturated fatty acids in their phospholipids, with a resultant doubling in the mean number of double bonds per fatty acid molecule. These data demonstrate that modification of cellular fatty acid composition may dramatically affect the sensitivity of a tumor cell to Adriamycin.

INTRODUCTION

Since its introduction into clinical use, Adriamycin has become one of the most frequently used antineoplastic agents utilized in the treatment of a variety of neoplasms including small cell lung cancer, breast cancer, sarcoma, lymphoma, and acute leukemia (35). Despite widespread application, however, knowledge concerning its mechanism of action is limited. Its ability to bind to DNA (12, 25) has frequently been used to argue that the mechanism involves a disruption in nucleic acid synthesis and function. While such effects on nucleic acid metabolism have been nonetheless shown to be cytotoxic (2, 26). Perhaps most damaging to the hypothesis that Adriamycin cytotoxicity is mediated by an effect on nucleic acid metabolism are studies on polymer-bound drug (32, 33). These studies indicate that Adriamycin does not need to enter the cell to exert its cytotoxic effect and suggest that a critical interaction may occur at the level of the cell membrane.

If the cytotoxic action of Adriamycin is mediated by its interaction with the cell membrane, modification of the cell membrane might be expected to result in a change in Adriamycin cytotoxicity. We reported recently that it is possible to modify the chemical and physical properties of L1210 lymphoblastic leukemia cell membranes by growing the cells in medium supplemented with the fatty acid docosahexaenoic acid (15). In this paper, we have used that system to investigate the effects of cellular fatty acid alteration on Adriamycin cytotoxicity.

MATERIALS AND METHODS

L1210 murine leukemia cells were grown at 37° in medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), 5% FBS\(^3\) (KC Biologicals, Inc., Lenexa, KS), and gentamicin sulfate (40 \( \mu \)g/ml; Grand Island Biological Co.) without constant mixing in a humid atmosphere of 5% CO\(_2\)/95% air. To produce modification in cellular and membrane fatty acid composition, cells were grown for 2 days under identical conditions in medium further supplemented with either docosahexaenoic acid (22:6; individual fatty acids are abbreviated as number of carbon atoms:number of double bonds) or oleic acid (18:1) (Nu Chek Prep., Inc., Elysian, MN). Known quantities of the sodium salts were added dropwise to the FBS used to prepare the growth medium. Unless otherwise stated, the fatty acids used were added so that their concentration in the final growth medium was 32 \( \mu \)M.

For survival studies, cells growing in the logarithmic phase of growth were harvested and resuspended at a concentration of 10\(^7\) cells/ml in 10 ml of medium consisting of RPMI 1630 with 5% unmodified FBS and 40 \( \mu \)g of gentamicin sulfate/ml. The use of RPMI 1630 rather than RPMI 1640 resulted in a more constant pH during the short incubations in closed submerged flasks. Adriamycin hydrochloride (Sigma Chemical Co., St. Louis, MO) was added at concentrations ranging from 0.1 to 1.6 \( \mu \)M, and the cells were then incubated at 37° in a water bath. At various intervals, the fraction of surviving cells was determined using a soft agar clonogenic assay as described previously (15).

For lipid analysis, cells were washed in phosphate-buffered saline (137 \( \text{mM} \), 8.1 \( \text{mM} \) Na\(_2\)HPO\(_4\), 1.5 \( \text{mM} \) KH\(_2\)PO\(_4\), 2.7 \( \text{mM} \) KCl, pH 7.25) and extracted with CHCl\(_3\)/methanol (2:1, v/v) (13). Neutral lipids and phospholipids were separated using silicic acid chromatography (6), and each fraction was then saponified for 60 min at 56° in 1.2 \( \text{nKOH}:80\% \) ethanol (1). Fatty acids in the saponifiable fraction were methylated for 10 min at 95° with 14% BF\(_3\)/CH\(_3\)OH (23), and their methyl esters were then separated by gas chromatography using 1.8-m column packed with...
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10% SP-2330 on 100/200 mesh Chromosorb (Supelco, Inc., Bellefonte, PA). Peak areas were quantified and identified by comparison of retention times to those of standards obtained from Supelco, Inc. Statistical analysis was done using the t test.

RESULTS

Growth Rate. It was first necessary to determine whether the 22:6 supplementation per se had any effect on cell growth. L1210 cells were incubated with medium containing 32 μM 22:6, and the doubling time during the logarithmic phase of growth was determined using a Model 2F Coulter Counter. As shown in Table 1, fatty acid supplementation had no effect on growth rate. This is similar to our previous observations that dietary alteration of L1210 cellular fatty acids in vivo has no effect on rate of growth when the cells are subsequently placed in culture (4). Likewise, fatty acid supplementation had no effect on cloning efficiency of cells which had been in their respective media for 48 hr.

Chart 1 illustrates the survival curves for L1210 cells exposed to Adriamycin at a concentration of 0.4 μM. Cells grown in medium supplemented with 22:6 at a concentration of 32 μM were more sensitive to the cytotoxic effects of Adriamycin than were cells grown in unsupplemented medium. Statistically significant differences in the surviving fraction were noted at all time points after 1 hr, and with a 5-hr exposure to Adriamycin, there was greater than a 1-log difference in the surviving fraction. The min of drug treatment required to reduce survival by 63% on the exponential part of the survival curve were 64.9 ± 4.2 min for cells grown in the 22:6-supplemented medium and 106 ± 9.7 min for cells grown in the unmodified medium (p < 0.005).

To examine the possibility that the difference in cytotoxicity was due to the addition of fatty acid in a nonspecific manner and not to the effect of the highly unsaturated 22:6, we tested the effect of 18:1. As seen in Chart 1, cells grown in 18:1-supplemented medium were similar in Adriamycin sensitivity to unsupplemented cells. The min of drug treatment required to reduce survival by 63% on the exponential part of the survival curve for 18:1-supplemented cells, 126.6 ± 9.1 min, was not significantly different from the value for cells grown in unsupplemented medium but was significantly greater than those grown in 22:6 (p < 0.001).

We next determined if these differences in Adriamycin sensitivity were a function of the concentration of 22:6. Cells grown in medium supplemented with 0 to 32 μM 22:6 were incubated for 2 hr with 0.4 μM Adriamycin, and the surviving fraction was then determined (Chart 2). The addition of 4 μM 22:6 to the growth medium did not alter Adriamycin sensitivity. However, at concentrations exceeding 8 μM, Adriamycin sensitivity increased as the concentration of 22:6 added to the growth medium increased. Cytotoxicity was also a function of drug concentration. The fraction of cells surviving a 2-hr exposure to concentrations of drug ranging from 0.1 to 1.6 μM is shown in Table 2. Differences in surviving fraction increased as the concentration of Adriamycin supplemented medium were significant at each concentration (p < 0.025).

Table 1

<table>
<thead>
<tr>
<th>Culture doubling time (hr)</th>
<th>Cloning efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22:6 supplemented</td>
<td>10.0 ± 0.3a</td>
</tr>
<tr>
<td>Unsupplemented</td>
<td>9.2 ± 0.9</td>
</tr>
</tbody>
</table>

a Mean ± S.E. of at least 9 individual determinations. Not significantly different.

Table 2

<table>
<thead>
<tr>
<th>Concentration of Adriamycin (μM)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22:6 supplemented</td>
</tr>
<tr>
<td>0.1</td>
<td>70.5 ± 5.0b</td>
</tr>
<tr>
<td>0.2</td>
<td>38.8 ± 4.0</td>
</tr>
<tr>
<td>0.4</td>
<td>23.7 ± 3.3</td>
</tr>
<tr>
<td>0.8</td>
<td>6.6 ± 2.4</td>
</tr>
<tr>
<td>1.6</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

b Differences between cells grown in the 22:6-supplemented and unsupplemented media were significant at each concentration (p < 0.025).

b Mean ± S.E. of 4 determinations.
was increased, such that at a concentration of 1.6 \( \mu \text{M} \), there was greater than a 1-log difference in surviving fraction. At each concentration of drug, the 22:6-supplemented cells were more sensitive than the unsupplemented cells (\( p < 0.025 \)).

**Cell Lipid Content.** We next determined if fatty acid supplementation of the medium altered the cellular lipids. Table 3 shows the fatty acid composition of the phospholipids isolated from the cells grown in the 2 media. When cells were cultured in 22:6-modified medium, it became the predominant fatty acid, making up over 20% of the fatty acids found in the phospholipid fraction. This differs from the value of less than 1% for cells grown in unmodified medium. This increase was fairly specific. Although 18:1 decreased, 16:0 increased slightly, and 18:0 remained unchanged.

The fatty acid composition of the neutral lipids is shown on Table 4. In contrast to the phospholipids, cellular neutral lipids were not significantly modified when 22:6 was added to the medium. This lack of modification of the neutral lipid storage sites indicates that the alteration occurs primarily in the phospholipids which are located mostly in membranes.

The overall changes in the fatty acid composition of the phospholipids and neutral lipids are summarized in Table 5. Within the phospholipid fraction, growth in 22:6-modified medium resulted in a greater than 3-fold increase in polyenoics, a 2-fold decrease in monoenoics, and a 1- to 2-fold increase in saturates when compared to cells grown in unmodified medium. These changes resulted in over a 2-fold increase in the average number of double bonds per fatty acid molecule in cells grown in 22:6 medium (1.81 \( \pm 0.3 \)) compared to unsupplemented medium (0.88 \( \pm 0.1 \)). Cholesterol levels and cholesterol-phospholipid molar ratios have been shown previously not to be altered significantly by 22:6 enrichment (15).

**DISCUSSION**

In this paper, we have demonstrated that growth of L1210 cells in medium supplemented with the highly polyunsaturated fatty acid, 22:6, increases their sensitivity to Adriamycin as compared to cells grown in unsupplemented medium. The heightened sensitivity to Adriamycin increased as the concentration of 22:6 in the growth medium increased and was apparent over a range of concentrations of drug from 0 to 1.6 \( \mu \text{M} \).

The mechanism whereby growth of L1210 cells in 22:6-supplemented medium increases their sensitivity to Adriamycin is not certain. The addition of 22:6 to the medium did not alter the growth rate or cloning efficiency of the cells in the absence of drug. However, there were changes in cellular lipid composition which might offer an explanation for the increased cytotoxicity.

The addition of 22:6 to the growth medium resulted in changes in the cellular fatty acid composition of particular interest was the increase in polyenoic fatty acids in the phospholipid fraction. When cells were grown in unsupplemented medium, polyenoic fatty acids comprised less than 10% of the fatty acids. This contrasts with the dramatic increase in polyenoic fatty acids of 28.5% seen when cells were grown in the 22:6-supplemented medium. Since phospholipids are important components of cell membranes, such changes in their fatty acid composition would be expected to influence a number of membrane properties. We have shown previously, for instance, that the incorporation of 22:6 into L1210 cell phospholipids has a membrane-fluidizing effect (15). The fatty acid composition and the fluidity of membranes have in turn been shown to effect such membrane properties as permeability (21), endocytosis (20), and the activity of membrane-associated enzymes (8, 11, 19) and transport systems (5, 17).

One explanation for our observations might be that growth of cells in the 22:6-supplemented medium leads to an increase in Adriamycin sensitivity by altering one of these or other similar membrane-related functions. For instance, with regard to drug transport, it has been postulated that Adriamycin uptake involves the diffusion of the drug through the lipid domain of the membrane (10) and that drug efflux is dependent upon an active energy-dependent process (16, 28). Either of these fluxes might be altered by the incorporation of large amounts of 22:6 into the membrane lipids and a resultant change in membrane fluidity.

Table 3

<table>
<thead>
<tr>
<th>Fatty acid composition of L1210 cell phospholipids</th>
<th>22:6 supplemented</th>
<th>Unsupplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual acids</td>
<td>22.0 ± 3.0(^a)</td>
<td>13.4 ± 1.8</td>
</tr>
<tr>
<td>18:0</td>
<td>18.7 ± 0.4(^a)</td>
<td>14.0 ± 0.4</td>
</tr>
<tr>
<td>16:1</td>
<td>3.0 ± 1.4</td>
<td>3.2 ± 1.2</td>
</tr>
<tr>
<td>16:2</td>
<td>25.0 ± 0.2</td>
<td>26.3 ± 1.7</td>
</tr>
<tr>
<td>18:2</td>
<td>3.7 ± 1.2</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>20:4</td>
<td>2.3 ± 3.7</td>
<td>15.3 ± 0.2</td>
</tr>
<tr>
<td>20:6</td>
<td>7.4 ± 0.7</td>
<td>5.0 ± 0.8</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.E. of determinations made on 4 individual samples of cells.


Table 4

<table>
<thead>
<tr>
<th>Fatty acid composition of L1210 neutral lipids</th>
<th>22:6 supplemented</th>
<th>Unsupplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual acids</td>
<td>23.2 ± 1.1(^a)</td>
<td>22.0 ± 3.4</td>
</tr>
<tr>
<td>18:0</td>
<td>10.3 ± 0.5</td>
<td>11.3 ± 2.3</td>
</tr>
<tr>
<td>16:1</td>
<td>18.2 ± 1.4</td>
<td>21.1 ± 1.2</td>
</tr>
<tr>
<td>18:1</td>
<td>19.1 ± 0.8</td>
<td>25.8 ± 5.2</td>
</tr>
<tr>
<td>18:2</td>
<td>6.5 ± 0.8</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>20:4</td>
<td>0.2 ± 0.1</td>
<td>ND(^a)</td>
</tr>
<tr>
<td>22:6</td>
<td>9.1 ± 3.8</td>
<td>1.6 ± 1.6</td>
</tr>
<tr>
<td>Other(^c)</td>
<td>13.8 ± 1.4</td>
<td>11.6 ± 2.4</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.E. of determinations made on 4 individual samples of cells.


\(^c\) ND, not detected.
Although it is generally believed that Adriamycin exerts its cytotoxic effect by binding to DNA, several observations suggest that it may also have a direct effect on cell membranes. Adriamycin is known to alter membrane-associated receptors (24), membrane glycoprotein composition (18), the transmembrane flux of sodium (29), and the exchangeability of membrane-bound calcium (34). Indeed, recent evidence indicates that Adriamycin need not enter the cell in order to exert its cytotoxic effect (32, 33). An alternative explanation for our observations might therefore be that the incorporation of large amounts of 22:6 into the membrane alters the interaction between the drug and a critical membrane-related target.

Such a target may in fact be the unsaturated fatty acids incorporated into membrane phospholipids. Adriamycin is known to bind to membrane phospholipids (14), and its metabolism by way of a semiquinone radical intermediate is believed to yield the superoxide radical (31). In the presence of an iron catalyst, this reaction has been shown to result in extensive decomposition of unsaturated fatty acids incorporated into phospholipid micelles (30). A cell whose membrane phospholipids have been enriched with the highly polyunsaturated fatty acid, 22:6, might be more susceptible to such lipid peroxidation with resultant membrane damage.

A change in Adriamycin sensitivity such as we have brought about here may have a therapeutic use. Dietary modification of tumor cell and normal tissue fatty acid composition has been demonstrated in animal models (3, 5, 7) and might be adapted for human use. Alternatively, techniques for the isolated perfusion of tumor-bearing body area with chemotherapy have been developed and could be adopted to allow delivery of a perfusate high in polyunsaturated fatty acid. In summary, we have shown that the incorporation of 22:6 into L1210 cell phospholipids increases their sensitivity to Adriamycin. This observation adds further evidence that the membrane may be an important site of anthracycline toxicity. The further determination of the metabolic reactions involved will yield important information as to the mechanism of action of Adriamycin at the cellular level.

REFERENCES


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