Modification by Adenosine of the Effect of Adriamycin on Myocardial Cells in Culture

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ABSTRACT

The cardiotoxic effects of Adriamycin (ADM) were studied utilizing mammalian myocardial cells in culture as a model system. ADM inhibited cell growth and the rhythmic contractions characteristic of these cells. Because a possible involvement of energy metabolism in the action of ADM was suggested previously, the adenylate energy charge and phosphocreatine mol fraction were determined in the ADM-treated cells. The adenylate energy charge was found to be significantly decreased, while the phosphocreatine mol fraction was not. Such disparity suggests an inhibition of creatine phosphokinase. The addition of 1 mM adenosine to the myocardial cell cultures markedly increased the adenosine triphosphate concentration but not the adenylate charge. In the ADM-treated cells, the addition of adenosine increased both the adenosine triphosphate concentration and the adenylate charge, and, concomitant with this increase, the functional integrity of the cells in terms of percentage of beating cells and rate of contractions was maintained.

INTRODUCTION

The use of ADM, a potent anticancer agent, for long-term maintenance chemotherapeutic treatment is limited because of a cumulative dose-dependent cardiomyopathy (e.g., Refs. 14 and 19). The toxicity seems to be very specific for the myocardium, but the pathogenesis has not been elucidated. Even with a restricted cumulative dose not to exceed 550 mg/sq m, a small percentage of patients still developed ADM cardiotoxicity, and the antibiotic had to be excluded from the chemotherapy (20). Numerous studies on cellular structure and function failed to reveal the pathogenesis (17).

A possible involvement of mitochondria in the cardiomyopathy has been suggested (5, 13) and, more specifically, the inhibition of coenzyme Q10 enzymes by ADM (11, 12). Recently, an antitoxic effect of coenzyme Q was reported (3). Isolated rabbit heart mitochondria were studied by Ferrero et al. (7), and an inhibition of State 4 and State 3 respiration resulting from ADM treatment was demonstrated, but not of the ADP/O ratio. Likewise, an inhibition of oxidative phosphorylation in bovine heart mitochondria and tumor cells was reported (16). After 56 days of increased doses of ADM, prominent mitochondrial lesions were observed in rabbits, but such changes were not thought to contribute to the genesis of the toxicity (4).

Gosalvez et al. (9) could not assign the ADM effect to any specific site of the respiratory chain. Based on the possibility that ADM cardiotoxicity is associated with the peroxidation of cardiac lipids, Myers et al. (21) reported that toxicity is reduced by prior treatment of mice with the free radical scavenger tocopherol.

Rat heart cells in culture also have been used as a model system for the study of ADM effect on cellular energy metabolism (26, 27). An inhibition of cell growth, particularly of the fast-dividing nonmuscle cells, in the ADM-treated myocardial cultures was reported (26). In this study, purified cultures were utilized to minimize the possible effects of changing cell population. The rhythmic contractions, characteristic of myocardial cells in culture, ceased with ADM treatment concomitant with significantly lower concentrations of ATP and PCr. It was suggested that a low energy charge might be a plausible direct underlying mechanism for the cessation of rhythmic contractions in culture (26, 27). In the present study, this possibility was further investigated, and it was demonstrated that ADM treatment decreased adenylate energy charge. Exogenous adenosine delays the ADM effect.

MATERIALS AND METHODS

Cardiac muscle cells were derived from neonatal Sprague-Dawley rats, 2 to 4 days old. The procedure for cell culture is based on that of Harary and Farley (10); however, the numerous modifications warrant a more detailed description of the method.

Hearts were excised, placed in a Petri dish containing cold saline A [140 mM NaCl, 5 mM KCl, 5 mM glucose, 4 mM NaHCO3, phenol red (20 mg/liter), streptomycin (1 mg/100 ml), penicillin (150 units/ml), Pfizer, Inc.] and squeezed to remove the blood. Hearts were then transferred to an empty Petri dish, pinched with scissors, rinsed 3 times with 5 ml 0.1% Bacto-trypsin (Difco Laboratories) in saline A, at 37°, and transferred to a trypsinizer flask (Belico Co.). The tissue was trypsinized repeatedly, 15 or 20 min each time, using 0.5 ml of 0.1% Bacto-trypsin per heart, and was centrifuged in a clinical centrifuge for 3 to 5 min. The first three 15-min supernatant extracts were discarded. Six 20-min extractions followed, and extracts were placed on ice for 10 min prior to centrifugation. Each pellet was suspended in 5 ml of growth medium [80% Ham’s Medium F-10, 10% fetal bovine serum, 10% horse serum, streptomycin (1 mg/100 ml), penicillin (150 units/ml), 1 mM CaCl2] and placed in the incubator at 37°, with water saturation and 95% air-5% CO2. The combined cell suspension was filtered through double-layer sterile gauze and centrifuged for 2 min. The pellet was resuspended in growth medium at a concentration of 1.5 hearts/ml. One-ml aliquots of cell suspension were placed in 60-mm Falcon No. 3002 Petri dishes containing 4 ml of growth medium and left in the incubator for 24 hr. The cell suspension was then aspirated, and the cells were rinsed once with 20 ml Ham’s Medium F-10. The cells were maintained in 4 ml Ham’s Medium F-10, 10% fetal bovine serum, 1% penicillin (150 units/ml), 1% streptomycin (1 mg/100 ml), 1 mM CaCl2, and 1 mM MgCl2. Each culture was maintained in a 5% CO2 incubator for 10 days at 37°.

0008-5472/79/0039-0000$02.00

Received November 17, 1978; accepted April 26, 1979.

1 This investigation was supported by Research Grant 572 from the American Heart Association, Greater Los Angeles Affiliate, and by research grants from the California Institute for Cancer Research and Cancer Research Coordinating Committee of the University of California.

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3 The abbreviations used are: ADM, Adriamycin; PCr, phosphocreatine; CPK, creatine phosphokinase.
2 hr. This last step separated out the epithelial and fibroblast cells which attach to the bottom of the dish during the 2-hr incubation. The suspension of myocardial cells from each plate was then transferred to an empty Petri dish and cultured. The highly homogeneous muscle cell cultures used in all experiments were approximately 90% pure at 24 hr in culture. Cultures were kept in the incubator at 37°, with water saturation and 95% air-5% CO₂; the medium was changed every 24 hr. The control and experimental plates were always from the same batch of cells, cultured for the same length of time.

The ADM-treated cells were cultured in the growth medium for 48 hr prior to a 3-hr treatment with 1.7 μM ADM (1 μg/ml) and then placed back in growth medium. The adenosine-treated cells were cultured in the growth medium containing 1 mM adenosine throughout the experiment. Cell density, percentage of beating cells, and rate of beating were determined and biochemical analyses were performed at 51, 72, and 96 hr in culture.

The extraction procedure for the chemical assays was that previously described (26). Protein was determined according to the method of Lowry et al. (15). The adenylate energy charge was calculated for each plate from the determinations of ATP, ADP, and AMP by the fluorometric enzyme assay technique (2). The mol fraction

\[
\frac{[ATP] + 0.5 [ADP]}{[ATP] + [ADP] + [AMP]}
\]

was calculated for each plate from the determinations of ATP, ADP, and AMP by the fluorometric enzyme assay technique (2). The mol fraction

\[
\frac{[PCr]}{[PCr] + [Cr]}
\]

was calculated from PCr, measured as previously described (26), and free creatine (Cr) (6). The significance of the observation was determined by paired t test.

RESULTS

The effect of ADM on myocardial cells in culture is density dependent. The observed effects are delayed with increasing age in culture, and cells cultured in medium containing 1.7 μM ADM at Time 0 fail to attach to the culture dish.4 A reproducible experimental protocol was met when myocardial cells were treated with ADM for 3 hr at 48 to 51 hr in culture, rather than at 24 to 27 hr in culture, as in previous studies with heterogenous cultures (26).

Chart 1A shows that both the average number of cells per field and the protein content per plate increased with time in culture. The addition of ADM to the cell culture inhibited cell division and also caused a slight decrease in number of cells per plate. The protein content per plate remained approximately constant from the time of ADM treatment.

Decreases in percentage of beating cells per total cell count and in the rate of beating with age in culture are typical of heart cells in culture and reflect the faster rate of nonmuscle cell division and, possibly, the crowding of cells (confluency), respectively (26). Chart 1B shows that, even in the myocardial culture that was originally 90% homogeneous, this variable was not eliminated. Furthermore, the addition of ADM resulted in a delayed (24 hr or more) cessation of beating of most cells; when occasionally beating cells were found, the rate was much slower.

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* M. W. Seraydarian and L. Artaza, unpublished data.
Table 1  Effect of ADM and adenosine on the concentration of adenine nucleotides

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ATP (nmol/mg protein)</th>
<th>ADP (nmol/mg protein)</th>
<th>AMP (nmol/mg protein)</th>
<th>Total adenine nucleotides (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51 hr</td>
<td>72 hr</td>
<td>96 hr</td>
<td>51 hr</td>
</tr>
<tr>
<td>Control</td>
<td>15.5 ± 0.9</td>
<td>15.7 ± 1.0</td>
<td>20.3 ± 0.8</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>ADM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.0 ± 1.3</td>
<td>10.7 ± 0.9</td>
<td>9.7 ± 1.8</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>1 mM adenosine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.7 ± 2.1</td>
<td>19.6 ± 1.5</td>
<td>24.1 ± 1.4</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>ADM, 1 mM adenosine</td>
<td>17.5 ± 1.8</td>
<td>14.6 ± 1.9</td>
<td>9.5 ± 1.9</td>
<td>4.8 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.E.
<sup>b</sup> ADM treatment at 48 to 51 hr.
<sup>c</sup> Adenosine treatment throughout the duration of the experiment.
but ADM had no further effect. It is not clear why the PCr mol fraction was decreased in these experiments, although it is plausible that in the course of the synthesis of ATP from adenosine the cytoplasmic CPK could catalyze the phosphor-ylation of ADP at the expense of PCr.

Chart 3 demonstrates that, concomitant with the increase in energy charge, the percentage of beating cells (Chart 3A) and the rate of beating (Chart 3B) of ADM-treated cells were also increased in the presence of adenosine. The energy charge of cells under physiological conditions is predicted to range between 0.85 and 0.90, and energy charge below 0.80 has been reported to be incompatible with normal function (1). An increase of adenylate energy charge in the myocardial cells in culture from 0.75 (ADM treated) to 0.80 (with adenosine) indeed maintained function; it is possible that the adenylate charge value for the beating cells exceeded 0.80. The increase in the rate of beating in the myocardial cells grown in the medium containing adenosine (Chart 3B, no ADM) without any increase of the already high adenylate charge might be partially attributed to a stimulation of the production of cyclic adenosine 3':5'-monophosphate (18). The latter might also contribute to the increased rate of beating in the ADM-treated cells grown in adenosine.

The addition of adenosine to the growth medium at the concentrations used in this study had no effect on either the number of cells per plate or the amount of protein per plate; however, 5 mM adenosine was toxic. There are reports in the literature on the toxic effects of adenosine on cells in culture (8).

DISCUSSION

The rat model system used in ADM studies was recommended by Mettler et al. (17) and Zbinden et al. (30) for use in the study of ADM cardiotoxicity.

The correlation of the cessation of beating of ADM-treated cells with a decrease in ATP concentration previously reported (26) was confirmed in the experiments summarized in Table 1. A decrease in adenylate charge at the functional site was suggested as the immediate cause, if high-energy charge is a prerequisite for energy-consuming reactions (1, 24). In this study, a decrease in adenylate charge in ADM-treated cells was demonstrated concomitant with cessation of rhythmic contractions. The PCr mol fraction was not decreased significantly. This was a somewhat surprising result, since in most instances a decline in muscle function is associated with a decrease in the PCr mol fraction (e.g., Refs. 23 and 24). However, if the contractile machinery indeed depends on the maintenance of the high energy charge by the myofibrillar CPK at the site of utilization, then a decreased CPK activity at that site could explain the observed results, i.e., the decreased adenylate charge, unchanged PCr mol fraction, and the cessation of contractions.

Immediately following the 3-hr ADM treatment (Chart 2A, 51 hr), the adenylate energy charge was not changed. Thereafter, at 72 and 96 hr, the adenylate charge dropped to a nonfunctional level and was not maintained at the functional site by the reverse CPK-catalyzed reaction

\[
\text{ATP} + \text{creatine} \rightarrow \text{ADP} + \text{PCr}
\]
even though the PCr mol fraction remained unchanged. Such inhibition of CPK would have a further consequence: because no creatine is generated, there is no stimulation of energy production, as expected if a change in PCr mol fraction functions in the feedback regulation of energy production in muscle (24). Whether both myofibrillar and mitochondrial CPK are inhibited as a result of ADM treatment is not known. Fluorodinitrobenzene, a known CPK inhibitor, was shown by Yang and Dubic (29) to inhibit CPK at both sites. The results reported in this study are compatible with ADM inhibition of myofibrillar CPK, predominantly. Since ADM does not directly inhibit CPK activity in vitro, it is conceivable that CPK synthesis is inhibited or degradation is enhanced by ADM treatment. This would also explain the delayed effect of ADM on the myocardial cells in culture and the particular susceptibility of the myocardium to ADM. The reverse CPK-catalyzed reaction (see above) is of particular significance in the heart, where anaerobic metabolism is limited.

The maintenance of ATP concentration and of the adenylate energy charge at the control level by adenosine spared the ADM effect. Functional integrity necessitates a high adenylate charge at the myofibrillar and/or membrane site. In the control cultures, exogenous adenosine does not affect the already high adenylate charge, even though the ATP concentration is increased. Exogenous adenosine in the ADM-treated cultures seems to have an access to the depleted functional compartment with a resulting delay in the decrease in adenylate charge correlated with a delay in the cessation of beating. Very little is known about the metabolic involvement of adenosine (8), but the existence of a pathway which leads to the synthesis of compartmentalized ATP pool from adenosine was indicated by Rapaport and Zamecnik (22). It is therefore possible that the generation of energy in the presence of adenosine is achieved at the site of depletion of the adenylate charge and can be maintained even if CPK is inhibited, as might be the case in the ADM-treated cells. It is also possible that the effect on beating is amplified by the stimulation of cyclic adenosine 3':5'-monophosphate production by adenosine, as suggested by Miles et al. (18).

REFERENCES


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*Cancer Res* 1979;39:2940-2944.

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