Pergamon

Biochemical Pharmacology, Vol. 50, No. 11, pp. 1943–1948, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02094-2

BIOCHEMICAL MODULATION OF TUMOR CELL ENERGY IV.

EVIDENCE FOR THE CONTRIBUTION OF ADENOSINE TRIPHOSPHATE (ATP) DEPLETION TO CHEMOTHERAPEUTICALLY-INDUCED TUMOR REGRESSION

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(*Received* 26 *April* 1995; *accepted* 7 *July* 1995)

Abstract—DNA-damaging agents, e.g. Adriamycin® (ADR), are reported to cause tumor regression by induction of apoptosis. A reduction in the intracellular content of ATP is part of the biochemical cascade of events that ultimately results in programmed death of the cell, or apoptosis. A chemotherapeutic three-drug combination (PMA) consisting of *N*-(phosphonacetyl)-L-aspartate (PALA) + 6-methylmercaptopurine riboside (MMPR) + 6-aminonicotinamide (6AN) significantly lowers levels of ATP in CD8F1 murine breast tumors *in vivo* and produces tumor regression by apoptosis. Addition of the DNA-damaging antitumor agent ADR to PMA was found to further significantly deplete ATP in CD8F1 murine breast tumors *in vivo* with a concomitant significant increase in the number of tumor regressions. The correlative biochemical and therapeutic results are consistent with, and support, the hypothesis that ATP depletion is a significant factor and, therefore, is a worthy therapeutic target in the production of apoptosis.

Key words: Adriamycin; adenosine triphosphate; apoptosis; tumor regression

ADR§ is a DNA-damaging agent [1] that has been shown to induce death by apoptosis in cancer cells *in vitro* [2]. PMA, a three-drug combination consisting of PALA + 6-MMPR + 6AN, has been shown to induce tumor regressions *in vivo*, and the combination of PMA + ADR has been shown to enhance markedly tumor regressions *in vivo* due to apoptosis when compared with either the three-drug combination without ADR or with ADR alone at its maximum tolerated dose [3].

A number of investigators [2, 4–12] have presented data that are supportive of the hypothesis that anti-cancer agent-induced DNA damage activates poly(ADP-ribose)polymerase, which lowers NAD pools and, in turn, leads to ATP depletion sufficient to effect apoptotic cell death. Since-PMA has been documented to depress tumoral ATP levels [13], and since ADR should (as a DNA-damaging agent) also produce ATP depletion, it seemed reasonable to administer the drugs in combination, (PMA + ADR), and complementary therapeutic activity was indeed documented [3].

The purpose of the present work was to measure the effect of the addition of ADR to the three-drug combination (PMA) on both intracellular concentrations of ATP in tumor, and on tumor regression. The addition of ADR to PALA + MMPR + 6AN was found to further significantly lower the intracellular concentrations of ATP in tumor when compared with that produced by the three-drug combination without ADR. Moreover, the further significant lowering of intracellular concentrations of ATP in tumor after treatment with the four-drug combination was correlated with a concomitant increase in the number of tumor regressions.

MATERIALS AND METHODS

CD8F1 spontaneous murine breast cancer system

This breast tumor was included in the murine tumor testing panel of the National Cancer Drug Screening Program [14]. CD8F1 hybrid mice bearing single spontaneous, autochthonous breast tumors were selected from our colony, which has been described previously [15, 16]. A tumor brei was made from 3–4 of these spontaneous tumors and employed to produce single first-generation subcutaneous transplants to syngeneic 3-month old Balb/c × DBA/8F1 (hereafter called CD8F1) mice. Six separate first-generation tumor transplant experiments are reported, each from a different brei (i.e. each from a different group of 3–4 spontaneous tumors) and, therefore, each transplant group contained a different number of chemotherapeutically sensitive and/or resistant tumor cells.

As in all spontaneous tumors, whether human or murine, each individual cancer has a heterogenous neoplastic cell population. For each experiment, the first-generation transplants of CD8F1 breast tumors were obtained from a tumor cell brei made by pooling 3–4 spontaneously arising tumors and, hence, the individual transplants in each experiment developed from a single brei that, although common to all the mice in that experiment, had a neoplastic cell composition that was likely somewhat different from that in another experiment. This makes for quantitative differences between exper-

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§ Abbreviations: ADR, Adriamycin®; PALA, *N*-(phosphonacetyl)-L-aspartate MMPR, 6-methylmercaptopurine riboside; 6AN, 6-aminonicotinamide; and PMA, a combination of PALA + MMPR + 6AN.

iments. For example, a single spontaneous tumor may have a large subset of cells resistant to a particular agent, and the pooling of this tumor with three more sensitive spontaneous tumors to make up a single tumor cell brei then results in the spread of these resistant cells throughout all the individual tumor transplants of the experiment. Consequently, the tumor-bearing mice in such an experiment are more refractory to tumor-regressing chemotherapy than mice in an experiment made from a tumor brei coming from 3–4 tumors all of which contain a paucity of resistant cells. Indeed, for this reason, an experiment comprised only of spontaneous tumors may yield therapeutic results that are more impressive than those of an experiment with first-passage spontaneous tumors. The need to employ first-passage spontaneous tumors is conditioned by economics as a tumor brei from 3–4 spontaneous tumors may yield 100–150 tumor-bearing animals only one transplant step away from the heterogeneity of spontaneous tumors. Quantitative measurements of any individual parameter (e.g. magnitude of biochemical response and antitumor sensitivity to a particular drug treatment) may be somewhat different from experiment to experiment with first-passage spontaneous tumors, but the findings will be quantitatively relevant within individual experiments, as will similar trends among experiments.

In approximately 3–4 weeks, when transplanted tumors were well advanced and measurable, all tumor transplants were measured, and the tumor-bearing mice were distributed among experimental groups (10 tumor-bearing mice/group) so that mice carrying tumors of approximately equal weight were represented in each treatment group. The average weight of all tumors used in this series of experiments was close to 150 mg at the beginning of treatment.

Tumor measurements

Two axes of the tumor (the longest axis, L, and the shortest axis, W) were measured with the aid of a Vernier caliper. Tumor weight was estimated according to the formula: tumor weight (mg) = L (mm) × W (mm)^2/2.

Chemotherapeutic agents

MMPR and 6AN were obtained from the Sigma Chemical Co., St. Louis, MO. ADR was obtained from Adria Laboratories, Columbus, OH. Each of these agents was dissolved in 0.85% NaCl solution immediately before use. Drug solutions were made fresh prior to injection. PALA, obtained from the Department of Health, Education, and Welfare, USPHS of the National Cancer Institute, Bethesda, MD, was dissolved in 0.85% NaCl solution, and the pH was adjusted to 7.2 to 7.5 with 1 N NaOH before adjustment to final volume. All agents were administered so that the desired dose was contained in 0.1 mL/10 g of mouse body weight. ADR was administered i.v., and all other agents were administered i.p. These drugs were administered in a timed sequence, with PALA administered 17 h before MMPR + 6AN, and ADR administered 2.5 h after MMPR + 6AN.

ATP assay

Intracellular ATP levels were measured in supernatants of individual tumors homogenized in 1.2 N perchloric acid and centrifuged at 10,000 *g* at 4°, for 10 min. ATP content was determined on a Turner model 20e luminometer using a luciferin/luciferase assay [17] as modified for neutralized extracts of solid tumors. The ATP assay was validated in each experiment using exogenous ATP standards. Background readings were subtracted from both standards and sample. A 100-μL aliquot of sample was mixed with 100 μL of luciferase, and the light output was recorded on the luminometer. All samples were run in triplicate; aliquots of diluted luciferase were used from the same lot number. Standard curves for ATP (0.02×10^{-8} to 10^{-7} M) were prepared for each experiment. The amount of ATP was quantitated by comparing light output from an unknown sample with that from a standard solution of ATP. Within this range, standard curves were linear and highly reproducible. The ATP content of samples was within the linear range of the standard curve. Protein content was assayed by the method of Lowry method *et al.* [18]. In all experiments, the intracellular levels for ATP in tumors from treated animals were compared with the intracellular levels of ATP in control tumors from saline-injected animals measured at the same time point. In each of the experiments, levels for intracellular ATP content in tumor were measured in saline-injected (untreated) control, PMA-treated and PMA + ADR-treated groups of tumor-bearing mice. Variations in intracellular ATP concentrations as a result of circadian rhythms were not a contributory factor in the experiments reported, as all of the experiments were performed on the same time schedule and animals were killed (by cervical dislocation) at the same time of day. Saline-treated control mice bearing tumors were killed at the same time as tumor-bearing mice that received drug treatment. Single agent controls for each of the drugs, as well as the rationale for the timing sequence, have been previously determined empirically and have been published previously [13].

Determination of chemotherapy-induced tumor regression rate

The initial size of each tumor in each treatment group was recorded prior to the initiation of treatment. Tumor size was recorded weekly during treatment and again at 7 days after the last (third) weekly course of treatment. For each experiment, a single observer made all measurements in order to avoid variation in caliper measurements from individual to individual. By convention, partial tumor regression is defined as a reduction in tumor volume of 50% or greater compared with the tumor volume at the time of initiation of treatment. The partial regression rate obtained from a particular treatment is expressed as a percentage, i.e. the number of partial regressions per group/total number of animals per group × 100.

Statistical evaluation

A paired Student's *t*-test was used for statistical evaluation. For all evaluations, differences between groups with P values ≤ 0.05 were considered to be significant. For each experiment, the mean (N = 8 tumors/group) ± SEM of values for intracellular ATP concentrations was calculated at each time point.

RESULTS

Tables 1 and 2 report intracellular ATP levels in tumors from two representative experiments. The effects on intracellular concentrations of ATP in tumors of the

Table 1. Effect of $PALA_{100}$ + $MMPR_{150}$ + $6AN_{10}$ ± ADR_6 on the intracellular concentration of ATP in tumors of CD8F1 mice: Significant depletion of ATP at 24 h

Group/Treatment	ATP (μg/mg protein)
(1) Saline control	1.85 ± 0.18 (100)
(2) PALA - (17 hr) → MMPR + 6AN	0.93 ± 0.1† (50)
(3) PALA - (17 hr) → MMPR + 6AN - (2.5 hr) → ADR	0.63 ± 0.06†‡ (34)

Intracellular concentration of ATP was measured 24 hr after the administration of MMPR + 6AN following one course of treatment. PALA [*N*-(phosphonacetyl)-L-aspartate] was administered 17 hr pirior to MMPR + 6AN; MMPR = 6-methylmercaptopurine riboside; 6AN = 6-aminonicotinamide. Subscripts in the title = mg/kg body weight. All agents were administered i.p. except for ADR (Adriamycin), which was administered i.v., 2.5 hr after MMPR + 6AN. Values are means ± SEM, N = 8; numbers in parentheses represent percent of the 24-hr saline control (Group 1).

* Experiment 131; male mice.

† Statistically significant from the saline control (Group 1), $P \leq 0.05$.

‡ Statistically significant from Group 2, $P \leq 0.05$.

three-drug regimen, PALA + MMPR + 6AN (PMA), and the four-drug regimen, PMA + ADR, are compared with each other as well as with the ATP content in the appropriate saline-injected control tumors measured at the same time point.

In Table 1, at 24 h, the intracellular concentration of ATP in tumors from animals treated with PMA, or with PMA + ADR, were both reduced significantly compared with the saline-treated control tumors, and the ATP concentration in tumors from animals receiving the four-drug regimen (group 3) was significantly lower than that in tumors from animals which receiving the three-drug regimen (group 2).

In the experiment in Table 2, at the 24-, 48-, and 72-hr time points the intracellular concentrations of ATP in tumors of group 2 (PMA), and of group 3 (PMA + ADR), were reduced significantly compared with the saline-treated control tumors. However, at 48 h the ATP concentrations in tumors treated with the four-drug regimen (group 3) were significantly lower than those in tumors from animals that received the three-drug regimen (group 2). At 72 h, the ATP concentration in animals receiving the three-drug regimen and the four-drug regimen were, respectively, further lowered significantly to 19% and 15% of saline control levels, but were not significantly different from each other.

In Table 3 the ATP depletion measured in tumors from six separate experiments (using different spontaneous tumors as the source for the six different first-generation transplants; see Materials and Methods) was compared between groups treated by the three-drug regimen (PMA), and those treated with the four-drug regimen (PMA + ADR). In addition, the percent of tumor regressions, after three weekly courses of PMA with or without the addition of ADR, are presented for each of the six separate experiments. Five of the six experiments evidenced greater and statistically significant ATP depletion in mice treated with the quadruple drug regimen, PMA + ADR, when compared with the respective three-drug control (PMA), in at least one of the time points measured (24, 48, or 72 h). Moreover, in *all* six experiments greater therapeutic activity was measured (as percent of tumor regressions) in mice treated with the quadruple drug combination, (PMA + ADR) over that found in the respective three-drug PMA-treated control group. Thus, although six somewhat quantitatively different anti-tumor responses (i.e. percent tumor regressions) were obtained (as would be expected in first-generation tumors from a spontaneous murine model system that exhibits marked tumor heterogeneity), a correlation of chemotherapeutically augmented tumor cell deaths (regressions) with greater depletion of tumoral ATP levels was demonstrated.

DISCUSSION

When viewed as a panel of responses in a heterogenous tumor model, these data exhibit the variation expected in such a system, but the correlation between enhanced therapeutic findings and greater depletion of ATP levels is clear. In *in vitro* studies with long-carried cell cultures, the homogeneity of the cells may be so great that there is "no quantitative variability among different experiments" [19]. Likewise, in common transplantable tumor models (such as Sarcoma 180), which tend to lose their heterogeneity due to repeated transplantation, the tumors may behave similarly in one individual syngeneic host compared with another, and therefore the results in one experiment compared with another may be very similar. By contrast, spontaneous tumors in CD8F1 mice, as in humans, have heterogeneous neoplastic cell populations and, therefore, differ in drug sensitivity and quantitative biochemical findings from one individual to another. Thus, quantitative measurements of any individual parameter (e.g. the control ATP level in Table 1 is 1.85 ± 0.18 μg ATP/mg protein, and in Table 2, it is 2.5 ± 0.24) may be somewhat different from experiment to experiment with first-passage spontaneous tumors (see Materials and Methods), but the findings will be quantitatively relevant within individual experiments (i.e. each experiment has its own control) as will similar trends among experiments. These findings and relevant conclusions were determined by the NCI prior to inclusion of the CD8F1 breast tumor model in the NCI's screening program [14].

Following chemotherapeutically induced damage to DNA, a biochemical cascade may ensue that results in programmed cell death, apoptosis [20]. This biochemical cascade may include the following: activation of chromatin-bound poly(ADP-ribose)polymerase, which results in a lowering in intracellular concentration of NAD, which, in turn, prevents the regeneration of ATP. The depletion of ATP, in turn, may disrupt many energy-dependent mechanisms, e.g. the functioning of ATP-dependent, membrane-bound pumps. The failure of the pumps may result in a rise in intracellular calcium levels and a fall in pH, which can activate intracellular endonucleases resulting in cleavage of DNA in specific patterns of nucleosome-length fragments, and apoptotic death of the cell. ATP depletion may be a significant factor in the production of chemotherapeutically induced cell death, a view previously expressed by many [2, 4–12]. The fact that the latter publications have stressed ATP is not the only reason ATP is singled out as a likely major contributor to the anti-tumor effects. PMA has a

Table 2. Effect of $PALA_{100}$ + $MMPR_{150}$ + $6AN_{10}$ ± ADR_6 on the intracellular concentration of ATP in tumors of CD8F1 mice: Significant depletion of ATP at 48 hr*

Group/Treatment	ATP (μg/mg protein) (A) 24 hr after MMPR + 6AN	(B) 48 hr after MMPR + 6AN	(C) 72 hr after MMPR + 6AN
(1) Saline control	2.50 ± 0.24 (100)	2.51 ± 0.21 (100)	2.42 ± 0.17 (100)
(2) PALA - (17 hr) → MMPR + 6AN	1.42 ± 0.12† (57)	1.17 ± 0.08† (47)	0.46 ± 0.07† (19)
(3) PALA - (17 hr) → MMPR + 6AN - (2.5 hr) → ADR	1.09 ± 0.17† (44)	0.86 ± 0.08†‡ (34)	0.37 ± 0.05† (15)

Intracellular concentration of ATP was measured at indicated times (24, 48 and 72 hr) after the administration of MMPR + 6AN following one course of treatment. PALA [*N*-(phosphonacetyl)-L-aspartate] was administered 17 hr prior to MMPR + 6AN; MMPR = 6-methylmercaptopurine riboside; 6AN = 6-aminonicotinamide. Subscripts in the title = mg/kg body weight. All agents were administered i.p. except for ADR (Adriamycin), which was administered i.v., 2.5 hr after MMPR + 6AN. Values are means ± SEM, N = 8; numbers in parentheses represent percent of the appropriately timed (24, 48 or 72 hr) saline control (Group 1).

* Experiment 148; male mice.

† Statistically significant from the appropriate saline control (Group 1), $P \leq 0.05$.

‡ Statistically significant from Group 2, 48 hr, $P \leq 0.05$.

major effect on intracellular ATP pools (e.g. MMPR, as an analog of adenosine, would be expected to exert its major effect on ATP levels; and, further, ATP as the most abundant of all the nucleoside triphosphates might be expected to be the most affected by a regimen containing MMPR). We have previously published data following treatment with PMA, indicating reductions in all four ribonucleoside triphosphates [21]. At 48 h following treatment with PMA, ATP levels reached a nadir of 15% of saline control ATP level and thus was the most severely depleted of all the ribonucleoside triphosphates (rNTPs). Following treatment with PMA, levels of UTP, GTP and CTP were lowered to 50, 48 and 46%, respectively, of saline control levels at either 24 or 48 h after MMPR + 6AN. The measurements for ATP depletion as well as the reduction of the other high-energy rNTPs are actually *overestimates* in measurements of *total* tumor rNTPs since we record only partial (not complete) tumor regressions. The biochemical changes thus measured represent the sum of the biochemical changes in chemotherapeutically induced cell death as well as normal biochemical events in chemotherapeutically refractory viable tumor cells. Moreover, as just discussed, although there is reason for PMA to affect intracellular ATP concentrations, there is no reason *a priori* to expect ADR to induce ATP depletion except for the depletion induced by poly(ADP-ribose)polymerase activation due to the ADR-induced DNA damage. Thus, if there is a possible relationship between ATP depletion and cell death (i.e. tumor regression), then the combination of these agents

Table 3. ATP-depleting and anti-tumor effects of the triple-drug combination, PALA + MMPR + 6AN (PMA), versus the quadruple-drug combination, PMA + Adriamycin (ADR)*

Expt. No.	Depletion of ATP by PMA + ADR compared with PMA after the first course	% Tumor regressions after three weekly courses† PMA	PMA + ADR
129	Significantly greater‡	0	60
131	Significantly greater‡	0	80
132	Significantly greater‡	0	10
147	Significantly greater‡	10	50
148	Significantly greater‡	70 (10% CR)	80 (40% CR)
152	Not significantly greater	10	80

* ATP was measured at each of three time points (24, 48, and 72 hr) after the initial administration of MMPR + 6AN. First generation transplants of CD8F1 spontaneous breast cancers were approximately 100 mg when treatment was initiated. $PALA_{100}$ was administered 17 hr prior to $MMPR_{150}$ + $6AN_{10}$. Subscripts = mg/kg body weight. All agents were administered i.p., except for ADR_6, which was given i.v., 2.5 hr after MMPR + 6AN.

† Partial tumor regressions = a reduction in tumor volume of 50% or greater compared with the tumor volume at initiation of treatment unless otherwise noted; CR = complete tumor regressions. Tumors were measured 1 week after the third course.

‡ Statistically significant (Student's *t*-test; $P \leq 0.05$) depletion of ATP in at least one of the three time points during the 72-hr period after the administration of MMPR + 6AN in the first course.

(PMA + ADR) might be expected to enhance both ATP depletion and the rate of tumor regressions; those indeed are the findings and support, but do not necessarily constitute, a cause-and-effect relationship. Relative to the relationship between the time (e.g. 24, 48 or 72 h after chemotherapy) of significant ATP depletion and the onset of apoptosis, the question may be asked, when does apoptosis set in? The activation of poly(ADP-ribose) synthesis [which is the initiating step for the depletion of ATP by poly(ADP-ribose)polymerase] following chemotherapy appears to be a cell-type specific phenomenon; also, there are variations in response among susceptible but different cell lines of the same histiotype [22]. These may be some of the reasons for the heterogeneity of the timing of therapeutic response. We have observed that different first-generation CD8F1 tumor transplants may undergo 50% or greater partial tumor regressions beginning 24, 48 or 72 h after effective tumor-regressing chemotherapy (data not shown). Consequently, we have made ATP measurements 24, 48, and 72 h after chemotherapy.

The finding of lowered intracellular concentrations of ATP is not an artifact due to necrosis, since microscopic analysis of the tumors after treatment by the quadruple combination has revealed extensive apoptotic (not necrotic) cell death [3]. Chemotherapeutically induced necrosis invariably presents as sheets of dead cells, and if ATP depletion is found in the presence of extensive numbers of dead cells, it is not clear whether the decrease in ATP is a cause for the dead cells or a result of biochemical analysis of mostly dead cells. Apoptosis is a discrete phenomenon that often is not conspicuous histologically. It is estimated that apoptotic bodies remain visible by light microscopy for only a few hours before disappearing by engulfment into, and digestion by, adjoining cancer cells [23]. However, as a continuously ongoing process for several days after each treatment, chemotherapeutically induced apoptosis can effect profound tumor shrinkage (i.e. tumor regression).

In conclusion, PMA + ADR produced a significantly greater depletion of intracellular concentrations of ATP compared with the triple drug combination (PMA). Further, the addition of ADR to PMA effected a significant increase in apoptotic antitumor activity as measured by both microscopic analysis and an increase in the rate of partial tumor regressions [3]. The correlative biochemical (i.e. ATP depletion) and therapeutic results are consistent with and support the hypothesis that ATP depletion appears to be a significant factor, and, therefore, a worthy therapeutic target in the enhancement of chemotherapeutically induced apoptosis in tumor cells.

However, although many investigators have demonstrated that high levels of DNA damage in tumor cells by chemotherapeutic agents effect cell death, which is preceded by activation of poly(ADP-ribose)polymerase, consumption of NAD^+ pools, and consequent ATP depletion, it is not proven that the latter two effects—NAD^+ and ATP depletion—are the cause of the cell deaths. Reports that chemotherapeutically induced cell death can ensue in the absence of NAD^+ depletion and/or ATP depletion [24–27] suggest that they may be epiphenomena. Whether or not these reports adequately refute the hypothesis that NAD^+ depletion can lead to ATP depletion and result in cell death requires further investigation, and indeed is being pursued in a number of laboratories as well as our own. Our newer findings demonstrate that ATP depletion occurred *prior* to the onset of apoptosis.* Thus, it is more likely that ATP depletion is causal to the apoptotic cascade. However, we do not believe that this finding conclusively demonstrates this point, as a still more *direct* causative relationship to apoptosis needs to be documented. It should be noted that Corcoran *et al.*, in their recent review of apoptosis [28], found that "the mechanisms involved in apoptosis differ in various cell types so that a single scheme cannot describe all the processes known to involve apoptotic morphology and death ... as a basic biologic phenomena that fulfills diverse needs for controlled cell death, apoptosis is undoubtedly subject to variation in mechanism."

In his recent review of apoptosis, Stewart [29] also noted that "findings may be specific to the cell line and/or the toxic agent." At this writing, it can only be said that the findings in this report support the initial hypothesis, but do not constitute proof. However, the therapeutic results (and biochemical correlation) obtained by pursuing this hypothesis-driven research remain valid and, since the majority of the employed agents (PMA) are not directly genotoxic, striking.

Acknowledgement—This work was supported by Public Health Service Grant CA25842 from the National Cancer Institute.

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