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Apoptosis induced in advanced CD8F1-murine mammary tumors by the combination of PALA, MMPR and 6AN precedes tumor regression and is preceded by ATP depletion

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Abstract The drug combination *N*-(phosphonacetyl)-L-aspartic acid (PALA), methylmercaptopurine riboside (MMPR) and 6-aminonicotinamide (6AN), referred to as PMA, induces regressions of advanced CD8F1 murine mammary carcinomas in vivo. We demonstrated that CD8F1 tumor regressions were preceded by the appearance of apoptotic bodies, as observed by microscopic examination of morphology and TUNEL end-labeling, and fragmentation of DNA into nucleosomal "ladder" patterns. These indications of apoptosis were present as early as 6 h after simultaneous administration of MMPR and 6AN and further increased by over fivefold during the next 3 to 6 h, then remained at 7 to 12.8% (0.6 to 2.4% in saline-treated controls) of the cell population for at least 24 h after MMPR + 6AN administration. The 5′-phosphate derivative of MMRP, MMPR-5P, which inhibits de novo purine biosynthesis, was present at a "steady-state" level, and significant (40%) depletion of ATP had occurred by 3 h and both of these events preceded the onset of apoptosis. In addition, MMPR-5P was retained in CD8F1 tumors at a high level over a prolonged period (>96 h) even as tumors were undergoing regression. The prolonged presence of MMPR-5P was important for optimal chemotherapeutic effect, since treatment with iodotubercidin (IodoT), an inhibitor of MMPR/adenosine kinase, 6 h after MMPR + 6AN administration prevented the prolonged accumulation of MMPR-5P and reversed the regression of CD8F1 tumors. In addition, compared to the PMA-treated group, there was a significant restoration of ATP levels after treatment with IodoT. In individual PMA-treated CD8F1 tumors the degree of ATP depletion was found to correlate with the degree of tumor shrinkage at 24 h, after tumors had sufficient time to respond to treatment. These results define the time-course of drug-induced apoptosis in CD8F1 tumors, show that ATP depletion occurs prior to apoptosis and demonstrate that prolonged retention of MMPR-5P is associated with optimal chemotherapy. Collectively, these results suggest that the depletion of ATP by PMA treatment may be a component of the biochemical apoptotic cascade in the CD8F1 tumor.

Key words Methylmercaptopurine riboside · *N*-(phosphonacetyl)-L-aspartic acid · 6-Aminonicotinamide · Mammary carcinoma · Tumor regression · Iodotubercidin · Apoptosis · Fluorouracil

Abbreviations *MMPR* methylmercaptopurine riboside · *MMPR-5P* the 5′-phosphate derivative of MMPR · *PALA* N-(phosphonacetyl)-L-aspartic acid · *6AN* 6-aminonicotinamide · *PMA* chemotherapeutic combination PALA, MMPR and 6AN · *PR* partial regression · *IodoT* iodotubercidin · *5FUra* 5-fluorouracil

Introduction

A combination of drugs consisting of *N*-(phosphonacetyl)-L-aspartic acid (PALA), methylmercaptopurine riboside (MMPR) and 6-aminonicotinamide (6AN), termed PMA, has been developed which induces regressions in advanced CD8F1 murine mammary carcinomas in vivo either as the PMA regimen alone [27] or in combination with other agents such as 5-fluorouracil (5FUra), adriamycin or radiation [20, 29, 30, 39]. PMA was originally designed to interfere with cellular energy production by inhibition of ATP synthesis [27]. While PMA has been observed to deplete ATP levels in

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regressing CD8F1 tumors [39, 40] and these regressions have been associated with apoptosis or programmed cell death within the tumors [29, 30], the relationship between tumor regression, ATP depletion and the induction of apoptosis has not been well characterized. However, we have recently shown that prolonged tumor retention of MMPR-5′ phosphate (MMPR-5P) is necessary for the optimal effect of PMA treatment [32] and now further define the timing and role of ATP depletion and apoptosis in CD8F1 tumor regressions following PMA treatment.

In the study of chemotherapy-induced cell death or "chemoapoptosis", a number of investigators have observed a cascade of events which is induced by DNA-damaging agents [2, 4, 12, 26, 35, 43]. According to this scheme, many anticancer agents induce a high degree of DNA breaks. DNA strand breaks then serve to activate the nuclear enzyme, poly(ADP-ribose)polymerase, which functions to inactivate certain proliferation-related proteins by attaching polymers of (ADP)-ribose at distinct sites on the protein at the expense of substrate NAD. If sufficient NAD is utilized, the shuttling of reducing equivalents is impaired, leading to inefficient regeneration of ADP to ATP with subsequent ATP depletion and eventually cell death. Since this cascade of events has been documented in numerous systems, it has been suggested to be a common pathway leading to apoptotic cell death [26].

In view of this consensus cascade of DNA-damaging agents, the tumor-regressing effect of PMA is of particular interest since none of the three agents is considered to have a primary or direct damaging effect on DNA. Thus, the main effect of PALA is inhibition of de novo pyrimidine biosynthesis at the level of aspartate transcarbamylase [5]; MMPR inhibits de novo purine biosynthesis as the 5′-monophosphate derivative, MMPR-5P, at the level of amido phosphoribosylpyrophosphate transferase [45, 47]; and 6 aminonicotinamide, as an NADP analogue, inhibits the pentose phosphate pathway at 6-phosphogluconate (6PG) dehydrogenase [18]. In addition, while each of the agents individually possesses some tumor growth *inhibiting* activity, combination treatment with all three agents is necessary for tumor *regressing* activity in the CD8F1 tumors [27]. Depletion of ATP alone is apparently not sufficient to initiate tumor regression by apoptosis, since administration of single de novo purine synthesis inhibitors may deplete ATP without causing regressions [16]. However, following treatment with PMA, where depletion of ATP is driven by prolonged MMPR-5P levels over an extended period of time [32], ATP depletion appears to be a significant contributor to apoptosis.

We have been interested in defining the mechanism of PMA-induced tumor regression in order to enhance chemotherapy and in defining biochemical parameters which will facilitate translation of PMA efficacy to the clinical setting. In the study reported here we showed that PMA induces CD8F1 tumor regression by the apoptotic process and that ATP depletion precedes this process. We also demonstrated that prolonged retention of MMPR-5P within the tumor is necessary for continued ATP depletion and tumor regression.

Materials and methods

Drugs

MMPR and 6-AN were purchased from Sigma Chemical Co. Iodotubercidin (IodoT) was purchased from Research Biochemicals International (RBI). PALA was provided by the NCI. Drugs were administered by intraperitoneal (i.p.) injection.

CD8F1 spontaneous mammary tumor

The CD8F1 spontaneous mammary tumor model has been previously described [28, 38] and is included in the murine testing panel of the National Cancer Drug Screening Program [14]. CD8F1 hybrid mice bearing single spontaneous, autochthonous breast tumors were selected from the mouse colony. A tumor brei was made from three to four of these tumors and transplanted subcutaneously in 3-month-old syngeneic Balb/C × DBA/8 (CD8F1) mice. In 3–4 weeks, tumor-bearing mice with equisized tumors were distributed among the treatment groups, and experiments were conducted in these first passage transplants. The average tumor weights were around 150 mg at the beginning of treatment.

All spontaneous tumors, whether human or murine, have a heterogeneous neoplastic cell population. Since each experiment consisted of a brei composed of several different spontaneous tumors, the neoplastic cell composition was likely somewhat different from experiment to experiment, resulting in certain quantitative differences between experiments. However, each experiment had its own control and the results are quantitatively relevant within individual experiments, as are trends among experiments.

Tumor measurements

Two axes of the tumor (the longest axis L, and the shortest axis W) were measured with a caliper. Tumor weight was estimated by the equation:

$$\text{Tumor weight (mg)} = L\ \text{(mm)} \times [W^2\text{(mm)}/2]$$

Determination of tumor regression rates

The initial size of each treatment group was recorded prior to initiation of treatment. Tumor size was recorded during treatment and at various times following 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 (PR) was defined as a reduction in tumor volume of 50% or more compared to the tumor volume at the time of initiation of treatment.

Statistical evaluation

Student's t-test was used for evaluation of measured biochemical differences between treatment groups, and these differences were considered to be significant with $P \leq 0.05$.

Treatment regimens

The standard PMA regimen consisted of administration of $PALA_{100}$ followed after 17 h with $MMPR_{150}$ and $6AN_{10}$ (subscripts refer to the dose in mg/kg). IodoT at 3 mg/kg was administered at 6 or 24 h after MMPR + 6AN treatment. $FUra_{75}$ was

given 2.5 h after MMPR + 6AN. Each of the agents was dissolved in 0.85% NaCl solution for i.p. injection. The desired dose was contained in 0.1 ml/10 g of mouse body weight.

HPLC methodology

Mice were anesthetized i.p. with pentobarbital (65 mg/kg). Tumors were excised free of gross necrotic tissue and immediately freeze-clamped using tongs cooled in liquid nitrogen. Frozen tumors were homogenized in ice-cold 0.4 *N* perchloric acid (1 ml/0.1 g tumor weight). The acid-insoluble fraction was removed by centrifugation (10 000 *g* for 5 min) and the acid-soluble fraction was neutralized by extraction with a mixture of tri-*n*-octylamine in Freon [17] and analyzed by HPLC. NTP (nucleoside triphosphate) values were normalized to the protein content of the acid-insoluble fraction and NTP values are expressed as nanomoles per milligram protein. Protein was solubilized in 0.5 *N* NaOH and quantitated [25]. HPLC analysis was performed using a Waters 840 HPLC, a WISP autosampler and tandem UV detection at 254 and 290 nm. Nucleotides were separated on a Whatman SAX column starting with 3 m*M* ammonium phosphate, pH 3.1, and proceeding in two steps to 70% of high-salt buffer, 0.5 *M* ammonium phosphate, pH 4.5. The injection volume of each sample was 200 µl. The run time was 70 min at a flow rate of 1.5 ml/min with a 20-min equilibration period between analyses. MMPR-5P levels are expressed as nanomoles per milligram protein.

Morphologic examination and TUNEL analysis of apoptotic bodies in CD8F1 tumor sections

Tumor-bearing CD8F1 mice were sacrificed at various times after PMA treatment. Tumors were excised and immediately placed in vials containing 20 ml phosphate-buffered formalin. Tumor tissues were paraffin-embedded and microscopically examined for apoptosis by the TUNEL end-labeling assay [13] as well as by morphological examination on two consecutive formalin-fixed paraffin-embedded sections. Areas with the highest rate of apoptosis were identified and a minimum of 500 cells per section were examined. Foci of geographic confluent necrosis were minimal and equivalent in both controls and treated tumors. Only cells with definite brown nuclear staining were counted as positive for TUNEL. H&E sections were examined for the range of morphological features associated with apoptosis. Cells with marked cytoplasmic condensation and nuclear pyknosis were included in the positive count as well as those showing well-developed apoptotic bodies (single or multiple ovoid eosinophilic mass containing one or more pyknotic chromatin fragments). All sections were examined by one pathologist. Corresponding TUNEL and H&E sections were scored independently and on two separate occasions to avoid any scoring bias. Treatment status was not revealed until all sections were scored. The results were analyzed using Student's *t*-test for comparison of apoptotic rates between treated and untreated groups.

Results

The effect of PMA treatment on ATP depletion and loss of tumor mass

The relationship of ATP depletion to tumor regression was studied. PMA-treated CD8F1 tumors were harvested at 10 and 24 h and individual tumors were examined for a correlation between ATP levels and tumor regression. As shown in Table 1, the average tumor weight for both the 10- and 24-h groups contained similar amounts of MMPR-5P and the levels of ATP depletion were similar for the 10- and 24-h PMA-treated groups, which were both significantly reduced compared to saline controls. In addition, a trend toward tumor mass reduction was seen in both groups, but was only significantly different from the saline tumors in the 24-h PMA-treated group. When the degree of ATP depletion was examined against the percentage change in tumor mass, there was no correlation for the 10-h sample; however, at 24 h there was a good correlation ($r = 0.7$) between ATP depletion and the decrease in tumor mass (Fig. 1).

It is also of interest that ATP was depleted to a greater extent (50% of the control value) than other metabolites at the earlier (10-h) time-point; for example, NAD 84%, UTP 90%, and GTP 109% of the control values. At 24 h, ATP was still depleted (54% of control) to the greatest degree, but there was a more uniform depletion of other metabolites: NAD 65%, UTP 66% and GTP 79% of control values. In addition, the ATP/ADP ratio at 10 h was 75% of control compared with a value of 83% at 24 h, suggesting that inhibition of ATP regeneration from ADP was important at the earlier time-point in addition to adenylate restriction. Collectively, these results indicate that the decrease in ATP was an early event which occurred in advance of the reduction in tumor mass. At the earlier time-point ATP was disproportionately depleted and tumor mass reduction was

Table 1 Harvested tumor weight, ATP and MMPR-5′ phosphate levels at 10 and 24 h after MMPR and 6AN treatment of tumors from PMA-treated CD8F1 mice. CD8F1-tumor bearing mice ($n = 7$) were treated with PALA 17 h prior to treatment with MMPR + 6AN. Thereafter at 10 and 24 h, tumors were excised and freeze-clamped for biochemical measurements. Tumors were subsequently weighed and the nucleotide pools were extracted for measurement of ATP, MMPR-5P and other nucleotides by means of HPLC. Tumors averaged 120 mg at initiation of chemotherapy (Exp. C2828/N143) as determined by caliper measurement (*ND* none detected).* Significant with respect to saline control

	10 hours		24 hours	
	Saline	Treated	Saline	Treated
Tumor weight (mg)	320 ± 170	270 ± 100 (84%)	360 ± 160	220 ± 90 (61%)*
Metabolite levels (nmol/mg total protein)				
MMPR-5P	ND	2.64 ± 0.94	ND	2.58 ± 1.01
ATP	11.06 ± 1.87	5.48 ± 1.22 (49.6%)*	9.98 ± 1.47	5.42 ± 0.09 (54.3%)*

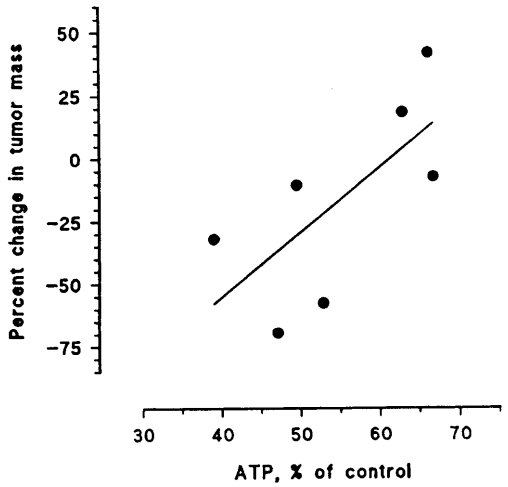

Fig. 1 Correlation of ATP levels with change in CD8F1 tumor mass at 10 and 24 h after PMA treatment. Individual CD8F1 tumors were measured prior to PMA treatment and again prior to excision. ATP and MMPR-5P were then measured in tumor extracts. The ATP level for each tumor was then plotted against the change in mass to determine correlation ($r = 0.7$) 24 h after MMPR + 6AN treatment (Exp. 2828/N143)

initiated. At the later time-point there was a more uniform depletion of cellular nucleotides and ATP depletion correlated with the extent of decrease in tumor mass.

Depletion of ATP and induction of apoptosis precede regression of PMA-treated CD8F1 mammary tumors

The degree of apoptosis occurring during CD8F1 tumor regression was studied in a similar experiment. Apoptosis was quantitated microscopically by both morphological criteria and by a DNA end-labeling (TUNEL) procedure at 10 and 24 h following PMA treatment, and these results were compared with tumor ATP levels and the decrease in tumor mass. As shown in Table 2, the ATP levels of PMA-treated CD8F1 tumors were similarly depleted to 48% and 44% of control values at 10 and 24 h, respectively, and both had similar high levels of MMPR-5P. However, at 10 h the tumor weights were not different from those of the saline control group, but by 24 h the tumors were significantly reduced to 52% of the saline-treated control tumors. The percent of apoptotic cells was about 10% compared with an average of 1.9% for control tumors, with some tumors showing a 20% incidence of apoptosis, and the incidence of apoptosis was more than fivefold higher in treated tumors than in control tumors at both 10 and 24 h, as assessed by either morphology or end-labeling. This confirmed that apoptosis was ongoing at 10 h in PMA-treated CD8F1 tumors, but raised the question of whether the observed depletion of ATP in regressing tumors could be caused by the apoptotic process. This question was addressed by determining the time-course of apoptosis following PMA treatment.

Significant depression of ATP prior to observable apoptosis in PMA-treated advanced CD8F1 mammary tumors

The relationship of ATP depletion to the onset of apoptosis was studied in CD8F1 tumors treated with PMA with the addition of 5FUra 2.5 h following PMA. The addition of 5FUra to PMA has been shown to provide a better chemotherapeutic effect than the PMA combination [39]. As shown in Fig. 2A, this treatment resulted in seven PRs at 72 h after the first course of MMPR + 6AN treatment. For biochemical and histological analysis, tumor samples were collected at 3, 6, 9, 15, and 24 h after MMPR + 6AN treatment. The response of the tumors to chemotherapy is shown in Fig. 2B. Although there were no PRs over this initial

Table 2 Tumor weight, ATP and MMPR-5′ phosphate levels and percent of apoptotic cells at 10 and 24 h after MMPR and 6AN treatment in PMA-treated CD8F1 mammary tumor-bearing mice. PMA-treated CD8F1-tumors were collected 10 or 24 h following MMPR + 6AN treatment. Tumors were excised, weighed and divided. One portion was immediately frozen for nucleotide pool analysis; the other was set into a vial with formalin for use in microscopic analysis. Thin sections were prepared and apoptosis was determined microscopically either following H&E staining or by means of the TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-X nick end-labelling) method. Tumors averaged 125 mg (caliper measurement) at the initiation of chemotherapy (Exp. C2852/N148) (*ND* none detected). $*P < 0.05$ with respect to saline control

	10 hours		24 hours	
	Saline	Treated	Saline	Treated
Tumor weight (mg)	270 ± 130	280 ± 140 (104%)	310 ± 160	160 ± 100 (52%)*
Metabolite levels (nmol/mg protein)				
MMPR-5P	ND	2.34 ± 1.10	ND	3.00 ± 1.63
ATP	10.11 ± 1.91	4.85 ± 1.53 (48%)*	14.09 ± 5.60	6.22 ± 1.95 (44%)*
% Apoptotic cells				
Morphology	1.5 ± 1.2	9.5 ± 5.9 (630%)*	2.1 ± 1.9	11.5 ± 6.0 (550%)*
TUNEL	2.4 ± 1.4	12.8 ± 7.0 (530%)*	1.4 ± 0.7	7.3 ± 3.2 (520%)*

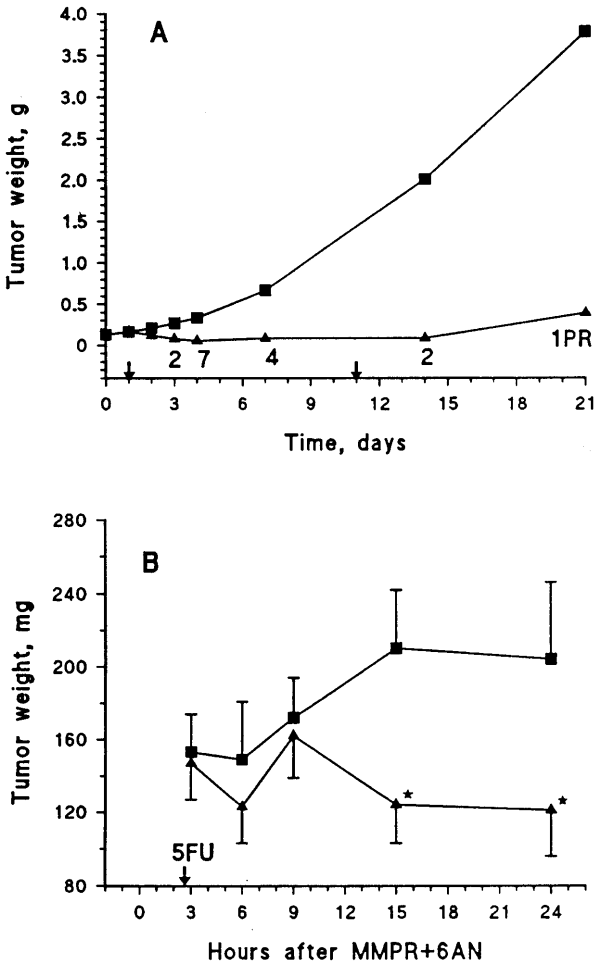

Fig. 2A,B CD8F1-tumor regression effect of treatment with PMA + 5FUra. CD8F1 tumors were treated with either saline (■) or the PMA regimen followed 2.5 h later with $5FUra_{75}$ (▲) (Exp. 2890/N156). **A** Tumor growth over the course of the chemotherapy experiment. The number of partial tumor regressions (*PR*) among each group of ten tumors is indicated next to the symbol for each group. The time of administration of MMPR + 6AN is indicated (↓) along the *x*-axis. **B** Comparison of the average weights of tumors collected for measurement of ATP and MMPR-5P levels and degree of apoptosis at 3, 6, 9, 15, and 24 h after MMPR + 6AN (see accompanying data in Table 3). $P < 0.05$ vs the saline treated group at the corresponding time point

period, the tumor regression induced by PMA treatment became significant 15 h after MMPR + 6AN treatment, and this was reflected by two PRs on day 3 of the experiment (48 h after the first course of MMPR + 6AN, Fig. 2A). As shown in Table 3, over the initial 24-h period shown in Fig. 2B, the incidence of apoptosis increased from 1.6% at 6 h after MMPR + 6AN treatment to 6.4%, 9.6% and 9.0% at 9, 15 and 24 h, respectively. The values, however, were not significantly different among the later three time-points. This general trend was similar to the time-course of apoptosis observed by the appearance of DNA laddering into nucleosome-length fragments (data not shown).

Over this time-course, ATP was significantly depleted at the earliest (3-h) time-point, a time when there was no apoptosis attributable to the PMA treatment. There was also a significant reduction in ATP at 6, 9, and 15 h. In addition, the levels of MMPR-5P achieved at 3 h persisted at the same "steady-state" level over the 24-h period. At the 9-h time-point, however, the levels were twofold higher and the ATP concomitantly decreased to a greater extent than for the other groups. These results clearly demonstrate that ATP depletion occurred prior to the onset of apoptosis and suggest that early ATP depletion may contribute to the onset of apoptosis in PMA-treated CD8F1 tumors.

Reversal of PMA-induced MMPR-5P accumulation, ATP depletion and CD8F1 tumor growth inhibition upon treatment with IodoT

The role of ATP depletion in relation to later periods of CD8F1-tumor regression were studied by reversing the accumulation of MMPR-5P with IodoT, a potent inhibitor of adenosine/MMPR kinase. As shown in Fig. 3A, there were three PRs among ten tumor-bearing mice in the PMA-treated group. A second group received IodoT 6 h after the simultaneous administration of MMPR + 6AN to allow an initial activation of MMPR to MMPR-5P. In the PMA + IodoT group

Table 3 Comparison of ATP and MMPR-5′P levels, percent of apoptotic cells and tumor weight for CD8F1 mammary tumors following treatment with MMPR + 6AN. CD8F1 tumor-bearing mice received MMPR and 6AN simultaneously 17 h after PALA. Time of tumor excision is referenced to the time of MMPR + 6AN treatment. 5FUra was given 2.5 h after MMPR + 6AN. Apoptotic bodies were determined by means of microscopic analysis using the TUNEL assay as described in the Materials and methods section. Saline controls showed an average incidence of apoptosis of 0.8 ± 0.5%. Tumors averaged 115 mg (caliper measurment) upon initiation of chemotherapy (Exp. C2890/N156). $*P < 0.05$ with respect to the group(s) indicated (C = saline control, number refers to the value corresponding to the indicated time-point). $**P < 0.05$ with respect to the MMPR-5P values at the 3, 6, 15 and 24 h time-points

Time (hr)	Tumor weight (% of control)	ATP (% of control)	MMPR-5′P (nmol/mg)	% apoptotic cells
3	96	$64^{*(C)}$	1.44 ± 0.47	0.6 ± 0.5
6	83	$79^{*(C)}$	1.83 ± 0.59	$1.6 \pm 1.0^{*(C,3)}$
9	94	$56^{*(C,6,15,24)}$	2.56 ± 0.65**	$6.4 \pm 4.0^{*(C,3,6)}$
15	$59^{*(C)}$	$75^{*(C)}$	1.34 ± 0.53	$9.6 \pm 5.7^{*(C,3,6)}$
24	$59^{*(C)}$	$90^{*(3)}$	1.26 ± 0.51	$9.0 \pm 3.9^{*(C,3,6)}$

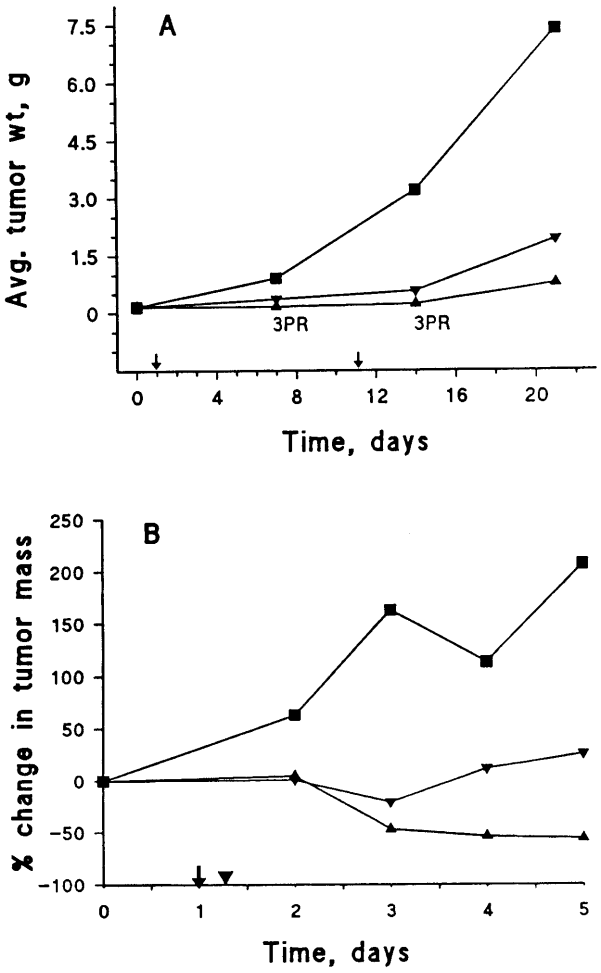

Fig. 3A,B Reversal of the PMA-induced antitumor effect by treatment with IodoT 6 h after MMPR + 6AN. CD8F1 tumor-bearing mice were treated with saline (■), PMA (▲) or with PMA followed 6 h later with IodoT (▲). **A** Tumor growth over the course of the chemotherapy experiment is shown. The times of MMPR + 6AN treatment are indicated (↓) along the time axis. The number of partial regressions (*PR*) among ten tumor-bearing mice are shown next to the symbol for each group (Exp. C2808/N142). **B** Changes in average mass of tumors collected for biochemical measurements on days 2, 3, 4, and 5. Tumor weights were estimated by caliper measurement at the onset of the experiment and again immediately prior to sacrifice at the indicated time-point (↓ time of MMPR + 6AN treatment, ▲, time of IodoT treatment 6 h after MMPR + 6AN)

good tumor growth inhibition was observed, but there were no PRs. In Fig. 3B, the growth of tumors collected for biochemical measurements at days 2, 3, 4 and 5 (MMPR + 6AN treatment was on day 1) is presented. The prevention of the PMA-induced antitumor effect by IodoT treatment, which was significantly different from that produced by PMA, is evident.

The corresponding biochemical data are presented in Table 4. PMA was found to produce a significant depletion of ATP over 96 h and was consistent with a prolonged retention of MMPR-5P over the same period. There was an initial depletion of ATP on day 1 for the PMA + IodoT group which was similar to that observed in the PMA group even though the MMPR-5P level was reduced to 30% of the PMA-treated value by treatment with IodoT 18 h earlier. On days 3, 4, and 5, however, little MMRP-5P was detected in the IodoT-treated groups and the ATP levels associated with these tumors were significantly higher than levels for the respective PMA-treated groups. On day 5, the ATP level for the PMA + IodoT group was not significantly different than that of the saline-treated group. These results show that prolonged MMPR-5P levels are important for an optimal PMA-induced antitumor effect and that reversal of MMPR-5P accumulation leads to the reversal of ATP depletion and subsequent tumor progression.

Discussion

Apoptosis is a physiological process whereby a cell is induced to participate in its own death. The apoptotic process is essential for proper growth and development of an organism, but impairment of appropriate apoptotic controls has been implicated in many neoplastic conditions. The apoptotic process has been reported to occur in a range of models where cells are induced to die by various means, including: (1) growth factor withdrawal (e.g. IL-2 withdrawal from IL-2-dependent T-cells [23], castration leading to prostate regression [22], nerve growth factor withdrawal from neuronal cells [8], serum deprivation, etc.), (2) treatment with cytotoxic drugs with diverse mechanisms of action (e.g. DNA-

Table 4 Effect of IodoT on ATP and MMPR-5′P levels when given 6 h after MMPR + 6AN in the PMA regimen. CD8F1 tumors were treated with PALA followed 17 h later with MMPR + 6AN (PMA) or with PMA followed 6 h later with IodoT to allow an initial period of synthesis of MMPR-5P prior to inhibition of adenosine kinase by IodoT. Tumors were collected on the day indicated after initiation of chemotherapy with the samples for day 2 being collected 24 h after treatment with MMPR + 6AN. Tumors averaged 153 mg (caliper measurement) at the initiation of chemotherapy (Exp. C2808/N142) $^{*}P < 0.05$ with respect to saline control, $^{**}P < 0.05$ with respect to the value for PMA treatment

Treatment	Day of tumor collection			
	2	3	4	5
PMA regimen				
ATP (% of control)	52*	38*	35*	50*
MMPR-P (nmol/mg)	3.2 ± 1.3	3.0 ± 0.8	1.5 ± 0.8	1.5 ± 0.3
PMA regimen + $IodoT_3$				
ATP (% of control)	56*	53*,**	74*,**	88*,**
MMPR-P (nmol/mg)	1.1 ± 0.7**	<0.1**	<0.1**	<0.1**

damaging agents [1, 26], protein kinase inhibitors [44], etc.), and (3) radiation treatment (membrane-associated mechanism involving the release of ceramide [11, 19, 34]). Thus, it is apparent that numerous stimuli ultimately lead to many of the same characteristic features of apoptosis (i.e. cell blebbing, loss of cell volume, nuclear DNA condensation and margination, and DNA fragmentation subsequent to nuclease activation; see reference 21 for a review).

These findings suggest a process that occurs in discrete phases, including an induction phase, an effector phase, a committed (or condemned) phase and an execution phase [10, 21]. Following a death stimulus (or withdrawal of a growth stimulus), the balance of pro-apoptotic factors and anti-apoptotic factors may in part determine whether a particular cell is competent to undergo apoptosis. Up to this point, the process may be reversible and may be modulated for enhancement or prevention of cell death. If the cell is capable of undergoing apoptosis, it then enters the committed (or condemned) phase which is irreversible and of variable duration. Ultimately the cell enters the execution phase (or apoptotic cascade) which is characterized by a relatively short, fixed duration during which the morphological features of cell blebbing, DNA condensation, etc., and the biochemical features of cell acidification, proteolysis, DNA fragmentation, etc., are apparent.

The ability of a cell type to undergo apoptosis following a given stimulus is dependent upon the balance between pro-apoptotic and anti-apoptotic factors [21] particular to that cell type. The critical factors or mechanisms which allow a cell to enter the committed phase following a given stimulus are currently unknown. However, several factors have been shown to be important. The p53 tumor-suppressor gene product, for example, normally functions to protect cells from DNA damage induced by radiation or cytotoxic insult [24] and inactivation of p53 by mutation allows tumorigenesis by prevention of apoptosis. In addition, a high level of the *bcl-2* gene product is associated with the prevention of apoptosis [31]. Proteases of the interleukin-1β-converting enzyme (ICE) protease family have been implicated in the apoptotic process [33, 46].

The emerging understanding of the process of apoptosis has led to a view that it is possible to modulate drug-induced cytotoxicity by affecting the cellular components which allow (or prevent) apoptosis. This approach is in contrast to the traditional efforts to modulate cytotoxicity by enhancing a particular drug–target interaction.

The PMA combination comprises a number of clinically relevant chemotherapeutic agents of vastly different primary drug–target interaction. The drug-induced regression of PMA-treated advanced CD8F1 tumors [29, 30] has been shown to occur via an apoptotic mechanism. In the present study, we defined the process of apoptosis in relation to PMA treatment. Thus, apoptosis was evident in advanced CD8F1 tumors as early as 6 h after MMPR + 6AN treatment and appeared to plateau at a rate of 9–10% at 9 h after MMPR + 6AN treatment. Treatment with PALA alone had no effect on induction of apoptosis since there was no increase of apoptosis in PALA-treated tumors prior to MMPR + 6AN treatment as compared to saline-treated tumors (data not shown). This early onset of apoptosis is similar to that observed in irradiated murine ovarian tumors by Meyn and coworkers [36, 37], and while the apoptotic rate appears to be somewhat lower, the duration of the process appears to be longer (beyond 24 h) in PMA-treated CD8F1 tumors.

While a 9% incidence of apoptosis may seem low, the execution phase of apoptosis, which was quantitated in this study, is of limited duration and occurs within several hours once this phase has been initiated [3, 42, 48]. As a result, the observed 9% incidence of apoptosis represents a ''steady-state'' level with cells continuously entering the execution phase in a stochastic manner, then being removed by neighboring cells some hours later. It is this continuously repeated process of tumor cell removal which leads to tumor regression [3, 42, 48].

PMA-induced enhancement of chemotherapeutically induced regressions of CD8F1 tumors have also been associated with enhanced depletion of ATP [6, 7, 40]. Here, we present evidence that ATP depletion occurs prior to the onset of apoptosis. Furthermore, after tumors started to regress, the extent of decrease in tumor mass after PMA treatment correlated with greater depletion of ATP, further indicating that ATP depletion correlates with the apoptotic process. In this regard, it is of interest that the effects of cellular energy have been shown to interfere with cell-cycle progression and in HL-60 cells two energy checkpoints have been identified [41]. Thus, treatment with energy inhibitors, including oligomycin, rhodamine-123, and dequalinium chloride, has been found to cause accumulation of cells at two points in the cell cycle depending on the degree of ATP depletion. Oligomycin is an inhibitor of the F_oF_1 ATPase located in the inner mitochondrial membrane [17, 18]. Thus, treatment with 0.05 μg/ml oligomycin causes a 20% inhibition of ATP and increases the proportion of cells in the G_1 phase by 20%. Dose escalation to 5.0 μg/ml causes a 40% depletion of ATP (60% of control) and this depletion is associated with a 60% increase of cells in the G_2-M phase. The other inhibitors show a similar effect [41]. The mechanism by which energy depletion relates to apoptosis is under current investigation. However, it is possible that altered protein phosphorylation may be involved. For example, it has been shown that a failure to dephosphorylate the retinoblastoma protein is associated with drug resistance [9, 15].

Among the drugs of the PMA combination, MMPR has been shown to have the greatest individual effect on ATP depletion [39]. We demonstrated in this study that prolonged retention of MMPR-5P in the CD8F1 tumor allowed for optimal therapeutic effects and was associated with prolonged depletion of ATP, since tumor regressions and ATP depletion were reversed when the

resynthesis of MMPR-5P was prevented by treatment with IodoT.

Collectively, these results suggest a causal relationship between PMA treatment, accumulation of MMPR-5P, depletion of ATP, induction of apoptosis and regression of CD8F1 tumors in vivo. While this scheme is consistent with the results presented, it must be emphasized that MMPR as a single agent is not sufficiently active to induce tumor PRs at host-tolerant doses, and the addition of PALA and 6AN contributes important additional tumor-regressing activity to the PMA combination [27].

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