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Research Article

Administration of the antitumor drug mitoguazone protects normal thymocytes against spontaneous and etoposide-induced apoptosis

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Abstract. The suggestion has been made that polyamines may be involved in the control of cell death, since exceedingly high or low levels induce apoptosis in different cell systems. For a deeper insight into the relationship between apoptosis and polyamine metabolism, we investigated in vitro the effect on rat thymocytes of mitoguazone (MGBG, which inhibits S-adenosylmethionine decarboxylase, i.e. a key enzyme in the polyamine biosynthetic pathway). Thymocytes were selected as an especially suitable model system, since they undergo spontaneous apoptosis in vivo and can be easily induced to apoptose in vitro by etoposide, used here as an apoptogenic agent. MGBG protected thymocytes from both spontaneous and drug-induced apoptosis, and this protective effect was associated with a decrease in polyamine oxidase activity and total polyamine levels.

Key words. Thymocyte; polyamine oxidase; polyamine; etoposide; mitoguazone; apoptosis.

Polyamines (i.e. putrescine, spermidine and spermine) are polycations present in all living cells where they play many important roles in cellular physiology. Their cellular levels are tightly controlled by synthesis, catabolism and transport, and are regulated by hormones, growth factors and feedback inhibition [1–5]. Since polyamines carry net positive charges they bind negatively charged molecules such as DNA and RNA, as well as acidic proteins; in particular, polyamines are involved in maintaining chromatin structure, and have been hypothesized to protect DNA from free-radical damage: spermine, in particular, was able to directly protect DNA from oxidative stress [6].

Recent studies suggest that polyamines are involved in controlling cell cycle progress and apoptosis [3, 7–10]. Although these results are sometimes contradictory, there is general agreement that the induction of polyamine catabolism, with consequent changes in the content and relative proportions of polyamines, can alter the balance between cell proliferation and cell death, which is of primary importance in tumor development and growth [2, 3, 9, 10].

Exceedingly high or low levels of polyamines dramatically affect their physiological functions, and have either inducing or protective effects on apoptosis [7, 8, 10]. Changes in ornithine decarboxylase activity and/or the levels of the three polyamines are observed in apoptosis induced by a variety of experimental conditions in several cell systems [2, 3, 8, 10]. A depletion of polyamines in thymocytes after dexamethasone [11, 12] or radiation and heat shock treatment was correlated to the appearance of apoptotic cells [13]. Etoposide-induced apoptosis

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of HL-60 promyelocytic leukemic cells was accompanied by an increase in polyamine efflux from the cells and a decrease in total polyamine content during the first 24 h of exposure to the drug [14]. On the other hand, an increase in polyamine levels has been found in apoptosis induced by the hepatocyte growth factor [15], and polyamine addition to the culture medium was able to induce apoptosis in different experimental conditions [16–18]. Thus, although a direct role of high polyamine levels in the induction of apoptotic cell death has yet to be established, excessive polyamine production clearly causes apoptosis, at least in some cell systems.

Several lines of evidence suggest that polyamines may also be involved in cancer cell growth. Therefore, compounds which interfere with polyamine metabolism and induce depletion of cellular polyamines have been used in the therapeutic treatment of cancer [1–5, 7, 10, 19–21].

The interest in polyamine catabolism is growing. Until recently, the polyamine catabolic pathway was thought to consist of two enzymes, the cytosolic acetyltransferase (SAT) and polyamine oxidase (PAO). PAO (E.C. 1.5.3.11) transforms acetylated spermidine and spermine into putrescine and spermidine, respectively, and also generates locally high concentrations of hydrogen peroxide as a by-product [2, 5, 20, 21]. Thus, the catabolism of polyamines by PAO has been suggested to be an important initiating reaction in cytotoxicity in different cultured cells, and that the regulation of the enzyme may have a role in facilitating apoptosis in cancer cells [reviewed in ref. 8]. In addition, our previous studies showed a positive correlation between PAO activity and apoptosis in rat thymus after hormonal treatments [22, 23].

Mitoguazone (MGBG) is a guanidino-containing compound, structurally similar to spermidine, which exerts an inhibiting action on S-adenosylmethionine decarboxylase, i.e. on a key enzyme in the polyamine biosynthetic pathway [24–26]. Clinical trials with MGBG as an antineoplastic drug were initiated in the early 1960s, but were soon stopped because of drug toxicity. Later, pharmacokinetic studies showed that MGBG has an extremely long half-life in vivo: this allowed the design of new low-dose schedules and MGBG is nowadays used (generally in combination with etoposide and other antitumor agents) in therapeutic protocols for relapsed and AIDS-related lymphomas [24, 27].

MGBG was found to induce apoptosis in tumor cell lines in culture [25, 28], and also in normal thymocytes in vitro, where it was used to deplete intracellular spermine [29]. In contrast, we have recently observed that MGBG, when administered to rats in vivo, reduced spontaneous apoptosis in the thymus, where a marked decrease in PAO activity was induced [30].

In the present research, we investigated in more detail the effect of MGBG on rat thymocytes in vitro, and attempted to elucidate whether variations in PAO activity and polyamine levels may correlate with either its apoptogenic or antiapoptogenic action on normal lymphoid cells; to this end, MGBG was also administered in association with the apoptogenic drug etoposide.

In fact, as mentioned above, the results reported in the literature for thymocytes in vitro and in vivo are still conflicting: one of the possible reasons for this inconsistency may be the use in vitro, in the incubation medium [29], of bovine serum which contains amine oxidases that can be an uncontrolled source of toxic and apoptogenic products; following suggestions by different authors [11, 31], we therefore used horse serum, which is devoid of amine oxidases, in our experiments.

Materials and methods

Cell culture and drug treatments

Thymuses were taken from 3- to 4-week-old rats, which had been euthanized by deep anesthesia with 35% chloral hydrate, followed by cervical dislocation. To obtain thymocyte suspensions, thymuses were cut into small fragments in prewarmed RPMI medium containing 10% heat-inactivated horse serum, 2 mM glutamine, and 100 units/ml of streptomycin and penicillin. To induce apoptosis, thymocytes were treated with 50 μM etoposide for 1 h, followed by a 3-h incubation in drug-free medium, since previous studies demonstrated that with this concentration, apoptosis massive occurs [32, 33]. The same treatments were performed in the presence of either 2.5 or 5 mM MGBG. Thymocytes samples were analyzed immediately after the isolation (untreated controls) or after being incubated in etoposide-containing medium.

To detect apoptotic cells and estimate their percentage (i.e. the apoptotic index), cytochemical and cytometric techniques were used.

DNA staining

Unfixed thymocytes were submitted to the single-step staining procedure suggested by Pellicciari et al. [34]. Briefly, 1-ml aliquots of thymocytes were collected from the culture flasks and dropped directly into 2 ml of 75 μg/ml propidium iodide (PI) solution in water, containing 100 Kunitz units/ml of RNase type A, 10 mM EDTA (to inactivate endogenous endonucleases) and 0.0015% Nonidet P-40 (all reagents from Sigma, St. Louis, Mo.); thymocytes were stained for at least 30 min.

Labelling of apoptotic cells by Annexin V and PI staining

To detect early and late apoptotic cells, control and etoposide-treated cells were incubated for 10 min in complete culture medium containing FITC-conjugated

Annexin V (3 µl/10^6 cells) and 2 µg/ml PI, as reported in Pellicciari et al. [34]. Early apoptotic cells are labeled by FITC-conjugated Annexin V but exclude PI, whereas late apoptotic cells are stained both green (FITC) and red (PI); non-apoptotic cells are not labelled by Annexin V and exclude PI.

Flow cytometry

Measurements of stained cell samples were taken with a FACStar cytometer (Becton-Dickinson, San José, Calif.) at 488 nm with 200-mW excitation power of the argon ion laser, using a 560-nm beam splitter, a 510- to 540-nm band pass filter for the green fluorescence detector, and a 610-nm long pass filter for the red fluorescence detector. PI-DNA content histograms and dual-parameter cytograms of green fluorescence (identifying Annexin V-labelled cells) versus red fluorescence (PI-labeled cells) were drawn. In each experiment, at least 20,000 events were measured in the histogram regions used for estimating the percentages of non-apoptotic and apoptotic cells.

Assay of PAO activity

Thymocytes were rinsed with PBS and ultrasonicated. PAO activity was assayed by the method of Suzuki et al. [35], modified as described by Wang et al. [21] (this modification was necessary because the acetylated polyamines are no longer available from a commercial source); all the reagents were from Sigma. The method measures the H_2O_2 formed due to the oxidation of spermine by converting homovanillic acid into a highly fluorescent compound in the presence of horseradish peroxidase. Each enzyme determination was performed in duplicate, and fluorescence was measured at 323-nm excitation and 426-nm emission.

Polyamine analysis

For polyamine analysis, extracts from cell samples were prepared in 300 µl of 0.6 N perchloric acid by ultrasonication and were centrifuged at 12,000 rpm for 20 min with an Eppendorf microcentrifuge. Aliquots of the supernatant were used to determine polyamine content by HPLC, as previously described [22]. Briefly, the polyamines were separated using a µBondapac C18 column and derivatized post-column by the O-phthalaldehyde method. A fluorescence detector was used at 340-nm excitation and 455-nm emission. Polyamine levels were then calculated based on external standard curves run within 24 h of the sample chromatograms.

Protein determination

Proteins were measured by the method of Geiger and Bessman [36] using bovine serum albumin as standard.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine differences between groups. A value of $p < 0.05$ was considered as statistically significant.

Results

Based on DNA histograms (fig. 1) or dual-parameter scattergrams of FITC-labeled Annexin V versus PI (fig. 2), the percentage of non-apoptotic and of early and late apoptotic thymocytes was estimated in untreated as well as etoposide-treated samples, before and after simultaneous administration of MGBG. The results are summarised in figure 3: as expected, apoptotic thymocytes were already present in untreated control cells, due to spontaneous thymocyte apoptosis occurring in vivo; the percentage of apoptotic cells increased following treatment with etoposide, whereas it progressively decreased upon simultaneous administration of MGBG at increasing concentrations.

Upon addition of either 2.5 or 5 mM MGBG only to the culture medium, a marked decreased in PAO activity was observed, compared to untreated control thymocytes (fig. 4). The apoptotic effect of etoposide was associated with an increase in PAO activity, which was prevented by the simultaneous administration of MGBG at either concentration (PAO activity was here about 50% of the basal levels).

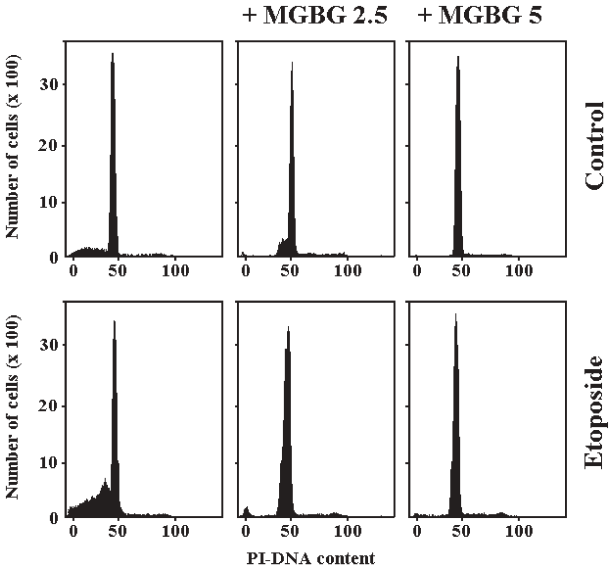

Figure 1. Cytofluorimetric histograms of DNA content after PI staining. The sub-G_1 peak demonstrates that apoptotic thymocytes are present both in untreated samples and 4 h after etoposide treatment, and that the simultaneous incubation with MGBG protects thymocytes from entering apoptosis.

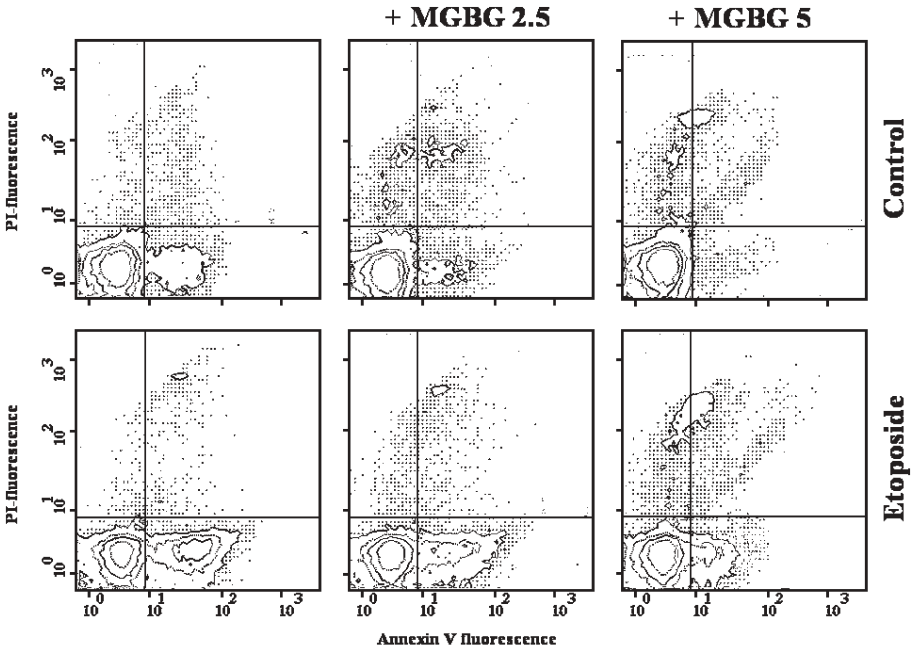

Figure 2. Dual-parameter cytograms of FITC-labeled Annexin V (in abscissa) versus PI staining (in ordinate) of thymocytes under the different conditions studied. Non-apoptotic (double-negative) cells fall in the lower-left quadrant, whereas early and late apoptotic cells fall in the lower- and upper-right quadrants, respectively. These cytograms confirm, consistent with the results in figure 1, that MGBG treatment caused a decrease in the apoptotic thymocyte fraction.

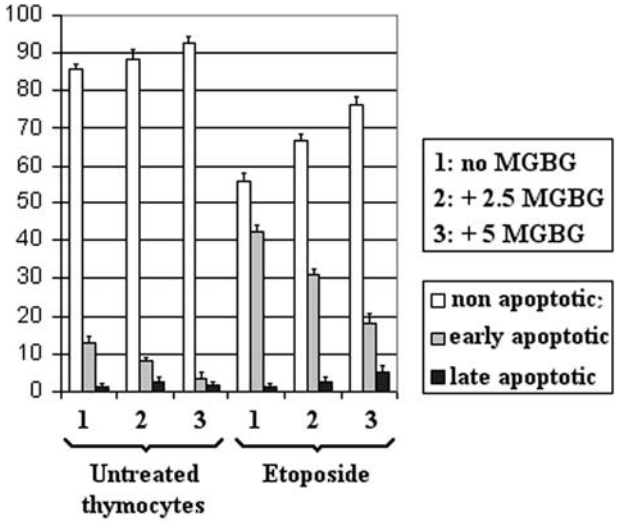

Figure 3. Percentage of non-apoptotic and early and late apoptotic cells under the different treatment conditions used. At least three experiments were considered for each treatment; the bars represent standard deviations.

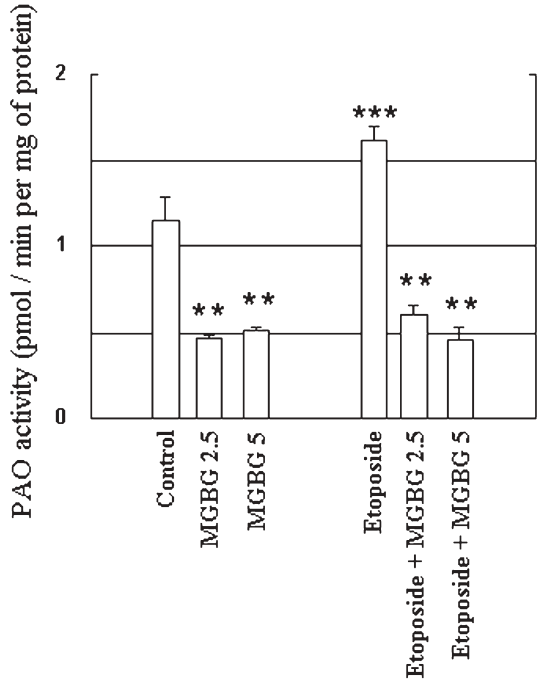

Figure 4. Effect of MGBG on PAO activity in thymocytes before or after being treated with etoposide for 4 h. All determinations were carried out in duplicate and data are the mean values; bars represent standard errors. The apoptotic effect of etoposide was associated with an increase in PAO activity, which was prevented by the simultaneous administration of MGBG at either concentration (**$p < 0.01$ or ***$p < 0.001$).

We also analyzed the effect of MGBG administration on the levels of polyamines in the absence or presence of etoposide (fig. 5). At the higher concentration, MGBG induced a significant decrease of total polyamine content, in particular of spermidine and putrescine; this was especially apparent in the presence of etoposide.

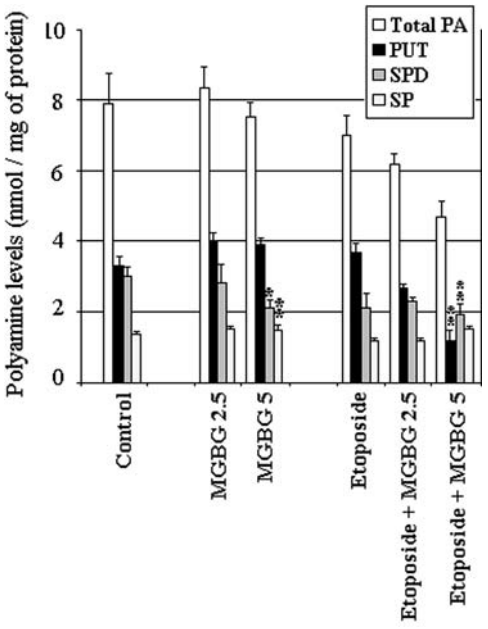

Figure 5. Effect of MGBG on polyamine levels in thymocytes before or after being treated with etoposide for 4 h. All determinations were carried out in triplicate; data represent the means, and the bars the standard errors. *$p < 0.05$ **$p < 0.01$ compared with untreated thymocytes or the corresponding controls (without MGBG).

Discussion

Reduction of intracellular polyamine levels using inhibitors slows or arrests growth of many cells in vitro, confirming that polyamines play a role in the cellular progress through the cell cycle [18]. Many data indicate that polyamines are also involved in apoptotic cell death [37, 38], although the results are sometimes contradictory. Spermine is required for caspase activation and this suggests a role in apoptosis induction [39]; actually, in some tumor cell models, polyamine depletion was found to protect cells from apoptosis [17]. However, low polyamine levels correlate with an enhanced sensitivity to dexamethasone-induced apoptosis [11], and polyamine depletion was sometimes found to induce apoptosis [3, 5, 8, 20, 40].

Based on these inconsistent results, polyamines have therefore been suggested, by different authors, as either promoting, modulating, or protective agents in apoptosis. Changes in the basal polyamine levels during apoptosis might depend on the (normal or tumor) origin of the cells considered, and on the apoptogenic stimulus applied.

In the normal thymocytes used in the present investigation, the induction of apoptosis after etoposide treatment was paralleled by the decrease in total polyamine content and an increase in the activity of PAO which is consistent with previous observations suggesting a direct role of PAO in apoptosis induction [5, 14]; our results also confirm that this enzyme is inducible also in thymocytes during apoptosis, as already shown by us [22, 23, 30] and others [4, 14, 40, 41] for other cell systems, both in vivo and in vitro.

Despite the lack of conclusive evidence about their role(s) in cell proliferation and apoptosis, polyamines have been taken as suitable targets for therapies which are specifically addressed to alter the cell cycle or induce cell death [3, 5, 7, 19, 20]. Several polyamine inhibitors actually proved to be effective in clinical trials [3, 4, 10, 19, 40], among them MGBG, whose intrinsic toxicity has been dramatically reduced with the recent introduction of low-dose protocols.

MGBG, when administered in vivo to rats, was observed to exert a protective effect against spontaneous apoptosis in the thymus, where a marked and permanent decrease in PAO activity was observed [30]. In contrast, MGBG induces apoptosis in vitro, in several human cancer cell lines [8, 28], or enhances the sensitivity of normal thymocytes to apoptosis induced in vitro by different treatments [29: as already recalled in the introduction, however, the results in this paper may have been affected by the use of bovine serum which is an uncontrolled source of toxic and apoptogenic products]. Again, the possibility exists that MGBG may have different effects on normal and tumor cells, consistent with the reports that low PAO activity may contribute to the relatively low level of apoptosis observed in tumor cells [42]. MGBG has recently been demonstrated to confer a dose-dependent protection against the damaging action of ethanol and other destructive agents on normal gastric mucosa of rats [43], whereas the MGBG-induced inhibition of polyamine synthesis results in growth inhibition (though without apoptosis) in Burkitt's lymphoma cells [44].

In agreement with our previous observation in vivo [30], and at least for the times of treatment considered here, MGBG alone does not interfere with thymocyte viability, and even determines a decrease in the percentage of apoptotic thymocytes in both untreated and etoposide-treated samples; we also observed a similar protective effect of MGBG against apoptosis of normal thymocytes in vitro after the application of completely different apoptogenic stimuli, i.e. actinomycin D treatment or growth in low-serum-containing medium (unpublished results, personal observation).

The antiapoptotic effect of MGBG was here observed to occur in parallel with a marked decrease in PAO activity as well as in the total polyamine levels, consistent with the evidence that high polyamine levels, which are essential for progression through the cell cycle, may possibly promote the apoptotic process [18, 37, 38]. However, MGBG acts in a complex way on cellular polyamine levels, since the drug increases ornithine decarboxylase and spermidine/spermine acetyltransferase activities [45], while decreasing S-adenosylmethionine decarboxylase [24–26], diamine oxidase [46] and PAO [30] activities:

therefore, that the extent of spermidine and spermine depletion in tissues or cells treated with MGBG has been quite variable from study to study, in the literature, is not surprising.

Further investigation will be necessary to confirm the antiapoptotic effect in vivo of MGBG on cells of lymphoid origin: this protective effect on lymphopoietic organs might be beneficial in the therapy of some tumors, such as relapsed or AIDS-related lymphomas [24, 27], for which MGBG is presently used in combination with etoposide or other antitumor drugs.

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