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MPA Increases Idarubicin-Induced Apoptosis in Chronic Lymphatic Leukaemia Cells via Caspase-3

Salvatore Florio,[1]* Luca Crispino,[1] Roberto Ciarcia,[1] Giovanni Vacca,[3] Ugo Pagnini,[1] Andrea de Matteis,[4] Carmen Pacilio,[4] Giuseppina D'Andrilli,[2] Christine Kumar,[2] and Antonio Giordano[2]

[1]Department of Structures, Functions and Biological Technologies and Department of Pathology and Animal Health, School of Veterinary Medicine, University of Naples ''Federico II'', Naples, Italy
[2]Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, 1900 N 12th Street, Philadelphia 19122
[3]Physiology Laboratory, Department of Medical Sciences, School of Medicine, University of Eastern Piedimont ''A. Avogadro'', Naples, Italy
[4]Department of Medical Oncology ''C'', National Tumour Institute, Naples, Italy

Abstract The caspase family of protease is speculated to have a crucial role in apoptosis. The effect of treatment with Idarubicin (IDA) and Medroxyprogesterone acetate (MPA), used alone or in combination, on the activation of Caspase-3 in canine Chronic Lymphatic Leukaemia (CLL) cells was investigated, in order to clarify the mechanism of chemo- and hormone-therapy mediated apoptosis. Caspase activity was determined by a quantitative fluorimetric assay. Apoptosis was monitored by propidium iodide (PI) and nucleosomes assay. Treatment of CLL cells for 24 h with MPA 5 μM did not significantly activate caspase-3 but its activity was increased almost 5-fold more with IDA 1 μM ($P < 0.05$) than control. Treatment of CLL cells with IDA 1 μM in equimolecular association with MPA was able to increase the activation of caspase-3 induced by IDA of the 61.2% ($P < 0.05$) in comparison with IDA alone. The activation of caspase-3 was confirmed evaluating apoptosis by PI and nucleosomes assay. Furthermore, both caspase-3 activation and apoptosis triggered by IDA alone or in combination with MPA were significantly inhibited by specific caspase-3 inhibitor AC-DEVD-CMK. These findings provide an explanation for IDA and MPA induced-apoptosis mechanism.
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Key words: idarubicin; medroxyprogesterone acetate; apoptosis; caspase-3

Anthracyclines are chemotherapeutic agents mostly used in the treatment of several malignant tumors. However, their clinical use has been restricted by a dose-limiting cardiotoxicity, which can lead to cardiomyopathy and heart failure [Young et al., 1981; Saltiel and McGuire, 1983; Kantrowitz and Bristow, 1984]. At the same time, resistance to chemotherapeutic agents is an important factor in treatment failure in leukemia. Therefore, the toxicity of anthracyclines-therapy as well as the failure of chemotherapy as a result of drug resistance are the major problems in the clinical management of neoplasm. Several drugs have been showed to effectively reverse drug resistance [Yusa and Tsuruo, 1989; Sonnevel et al., 1992; Merlin et al., 1994; Reichle et al., 1994; Haberl et al., 1998]. However, the clinical use of such chemosensitizers remain questionable and the identification of more active but less toxic compounds is needed [Ozols et al., 1987; Dalton, 1993; Kave, 1993; Sikie, 1993].

Steroid hormones and anti-hormones were shown to enhance, in vitro, drug toxicity of Multi Drug Resistant-positive cell lines [Fleming et al., 1992; Wang et al., 1994]. Medroxyprogesterone Acetate (MPA) is a semisynthetic progestin mostly used in advanced breast cancer therapy [Cavalli et al., 1984]. In fact in

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*Correspondence to: Prof. Salvatore Florio, Department of Structures, Functions and Biological Technologies, University of Naples "Federico II", Via Veterinaria, 1 80137 Naples, Italy. E-mail: florio@unina.it

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advanced breast cancer either hormone receptors positive or negative, MPA increases anthracyclines activity and reduces also the metastatic risk [Hupperets et al., 1993]. Also in prostate cancer, MPA sensibly improves anthracycline activity [Anderstrom, 1994]. Our previous studies demonstrated that MPA significantly increases the intracellular uptake of anthracyclines and is able to modulate the balance between lipid peroxidation products and nitric oxide production, which in turn may play a key role in increasing anthracyclines-induced citotoxicity [Pacilio et al., 1998, Pagnini et al., 2000].

Apoptosis, or programmed cell death, is distinguished from lysic or necrotic cell death by specific biochemical and structural events. The apogenic signals trigger specific signaling pathways, including protease activation, which is followed by the appearance of morphologic changes of cells undergoing apoptosis such as condensation of nuclei and cytoplasm, blebbing of cytoplasmic membranes, and finally fragmentation into apoptotic bodies that are phagocytosed by neighboring cells [Steller, 1995]. A central theme of cancer research today is the ability of tumor cells to resist from apoptosis in response to triggers which typically induce cell cycle arrest or death in their untransformed counter parts.

The caspase family of proteases is speculated to have a critical role in apoptosis and anthracycline has been proved to induce apoptosis through caspase activation. In fact, it has been shown that the effect of treatment with anthracyclines might induce apoptosis in Renal Carcinoma Cell mainly trough the activation of caspase-3 [Wu et al., 2001]. It has also been demonstrated that Idarubicin (IDA), a high lipophilic anthracycline, is able to induce the generation of reactive oxygen species (ROS) and apoptosis in human leukaemia cells and these are separate events. In fact an inhibitor of caspases, the benzyloxycarbonil-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), facilitates IDA-mediated generation of ROS [Liu et al., 2001]. Moreover, it has been shown that clinically attainable concentrations of MPA can inhibit the growth of some human pancreatic carcinoma cells in vitro by inducing apoptosis in association with the phosphorylation of bcl-2 [Abe et al., 2000]. However, the MPA pro-apoptotic activity, remains ambiguous; in fact it has been demonstrated that progesterone exhibited a protective effect against the apoptotic process during mouse mammary gland involution in vivo [Ory et al., 2001].

Thus the aim of the present study was to evaluate the chemosensitizing activity of MPA on IDA-induced apoptosis in canine Chronic Lymphatic Leukaemia (CLL) cells and the involvement of caspase-3.

MATERIALS AND METHODS

Chemicals

IDA and MPA were obtained from Pharmacia-Upjohn (Milan, Italy), Phitoemoagglutinine (PHA), RPMI-1640, Fetal Calf Serum (FCS), Hystopaque, Propidium Iodide (PI) were obtained from SIGMA (Milan, Italy).

Leukocytes Preparation

Heparinized blood was obtained from an irish setter dog affected by CLL. Leukocytes were isolated using the modification of a method of Boyum as described [Bass et al., 1978]. Briefly, 10-ml blood samples were layered over Hystopaque (density 1.077) gradient centrifugation at 600*g* per 30 min at room temperature. The ring of leukocytes at the interface was collected and washed two times with 5 ml of PBS. The preparation was found to be 89% pure (range 83–92%; red blood cells 6%; polymorphonucleates 2.2%; platelets 2.3%). Cells were cultured in RPMI 1640 containing 10% FCS, 1% penicillin and streptomycin, and 2 mM glutamine, at 37°C in humidified atmosphere of 5% CO_2 in air. Leukocytes were stimulated with 10 nM L-PHA and observed daily with an inverted microscope (Diaphot, Nikon). The culture medium was changed every 4 days and the cultured cells replaced with newly/freshly isolated CLL cells every 21 days.

Viable cells (as assayed by means of the Trypan blue exclusion test), were seeded at 3×10^5 cells in six well tissue culture plates. After 24 h, the medium was removed and fresh medium along with various concentrations of IDA, in presence or absence of MPA was added to cultures in parallel. IDA was used at a final concentration of 0.3–10 μM. Solutions stock of MPA in ethanol were diluted with medium to give a final concentration of 10 μM. The final ethanol concentration in the medium was below 0.1% (v/v). Control cultures received fresh medium containing 0.1% ethanol and were incubated in parallel.

Apoptosis Assay by PI

At different times according to different experimental protocols the percentage of apoptotic cells from treated or control cultures was evaluated using PI assay. Flow cytometry analysis of isolated nuclei was performed using a method described for lymphoid cells by Matteucci et al. [1999]. Briefly, CLL cells were washed twice in PBS in 15 ml polypropylene tubes (Falcon/Becton Dickinson Labware, Lincoln Park, NJ). The cell pellet was immediately resuspended gently at room temperature in 1 ml of hypotonic solution consisting of 50 μg/ml PI (SIGMA), 0.1% sodium citrate (SIGMA), and 1% Triton X-100 (SIGMA) in distilled water. The tubes were placed at 4°C in the dark overnight. Flow cytometry analysis was subsequently performed using a FACscan flow cytometer (Dako Parteck). A correct threshold value was experimentally selected to exclude the majority of cell debris. Data collection was gated using adequate values of forward- and side-angle scatter to exclude remaining cell debris and large nuclei aggregates, and to include nuclei from apoptotic, necrotic, and living cells. For each sample 5,000 events were acquired.

Mono and Oligo-Nucleosomes Assay

To detect mono and oligo-nuclesomal DNA fragmentation, the Cell Death Detection ELISA Plus (Boehringer Mannheim, Indianapolis, IN) was used. The assay is based on a quantitative sandwich-immunoassay principle using mouse monoclonal antibodies direct against DNA and histones, respectively. Canine CLL cells (5×10^6) treated according the above cited experimental protocols were collected by centrifugation, washed, resuspended in 500 μl of incubation buffer to lyse the cells, and incubated for 30 min at 4°C. The lysate centrifuged at 15,000 rpm for 5 min, and 400 μl of the supernatant, containing the fragmented DNA, was removed. The supernatant was diluted with incubation buffer to a final concentration of 10^6 cell equivalents/ml, and 100 μl was added to each well of 96-well plates coated with anti-histone antibody. After 90 min of incubation at room temperature, the wells were washed three times with PBS. Peroxidase conjugated anti-DNA antibody was added to the wells, and incubated for 90 min at room temperature. After the wells were washed three times, 100 ml of colorimetric substrate was added and serial readings of optical density at 405 and 490 nm was taken at 5 min intervals.

Caspase-3 Assay

Caspase-3 activity was determined by a quantitative fluorimetric assay according to the manufacturer's instruction described in Fluor-Ace Apopain assay kits (Bio Rad, Hercules, CA). Briefly: Apopain/Caspase-3 is derived from the proenzyme CPP32 at the onset of apoptosis and plays a pivotal role in programmed cell death. Apopain activity has been monitored in vitro using the fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-Trifluorimethyl coumarin (AC-DVED-AFC), [Nicholson, 1996]. Canine Chronic limphatic leukaemia cells (5×10^6) treated according the above cited experimental protocols were collected by centrifugation, washed, resuspended in 500 μl of incubation buffer to lyse the cells, and incubated for 30 min at 4°C. The lysate centrifuged at 15,000 rpm for 5 min, and 400 μl of the supernatant was removed. The supernatant was diluted with incubation buffer to a final concentration of 10^6 cell equivalents/ml, and 100 μl was added to each well of 96-well plates containing Apopain substrate (Z-DEVD-AFC) in presence or without 200 μM Apopain inhibitor (AC-DEVD-CMK) with the aim of verifying the interference from non-specific protease activity. After incubation at 30°C for 60 min the AFC substrate was detected by a fluorometer SPEX-Fluoromax using 370 nm excitation and 530 nm emission. The Units Apopaine (AI) in sample were calculated: $\Delta F/\text{min} \times 1{,}000$, where the fluorescence signal as recorded from the fluorimeter and the factor of 1,000 converts nM AFC for min to activity units.

Statistical Analysis

All determinations were repeated three times, and results expressed as mean $\pm$ SD. Quantitative experiments were analyzed by the Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Activity on Apoptosis

In the experiments in Figure 1, the percentage of hypodiploid nuclei obtained from CLL cells treated with MPA and IDA alone or in association are reported. As shown, the treatment

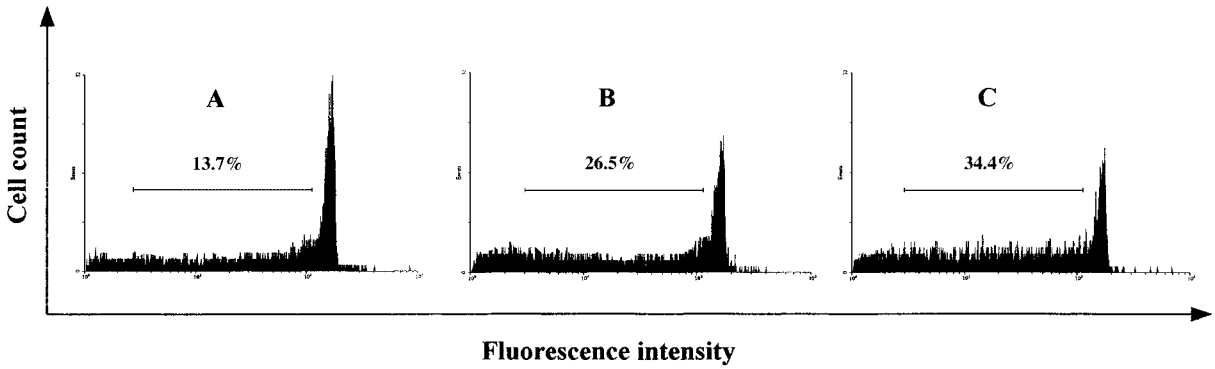

Fig. 1. Induction of apoptosis in CLL cells, detected by flow cytofluorimetry, after exposure for 24 h with MPA 5 μM (**A**) or IDA 5 μM (**B**) or MPA 5 μM + IDA 5 μM (**C**).

with MPA (5 μM) for 24 h induced apoptosis in only 13.7% of cells (A), while the treatment with IDA (5 μM) induced apoptosis in 26.5% of CLL cells (B), and equimolecular association of IDA plus MPA iduced apoptosis in 34.4% of cells (C) by cytometric analysis.

Activity on Nucleosomes

Figure 2 shows apoptosis induced in CLL cells treated for 24 h with increasing doses of IDA or MPA evaluated by assaying mono and oligonucleosomes. IDA significantly increased apoptosis and such effect was dose-dependent. In fact IDA at 0.3 μM increased intracytoplasmic levels of nucleosomes and the Absorbance shifted from 0.091 ± 0.02 to 0.122 ± 0.033. At concentration of 10 μM the Absorbance shifted to 0.205 ± 0.041, and such increase was dose-dependent. By the contrary, MPA showed a significant effect only at dose of 10 μM and Absorbance shifted from 0.091 ± 0.02 to 0.134 ± 0.04.

Intracytoplasmic levels of nucleosomes obtained in CLL cells treated with IDA at

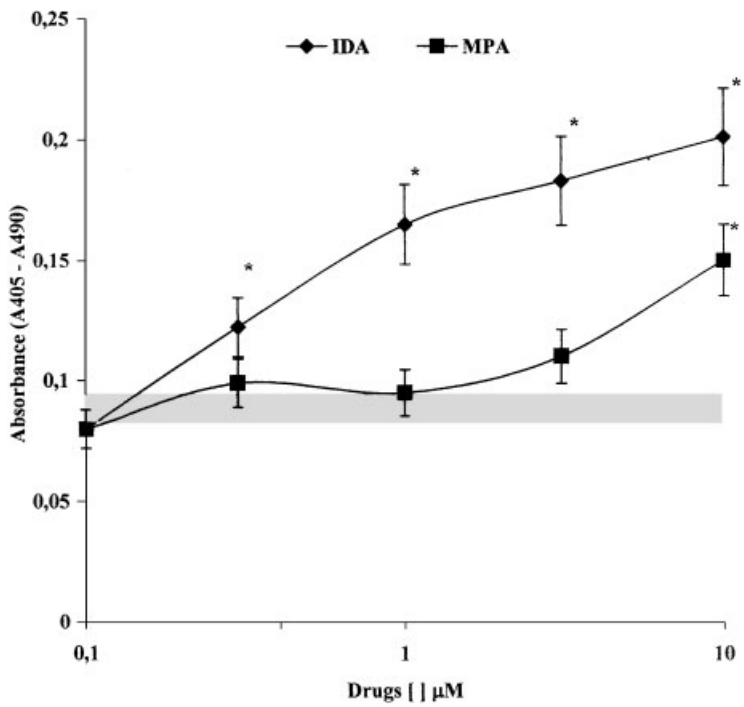

Fig. 2. Effect of IDA and MPA used alone on intracytosolic levels of nucleosomes. Canine Chronic Limphatic Leukaemia cells were treated with drugs for 24 h. The **grey zone** represent the basal levels of intracytosolic nucleosomes. Data represent the mean of Absorbance ± SD of three experiment conducted in duplicate. *$P < 0.05$ versus basal values.

concentration of 1 μM alone or in presence of increasing concentrations of MPA for 24 h are shown in Figure 3. When the two drugs were used in association using IDA at the dose of 1 μM in presence of MPA (0.3–10 μM), the percentage of intracytoplasmatic levels of nucleosomes significantly increased in a dose-dependent manner. Particularly, the percentage analysis demonstrated that these levels increased about 20.1% when the cells were treated with equimolecular dose of 1 μM. MPA at the concentration of 10 μM increased IDA-induced increase nucleosomes of 63.6%.

Activation of Caspase-3

In Figure 4 the results of the Caspase-3 activity are reported. As shown, while Caspase-3 activation significantly increased in presence of IDA in a dose-dependent manner, MPA, up to concentration 5 μM, did not modify the Caspase-3 activity. In fact the treatment with IDA at 1 μM increase the AI formation from 3.02 ± 0.61 at 5.3 ± 1.7 AI U/10^6 cells. The treatment with MPA at 10 μM increase the AI formation from 3.02 ± 0.61 at 4.4 ± 2.9 AI U/10^6 cells.

The percentage variations of the activation of Caspase-3 obtained in Canine CLL cells cultured for 24 h in presence of IDA alone or associated with increasing doses of MPA are reported in Figure 5. MPA was able to increase the activation Caspase-3-induced IDA and such effect was dose-dependent. In fact the percentage activation of Caspase-3 shifted from $58.7 \pm 5.8\%$ (IDA 1 μM alone) to $94.6 \pm 8.9\%$ (IDA 1 μM + MPA 10 μM).

Inhibition of Apoptosis by Caspase-3 Inhibitor

The ability of AC-DEVD-CMK to inhibit the activation of apoptosis triggered by chemotherapeutic agents was then analyzed. As Figure 6 shows, IDA induced apoptosis, evaluated cytofluorimetrically, in canine CLL cells and the association with the equimolecular dose MPA increase the percentage of apoptotic cells. Apoptotic activity of single drugs was inhibited significantly by co-treatment of inhibitor of Caspase-3 (AC-DEVD-CMK) at the

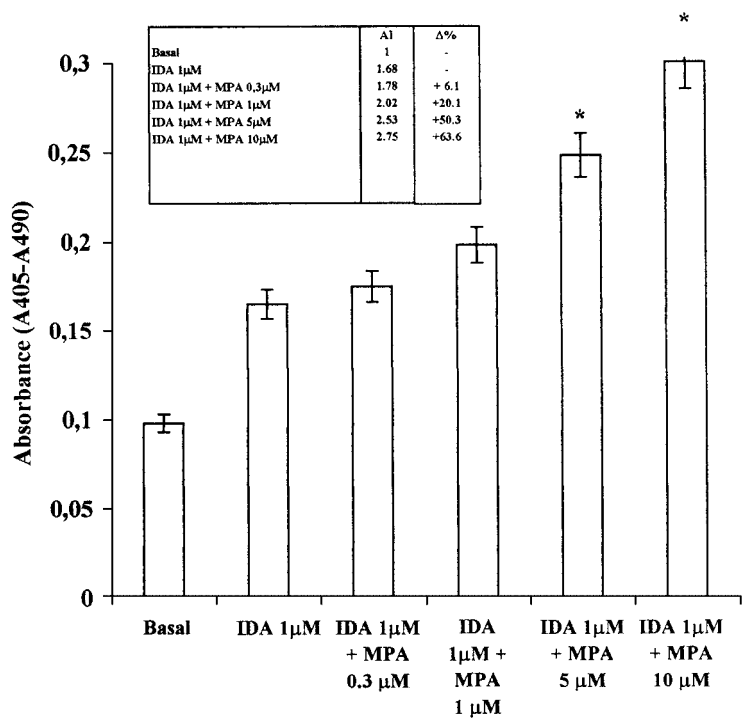

Fig. 3. Effect of IDA at the concentration of 1 μM alone or in association with increasing concentrations of MPA on intracytosolic levels of nucleosomes. Canine Chronic Limphatic Leukaemia cells were treated with drugs for 24 h. Percentage analysis: the MPA at dose 5 μM was able to increase by 50% of the absorbance-induced 1μM IDA. Data represent the mean of Absorbance ± SD of three experiments conducted in duplicate. *$P < 0.05$ compared with IDA 1 μM treatment.

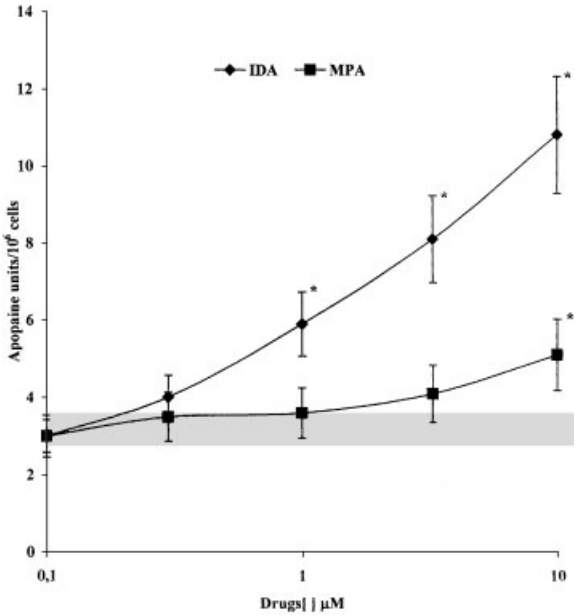

Fig. 4. Effect of IDA and MPA on the activation of Caspase-3. Canine Chronic Limphatic Leukaemia cells were treated with drugs for 24 h. The **grey zone** represent the basal levels of Apopain units. Data represent the mean of Apopain U/10^6 cells ± SD of three experiment conducted in duplicate. *$P < 0.05$ compared with basal values.

concentration of 200 µM. In particular, the percentage of apoptotic cells shifted from 25.5 ± 4.5% (IDA 5 µM alone) to 13.1 ± 2.8% (IDA 5 µM + AC-DEVD-CMK) and from 12.6 ± 3.5% (MPA 5 µM alone) to 8.3 ± 1.5%

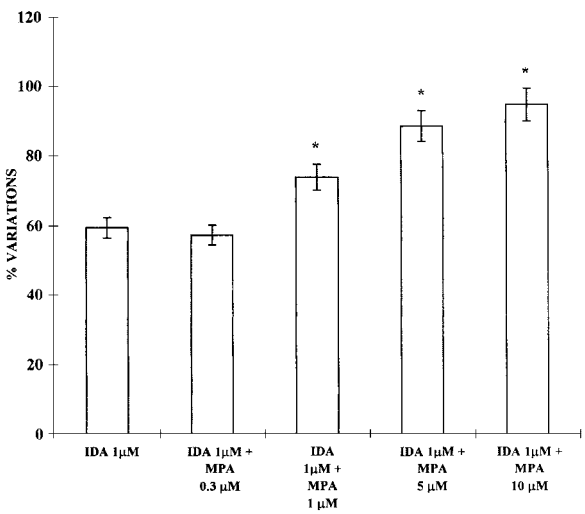

Fig. 5. Effect of IDA at the concentration of 1 µM alone or in presence of increasing concentration of MPA on the activation of Caspase-3. Canine Chronic Limphatic Leukaemia cells treated with both MPA and IDA for 24 h. Data represent the mean of percentage variations ± SD of three experiment conducted in duplicate. *$P < 0.05$ compared with IDA 1 µM treatment.

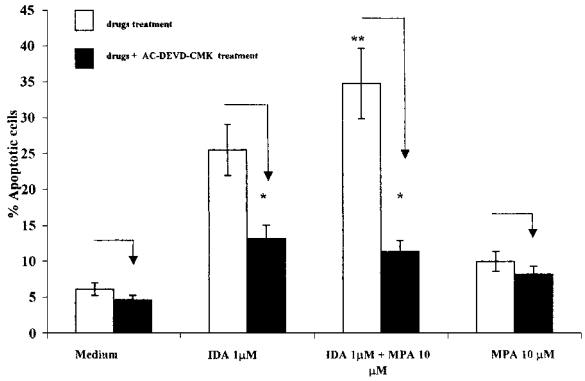

Fig. 6. Apoptosis cytofluorimetrical analysis. Canine Chronic Limphatic Leukaemia cells were treated or not with drugs for 24 h. **White columns**: percentage of apoptotic cells obtained after exposure with Medium or IDA 5 µM or MPA 5 µM alone or (IDA 5 µM+MPA 5 µM). **Black columns**: percentage of apoptotic cells obtained after exposure with Medium or IDA 5 µM or MPA 5 µM or (IDA 5 µM + MPA 5 µM) in association with 200 µM of AC-DEVD-CMK. Data represent the mean ± SD of three experiment conducted in duplicate. *$P < 0.05$ compared with IDA or IDA + MPA, respectively. **$P < 0.05$ compared with IDA alone.

(MPA 5 µM + AC-DEVD-CMK). When the two drugs were used in association the percentage of apoptotic cells shifted from 34.7 ± 4.2% to 11.3 ± 0.9% (IDA 5 µM + MPA 5 µM) to 11.3 ± 2.7% (IDA 5 µM + MPA 5 µM + AC-DEVD-CMK).

DISCUSSIONS

Our results demonstrate that IDA induces apoptosis in Canine CLL cells and that MPA is able to chemosensitise tumor cells to IDA-induced apoptosis. Apoptosis is defined as a cascade of biochemical events leading to nuclear fragmentation and cell death. The process is characterized by a variety of biochemical and morphological changes in the cell, including activation of proteinases, loss of membrane asymmetry, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation—currently the key marker for apoptosis.

The cytotoxic effect of most chemotherapeutic agents in vitro and in vivo depends on the induction of apoptosis in susceptible tumor cells [Schmitt and Lowe, 1999]. The apoptosis induced in tumor cells by anticancer drug require the action of the members of the caspase family [Los et al., 1997; Wesselborg et al., 1999]. Several studies demonstrated that Anthracyclines induce apoptosis in human T-cell leukaemia

cells [Gamen et al., 1997], neuroblastoma cells [Fulda et al., 1998], breast cancer cell lines [Keane et al., 1999], colorectal adenocarcinoma cells and human leukaemic cells [Liu et al., 2002] trough the activation of caspase-3 and/or 8. These findings indicate that the activation of caspase is critical for chemotherapeutic drug-mediated apoptosis.

Little is still known about the chemoterapeutic effect of MPA on the activation of caspases. Furthermore, MPA has been known to act directly on the growth of breast carcinoma and/or endometrial carcinoma trough its inhibitory effect on DNA and/or RNA synthesis via the Progesterone-receptors [Blossey et al., 1984]. Moreover it has been demonstrated that MPA acts directly on human pancreatic carcinoma cells growth trough bcl-2 phosphorilation [Abe et al., 2000]. In fact, the drug-induced phosphorilation has been associated with bcl-2 function decrease and the induction of apoptosis in a number of malignant cell types [Blagosklonny et al., 1996; Haldar et al., 1996]. Our results showed that IDA (0.3–10 μM) was able to significantly activate caspase-3, while MPA, used alone, didn't show a significant effect. However MPA was able to synergyze IDA effect when it was used at equimolecular doses. These results, in agreement with our previous investigations that demonstrated a synergistic activity of MPA on anthracyclines intranuclear uptake in human CLL cells [Pagnini et al., 2000], suggest that the modification in the balance between lipid peroxidating products and NO productions may play a key role to modulate anthracyclines activity. This results suggest, according to Liu et al. [2001], that IDA-induced apoptosis is mediated via caspase-3 and that IDA-induced ROS generation and apoptosis are separate events in the citotoxic activity of anthracyclines.

The present study showed that both activation caspase-3 and apoptosis induced by IDA in Canine CLL cells were inhibited significantly by the specific Caspase-3 inhibitor AC-DEVD-CMK. Besides, the synergic action of MPA on IDA-induced apoptosis seems to involve the activation on Caspase-3. These findings suggest that activation of Caspase-3 is essential at least for IDA mediated apoptosis in canine CLL cells. In conclusion both IDA-induced apoptosis and the synergistic action of MPA in canine CLL cells are mediated by caspase-3 activation.

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