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MDP desmuramyl analogue LK-404 protects bone marrow and spleen cells from cyclophosphamide induced apoptosis

Rok Kostanjšek[1], Petra Kuralt[1], Tadej Malovrh[1], Mojca Škoberne[1], Anton Štalc[2], Vladimir Kotnik[1]

[1]*Institute of Microbiology and Immunology, Medical Faculty, Ljubljana,*
[2]*Research and Development, Lek d.d., Ljubljana, Slovenia*

In this article, we present the data on induction of apoptosis in mouse bone marrow cells and spleen cells after treatment with different concentrations of cytostatic cyclophosphamide in vivo and in vitro. Increasing apoptosis rate of the cells was observed with the increasing concentration of cyclophosphamide, and with the prolongation of incubation time after in vivo as well as in vitro administration of the drug. When apoptosis inducing activity was established, the immunomodulatory and feasible protective effect of desmuramyl analogue of MDP (LK-404) against cyclophosphamide induced apoptosis in mouse bone marrow and spleen cells was studied. Cultivating the cells with cyclophosphamide and LK-404 simultaneously revealed the same apoptosis rate as cultivating with cytostatic only. Treatment of cells with LK-404 prior to treatment with cyclophosphamide decreased apoptosis of bone marrow and spleen cells which suggests potential protective role of LK-404 against cyclophosphamide induced apoptosis.

Key words: apoptosis, bone marrow, spleen, cytostatic, immunomodulator

Introduction

Apoptosis is a genetically controlled process of cell death.[1-5] It has significant value as counter-weight to cell division and proliferation, thus keeping the number of cells in tissue constant.[6-9] Apoptotic cells present unique changes of DNA molecule. Apoptotic process occurs spontaneously, but can also be induced. Important inducers of programmed cell death are deduction of nutrients, regulatory molecules as for instance cytokines, hormones and growth factors, switching on of several distinctive genes, treatment with radiation or with cytostatics.[10,11] Abnormalities in the process of apoptosis may have a large influence on beginning and development of diseases like cancer, viral infections, autoimmune disease and central nervous system disease.[12-18]

Apoptotic cell death can be detected by several methods.[19] In the presented experiments, the apoptosis rate was determined using a specific ELISA (Boehringer Mannheim, Germany) detecting apoptosis specific

Correspondence to: Prof. Vladimir Kotnik, MD, PhD, Institute of Microbiology and Immunology, Medical Faculty, Zaloška 4, SI-1000 Ljubljana, Slovenia. Tel: +386 61 14-03-042; Fax: 14-23-518; E-mail: vladimir.kotnik@mf.uni-lj.si

DNA fragments with enzyme labelled antibodies.

Cyclophosphamide is a cytostatic drug widely used in anti-tumor therapy. It has cytotoxic effect in all phases of the cell cycle, although it has stronger influence on activated or already dividing cells. Cyclophosphamide is a DNA-alkylating drug able to cross-link DNA chains, thus causing cell death.[20] It is generally known that also the cells of the immune system die as a side effect of such tumor treatment. The reduction in number of immune cells results in a decreased immune response which can lead to severe and more frequent infections.

Apoptosis in mouse marrow and spleen cells was studied in described experiments using cyclophosphamide as the inductor of cell death . [21,22] The aim of the study was to examine the apoptosis of bone marrow and spleen immune cells as an inducible process which can be stopped by appropriate treatment. It would be well appreciated, if selective reduction of apoptosis of immune cells and potentiation of toxicity for the tumor cells could be achieved. Using immunomodulating MDP derivatives (N-acetyl-L-alanyl-D-isoglutamine) with hemopoiesis restoring activity may be effective.[23-25] Therefore the attempt was done to restore the cyclophosphamide induced apoptosis in bone marrow and spleen cells with LK-404 one of MDP analogues. LK-404 (N-(7-oxododekanoyl)-L-alanine-D-isoglutamine) an desmuramyl analogue synthesized at Faculty of Pharmecy, University of Ljubljana, in cooperation with Lek d.d., it is in opposite to original MDP molecule without disadvantageous pyrogenicity, and less toxic. The potential protective effect of LK-404 against cyclophosphamide induced apoptosis was studied using LK-404 simultaneously and consecutively to treatment with cyclophosphamide.

Materials and methods

Animals and the preparation of cells

Han NmRi mice were used in all the described experiments. The animals were provided by Lek Research and Development Animal Care, Ljubljana, Slovenia. After delivery from Animal Care they were kept under standard conditions in our facilities. The mice, we used as a source of bone marrow and spleen cells, were at the beginning of the experiment, sacrificed with an ether overdose anesthesia. The femurs and spleens were isolated. The epiphyses were cut off the femur and 5 ml RPMI 1640 Medium (Sigma Chemicals; St. Louis, USA) was squirted through the bone. Washed out cells were sucked into the syringe again and then flushed to the wall of Petri dish. Spleen cells were isolated from the spleen using sintered microscope slide glass plates and afterwards suspended in growth medium RPMI 1640. Contaminating erythrocytes were removed from both cell suspensions by adding 2 ml of 0.85% TRIS buffered ammonium chloride (NH_4Cl). The remaining mononuclear cells were washed three times with MEM medium (Sigma Chemical Co., St.Louis, USA). Cells were suspended (1:10) in trypan blue (0.1%) and then counted in haemocytometer. A concentration of $5x10^5$ cells per ml was prepared for the experiment.

Apoptosis induction

Lyophilized cyclophosphamide (Endoxan, Asta Medica AG Frankfurt, Germany) was dissolved in sterile bidistilled water and solutions giving concetrations of 6.25, 12.5, 25, 50 and 100 mg of cyclophosphamide per kg of mouse weight were prepared.

In *in vivo* experiments, 200 µL of cyclophosphamide at a dose of 50 mg/kg of mouse body weight or 100 mg/kg of mouse body

weight was injected intraperitoneally. Treatment was repeated on days one, four and six. On the seventh day of experiment, the mice were sacrificed. The control group of mice was injected with sterile physiological saline instead of with cyclophosphamide at the same days.

In *in vitro* experiments, cyclophosphamide was used in appropriate equivalents at concentrations of 6.25 mg/kg, 12.5 mg/kg, 25 mg/kg and 50 mg/kg of mouse weight. The cell samples were taken 5, 10, 15, 30, 60 and 90 minutes after the addition of cyclophosphamide to the cells used in the experiment.

LK-404

The compound LK-404 was prepared in RPMI 1640 at concentrations of 0.525 µM, 5.25 µM and 52.5 µM and then used in the experiments described below.

In first series of experiments, isolated bone marrow and spleen cells were treated with LK-404 and cyclophosphamide simultaneously. Aliquoted samples were taken for testing the apoptosis 5, 10, 15, 30, 60 and 90 minutes after incubation. All three concentrations of LK-404 were tested.

In the second series of experiments, consecutive applications of LK-404 and cyclophosphamide were studied. The cells were first incubated at all three concentrations of LK-404 for 90 minutes. Ninety minutes after the beginning of the experiment, a 25 mg/kg of cyclophosphamide was added to each of the samples. Aliquots were taken for testing 5, 10, 15, 30, 60 and 90 minutes after the cyclophosphamide addition.

Cell death detection ELISA

The apoptosis rate was determined using Cell Death Detection ELISA (Boehringer Mannheim, Germany). The assay is based on the quantitative sandwich-enzyme-immunoassay principle using monoclonal antibodies directed against DNA and histones, respectively. This permits a specific determination of mono and oligonucleosomes in the cytoplasmic fraction of cell lysates. In the first step, anti-histone antibody is fixed to the wall and the bottom of the microtiter plate module. During the second step, the nucleosomes contained in the sample bind, via their histone components, to the immobilized anti-histone antibody. In the third step, anti-DNA-peroxidase labeled antibody (POD) reacts with the DNA part of the nucleosome. The amount of peroxidase retained in the immuncomplex is determined spectrophotometrically with ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)) as substrate.

Design of the experiments performed

Treatment of bone marrow and spleen cells with cyclophosphamide in vivo: 9 mice were divided into 3 subgroups. Three mice from the first subgroup were injected with 200 µl of cyclophosphamide at a dose of 100 mg/kg, three from the second subgroup with 200 µl of cyclophosphamide at a dose of 50 mg/kg, and the remaining three with 200 µl of physiological saline on the first, fourth and sixth day. The mice were sacrificed on the seventh day. Bone marrow cells and spleen cells were isolated and divided into two portions. Apoptosis of the cells from the first aliquot was measured the same day. The second aliquot was first incubated for 24 hours at room temperature; the apoptosis was measured on the second day.

Treatment of bone marrow and spleen cells with cyclophosphamide in vitro: Isolated bone marrow and spleen cells were treated with the following concentrations of cyclophosphamide: 25 or 50 mg/kg. The sample treated with RPMI 1640 alone instead of with cyclophosphamide; was regarded as a

control sample. The samples were incubated for 15, 30, 60 or 90 minutes. Aliquots were taken at indicated times. The number of live cells was determined and amount of apoptosis measured in each of the samples at each time point.

Simultaneous application of LK-404 and cyclophosphamide to bone marrow and spleen cells in vitro: Bone marrow and spleen cells were isolated. Cyclophosphamide at a concentration of 25 mg/kg and LK-404 at concentrations of 0.525 μM, 5.25 μM or 52.5 μM were added to cell suspensions. The first control sample was incubated only with 25 mg/kg cyclophosphamide, whereas the second one only with medium RPMI 1640. Aliquots for testing were taken 5, 10, 15, 30, 60 and 90 minutes after the beginning of the experiment and the amount of apoptosis was measured.

Consecutive application of LK-404 and cyclophosphamide to bone marrow and spleen cells in vitro: Bone marrow and spleen cells were isolated and incubated with LK-404 at concentrations of 0.525 μM, 5.25 μM or 52.5 μM. After 90 minutes, a 25 mg/kg of cyclophosphamide was added to each of the samples. Aliquots were taken for testing 5, 10, 15, 30, 60 and 90 minutes after adding the cyclophosphamide.

Two samples of cells were prepared without LK-404 and, after 90 minutes of incubation, the cells of the first sample were treated with 25 mg/kg of cyclophosphamide. The cells of the second sample were treated neither with LK-404 nor with cyclophosphamide. Aliquots for testing were taken after 5, 10, 15, 30, 60 and 90 minutes.

Statistical analysis

Student's t-test was used to determine the difference between samples and $p<0.05$ was regarded as significant.

Results

Treatment of bone marrow and spleen cells with cyclophosphamide in vivo

The apoptosis of bone marrow cells and spleen cells depends on the concentration of cyclophosphamide and time of incubation. The bones from the mice treated with cyclophosphamide at a dose of 50 and 100 mg/kg contained less marrow and the weight of the spleen was reduced as compared to the control nontreated mice.

Treatment of bone marrow and spleen cells with cyclophosphamide in vitro

In previous experiment, we noticed that cyclophosphamide reduced the number of bone marrow and spleen cells during the incubation. To get a real insight in the process of apoptosis, we compared the number of bone marrow and spleen cells with the amount of apoptotic products in each of the sample. The rate of apoptosis in Figure 1 is therefore presented as a quotient between the apoptosis (O.D.) and the number of cells in the sample. Apoptosis of bone marrow and spleen cells treated with cyclophosphamide depends on the concentration of cyclophosphamide and incubation time.

Influence of simultaneous application of LK-404 and cyclophosphamide on apoptosis in bone marrow and spleen cells in vitro

Bone marrow and spleen cells were treated in four different ways. In the control group, the cells were treated with cyclophosphamide at a dose of 25 mg/kg. The first group was treated with 25 mg/kg of cyclophosphamide plus 0.525 μM of LK-404, the second with 25 mg/kg of cyclophosphamide plus 5.25 μM of LK-404, and the third with 25 mg/kg of cyclophosphamide plus 52.5 μM of LK-404. In all eight groups

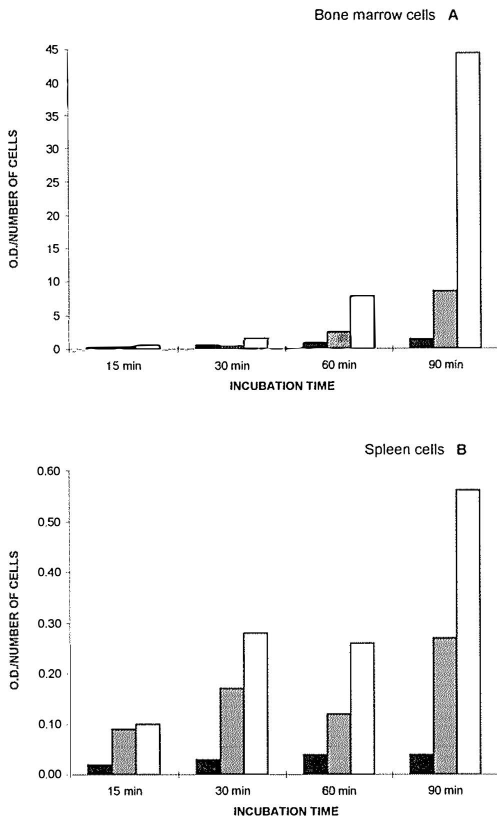

Figure 1. Panel A: Treatment of bone marrow cells with cyclophosphamide *in vitro*. In the control group (cells were not treated with cyclophosphamide), the apoptosis quotient was slowly increasing with the prolongation of incubation time. In the group where bone marrow cells were incubated with 25 mg/kg of cyclophosphamide, the apoptosis quotient was steeply increasing during the prolongation of incubation time. In the group where cells were incubated with 50 mg/kg cyclophosphamide, the apoptosis quotient was exponentially increasing with the prolongation of incubation time. **Panel B:** Treatment of spleen cells with cyclophosphamide in vitro. The apoptosis quotients of the control group (cells not treated with cyclophosphamide) were slowly increasing with incubation time. The apoptosis quotients of the group where cells were treated with 25 mg/kg of cyclophosphamide shows a tendency towards a steep increment with incubation time. The apoptosis quotients of the cells in the group where cells were treated with 50 mg/kg of cyclophosphamide increase rapidly with incubation time.

Legend: ■ control, ▨ cyclophosphamide 25 mg/kg, □ cyclophosphamide 50 mg/kg

(four groups of bone marrow cells and four groups of spleen cells), apoptosis increased by the same rate with the prolongation of incubation time without significant influence of treatment with LK-404.

Influence of consecutive application of LK-404 and cyclophosphamide on apoptosis in bone marrow and spleen cells in vitro

The rate of apoptosis of bone marrow and spleen cells in the first control sample (cells incubated in RPMI 1640 only) was slowly increasing during the incubation time due to spontaneous apoptosis of incubated bone marrow and spleen cells. In the second control sample (cells treated with cyclophosphamide after 90 minutes of incubation, and not pretraeted with LK-404) apoptosis of bone marrow and spleen cells rapidly increased after cyclophosphamide had been added, due to cyclophosphamide induced apoptosis.

The difference in apoptosis between both control samples was significant (p=0.015). Apoptosis in other samples (treated with LK404 prior to cyclophosphamide) increased only slowly in comparison to the control samples. The treatment of bone marrow and spleen cells with the highest concentration of LK-404 (52.5 μM) slowed down the apoptosis. The decreasing effect calculated between the second control sample (cells treated with cyclophosphamide after 90 minutes of incubation and not pre-treated with LK-404) and the sample pre-treated with 52.5 μM of LK-404 was significant (p=0.004 for bone marrow cells and p=0.042 for spleen cells). The results of these experiments are shown in Figure 2.

Discussion

The importance of apoptosis in the development and differentiation of immune cells is

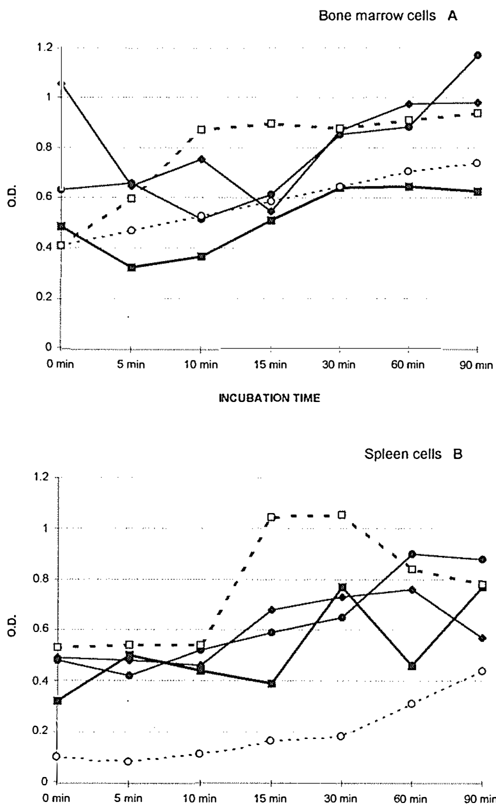

Figure 2. Panels A and B: Influence of consecutive applications of LK-404 and cyclophosphamide on apoptosis in bone marrow and spleen cells in vitro. Apoptosis of bone marrow (A) and spleen (B) cells incubated with RPMI 1640 only (- - O - -) was slowly increasing with the prolongation of incubation time. After addition of cyclophosphamide in the second control sample (- - □ - -) (cells treated with cyclophosphamide after 90 minutes, and not pre-treated with LK-404) the apoptosis increased steeply. Apoptosis in other samples (of both types of cells) treated with 0.525 µM (--●--), 5.25 µM (—◆—), and 52.5 µM LK-404 (--■—) prior to cyclophosphamide addition, decreased in comparison to the second control sample (- - □ - -) of bone marrow and spleen cells. The most effective was pre-treatment with 52.5 µM LK-404 (--■—).

not doubtful any more. At present, the focal question concerning the immune system is the involvement of apoptosis in regulation and functioning of the immune cells.[5,10, 11,21,22] However, the immune response is a self-limiting process under the influence of antigen burden, stability of the antigenic structure, activity of macrophage phagocytic system, antigenicity, capability of the antigen presentation and recognition, function of B and T cells and competence of effector functions of antibodies and cytotoxic lymphoid cells, it may also be limited by induction of apoptosis. Interference with apoptosis leaving cells alive for longer period of time, as determined by the genetically determined cell life span, would give an interesting tool to manipulate disease which depends also on immune system activity.[15,18] In malignant disease treated with cytostatics, immune cells are not prone to respond effectively. The consequences are oportunistic infections which may be the cause of death of the patient. Our experimental approach to study the importance of apoptosis in regulation of immune system function is a copy of the treatment of patients with alkylating drugs. Cyclophosphamide treatment is accompanied by a decreased function of immune system and followed by increased susceptibility to infection.[20]

Protection of the immune cells against cytostatics or adjuving depressed lymphocyte function would be of great value. MDP molecule, an important component of the peptidoglycan, has been proved to have an immunoadjuvant activity. Unfortunately, the original MDP molecule is highly pyrogenic and a good inducer of autoimmunity and as such of no use as an immuno-adjuvant. By changing the structure of MDP molecule, analogues may be prepared with a preserved adjuvant activity with no unpleasant side effects.[26] Desmuramyl analogues of MDP are able to increase the functioning of the immune cells.[27] The exact way how they do it is unknown. It would be therefore interesting to know whether such preparations would be able to interfere with the process of apoptosis.

For the induction of apoptosis we applied the widely used cyclophosphamide, a cytostatic with not yet describet apoptosis activity.

We were able to induce apoptosis with cyclophosphamide as presented in the results section. Apoptotic function of cyclophosphamide was time and dose dependent. Therefore, in patients treated with cyclophosphamide, it is expected that at least some of the immune cells will die because of the induced apoptosis. This was proved to be true in our experimental model of mice. *In vivo* treatment with cyclophosphamide resulted in increased apoptosis rate in bone marrow and spleen cells. The same was demonstrated also *in vitro* when the isolated cells were treated with cyclophosphamide. Isolated immune cells fall to apoptosis spontaneously, however, the process itself is forced by cyclophosphamide treatment.

To study apoptosis protective effect of MDP analogues (potent drugs for restoring mielosuppression) bone marrow and spleen cells were treated with MDP desmuramyl derivative LK-404. It was shown that LK-404 did not substantially increase spontaneous apoptosis at doses proposed to have an immunoadjuvant activity. Therefore, MDP treated cells were exposed to cyclophosphamide. To our surprise, apoptosis of LK-404 treated cells wasdose and time dependent and cells were less apoptotic than those treated with cyclphosphamide alone. The same result was achieved with bone marrow and spleen cells. The extent of apoptosis inhibition was greater when bone marrow cells were used as the target cells, suggesting that anti-apoptotic effect of LK-404 could be advantageous in treating the patients with the affected bone marrow.[28] The mechanism how LK-404 protects cells from the apoptosis induced by cyclphosphamide is not clear.

The potential use of anti-apoptotic phenomenon of LK-404 requires further investigations. The substance could be beneficial in the treatment of patients with suppressed activity of bone marrow as the consequence of cytostatic or radiation treatment, viral infections, prolongation of the specific immune response in the extinguishing phase of immune response, treatment of the immune cells in aged (senile) organisms. A more challenging use of LK-404 could be its application in treating Alzheimer's and Parkinson's diseases.

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