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Induction of programmed cell death (apoptosis) in mature lymphocytes

Miguel Lucas, Francisca Solano and Aureo Sanz

Dpto de Bioquímica, Hospital Universitario V. Macarena, Facultad de Medicina, Avda Sánchez Pizjuán 4, 41009 Sevilla, Spain

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The apoptosis of human peripheral blood lymphocytes was analyzed by the breakdown of DNA into oligonucleosome-sized fragments. The mature lymphocytes were rendered sensitive to apoptosis by either the omission of fetal bovine serum in the culture medium or the addition of polymyxin B. In the first case it was counteracted by phorbol myristate acetate. The possible involvement of protein kinase C in cell survival is pointed out.

Polymyxin B; Tumor promoter; Apoptosis; Nucleosome

1. INTRODUCTION

Apoptosis, also called programmed cell death to discriminate it from necrosis, is characterized by: chromatin condensation, fragmentation of DNA into oligonucleosome-sized pieces, swelling and progressive cell degradation. It occurs in a number of processes, e.g. embryo morphogenesis, T-cell clone maturation, senescence of neutrophils, etc. Apoptosis has been described as a form of cellular 'suicide', since the cell death appears to result from the induction of active processes [1,2]. Little is known of the intracellular effector mechanisms in apoptosis and in particular of the DNA cleavage although endonucleases seem to be clearly involved [3]. Possible intracellular signaling mechanisms in the initiation of apoptosis include influx of calcium [4] and altered expression of oncogenes *c-fos* and *c-myc* [5].

The promotion of cell survival by suppression of the process of apoptosis, an interesting view described for haemopoietic colony stimulating factor [6], may be crucial for cell differentiation and tumorogenesis. Interestingly, apoptosis is inhibited by tumor promoters [7]; moreover, those agents that increase cAMP concentrations stimulate DNA fragmentation in thymocytes when it occurs without activation of protein kinase C [8], a property which has been also outlined for increases in $[Ca^{2+}]_i$. In addition, IL-2 has been described to block glucocorticoid-induced apoptotic death of IL-2-dependent T lymphocytes [9].

Although poorly documented, mature lymphocytes appear to be relatively insensitive to apoptosis. We present here the induction of DNA-fragmentation in peripheral blood lymphocytes by polymyxin B, a putative inhibitor of protein kinase C.

Correspondence address: M. Lucas, Dpto de Bioquímica, Hospital Universitario V. Macarena, Facultad de Medicina, Avda Sánchez Pizjuán 4, 41009 Sevilla, Spain

2. MATERIALS AND METHODS

Peripheral blood lymphocytes were obtained from freshly venesected blood by the method of Boyum [10], resuspended and cultured at 2×10^6 cells/ml in RPMI 1640 medium supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), 20 mM Hepes buffer, pH 7.3, and 10% heat-inactivated fetal bovine serum.

DNA was extracted [11] from the lymphocytes, pelleted at approximately $500 \times g$, after washing out the incubation medium. Cells were resuspended in Tris-EDTA medium (50 mmol/l Tris, 10 mmol/l EDTA, pH 8) and supplemented with 0.5% (w/v) *N*-laurylsarcosine and 0.5 mg/ml proteinase K. After 1 h at 50°C the incubation medium was supplemented with 0.25 mg/ml heat-treated ribonuclease A and further incubated in the water-bath at 50°C for 1 h. The tubes were supplemented with 2.5 vols of TE (10 mmol/l Tris, 1 mmol/l EDTA, pH 8). After addition of 2.5 vols of 95% ethanol DNA was precipitated overnight at −60°C. The DNA precipitates were pelleted by centrifugation and dissolved in TE buffer. Samples were heated at 65°C and supplemented with loading buffer (10 mM EDTA, pH 8, containing 0.25% Bromophenol blue, 1% low-gelling-temperature agarose, 40% sucrose) at a 1:5 (v/v) ratio. Electrophoresis was carried out in 80 mM Tris/20 mM phosphate/2 mM EDTA, pH 8.

2.1. *Chemicals*

Agarose was from BRL; the λ-DNA *Hin*dIII digested molecular weight markers, proteinase K and ribonuclease A were from Boehringer-Mannheim; bleomycin was from Almirall (Spain); phorbol myristate acetate (PMA), A23187, polymyxin B, *N*-laurylsarcosine and ethidium bromide from Sigma; RPMI, fetal bovine serum, streptomycin and penicillin from Flow.

3. RESULTS AND DISCUSSION

The incubation of lymphocytes in RPMI medium (see section 2) lacking fetal bovine serum produced a pattern of apoptotic DNA degradation which was hindered by the presence of either PMA (see Fig. 1) or A23187 (data not shown), although in the latter case lymphocytes were rendered permeable to vital dyes indicating a lytic effect of the calcium ionophore under the described culture conditions. The effect of calcium ionophore can be interpreted as a requirement of lymphocyte integrity to enable the induction of DNA breakdown. The protective effect of PMA is in agree-

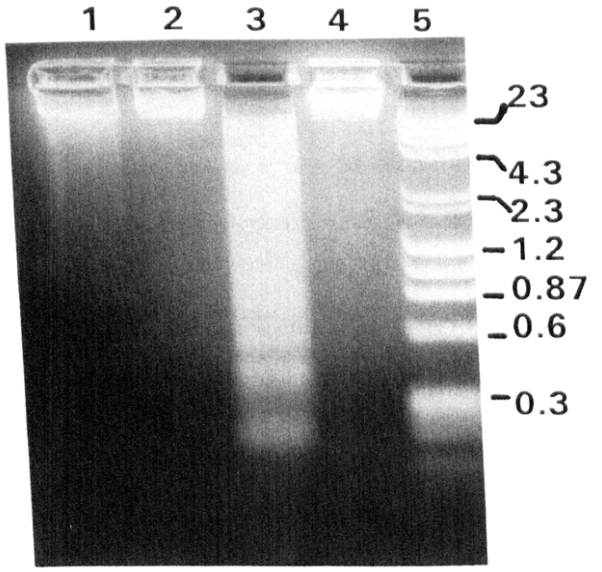

Fig. 1. Apoptosis produced by fetal bovine serum deprivation. Effect of PMA. Lymphocytes were cultured for 72 h and DNA was extracted (see section 2). Electrophoresis was carried out in 2% agarose. The culture conditions were as follows: (lane 1) complete medium; (lane 2) complete medium plus 50 nM PMA; (lane 3) medium lacking fetal bovine serum; (lane 4) medium lacking fetal bovine serum plus 50 nM PMA. A mixture of λ-DNA/*Hin*dIII and φX174/*Hae*III in lane 5 refers to molecular size markers. Kbp values are given on the right of the gel.

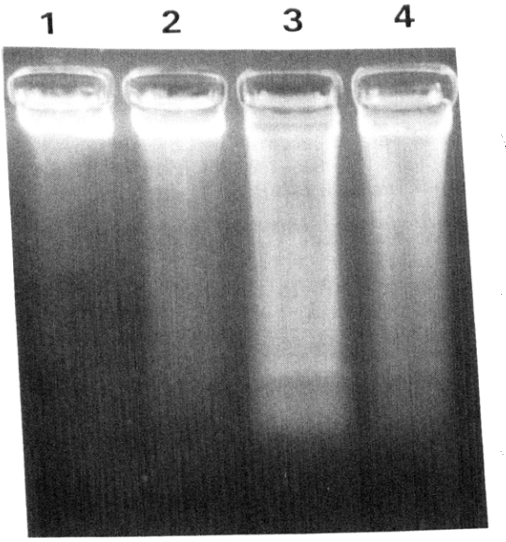

Fig. 2. DNA cleavage by polymyxin B and bleomycin. Human lymphocytes were cultured for 24 h in complete RPMI medium, i.e. with fetal bovine serum, in the absence (lane 1), and in the presence of 10 μM polymyxin B (lane 2), 300 μM polymyxin B (lane 3), 1 mg/ml bleomycin. DNA was extracted and analyzed in 2% agarose gel electrophoresis.

ment with data previously reported on inhibition of apoptosis in vitro by tumor promoters [7].

Unlike immature thymocytes [12], mature lymphocytes were insensitive to treatment with calcium ionophore and PMA when cultured in the complete medium, i.e. containing fetal bovine serum, but indeed apoptosis was induced by treatment with bleomycin (see Fig. 2), an antibiotic which is known to release nucleosomes from chromatin and chromosomes [13]. The effective concentration of bleomycin for DNA breakdown in intact lymphocytes was in the range 0.3–3 mg/ml which is comparable, although relatively higher, to that described for isolated nuclei [13]. With bleomycin as positive controls for the experimental procedure, we could detect the induction of DNA degradation upon treatment with polymyxin B, in a concentration range of 10–300 μM, leading to the electrophoretic pattern characteristic of DNA fractions of integer multiples of about 200 base pairs (see Fig. 2).

The effect of bleomycin was explained [13] as a non-enzymatic chemical mechanism which affects only the DNA between nucleosomes, i.e. the linker DNA. The described effect of polymyxin upon intact lymphocytes is not explained in the present work. It is tempting to argue on mechanisms relying on protein-kinase C inhibition, as described in neutrophils [14]; however, it could also be explained by a direct effect on gene activation which, as has recently been described [15], is required for programmed cell death. Anyway, polymyxin B appears to be an interesting tool to study the balance between apoptosis and cell survival in mature lymphocytes.

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