THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 16, Issue of June 5, pp. 10939-10941, 1992
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Mitochondrial DNA Is Not Fragmented during Apoptosis*

(Received for publication, December 27, 1991)

Marta Murgia‡§, Paola Pizzo‡, Dorianna Sandoná‡, Paola Zanovello¶, Rosario Rizzuto‡, and Francesco Di Virgilio‖

From the ‡National Research Council for the Study of the Physiology of Mitochondria and Institute of General Pathology, via Trieste 75 and ¶Chair of Immunology, via Gattamelata 64, University of Padova, Padova, Italy and the ‖Institute of General Pathology, University of Ferrara, via Borsari 46, Ferrara, Italy

We have exposed mouse thymocytes and P-815 mastocytoma cells to four different conditions reported to cause apoptosis: 1) incubation in the absence of mitogenic factors; 2) incubation in the presence of dexamethasone; 3) stimulation with external ATP; 4) treatment with high concentrations of the K^+ ionophore valinomycin. These treatments caused DNA fragmentation to a varying extent in the two cell types. High stringency hybridization with a cDNA probe specific to a mitochondrial DNA sequence revealed that during apoptosis induced by lack of mitogenic factors, dexamethasone, or extracellular ATP, mitochondrial DNA was not fragmented. On the contrary, valinomycin caused extensive degradation of mitochondrial DNA. These results support the notion that DNA fragmentation during apoptosis is a specific nuclear event and suggest that other agents, such as valinomycin, may act less selectively.

There is increasing interest in the mechanism of apoptosis, the active process whereby cell death occurs during embryogenesis, hormone and growth factor deprivation, thymic selection, cell-mediated cytotoxicity, and glucocorticoid treatment (1). Apoptosis is understood to require gene expression and activation of nuclear endonucleases, these latter responsible for the pattern of DNA degradation into nucleosome-size fragments considered to be the hallmark of apoptosis (1, 2). DNA-degrading events are believed to occur early after the delivery of the lethal hit and before intracellular organelles and the plasma membrane are damaged (3). Thus, according to this hypothesis, DNA degradation should be a specific nuclear event. However, whether DNA contained within other subcellular organelles, *e.g.* the mitochondria, also undergoes fragmentation or rather remains intact, is unknown. In the present study we have induced apoptosis of two cell types, mouse thymocytes and P-815 mastocytoma cells, by three means: 1) culture in the absence of mitogenic factors (4); 2) incubation in the presence of dexamethasone (5); 3) stimulation with external ATP (6, 7). By using a cDNA probe for mitochondrial DNA, we show that in these cells mitochondrial DNA does not undergo fragmentation during apoptosis and runs as intact molecule. This evidence support the specific nuclear location of DNA fragmentation during apoptosis.

MATERIALS AND METHODS

Thymocytes, obtained from 3-week-old BALB/c mice, were resuspended at a final concentration of 5×10^6/ml in RPMI 1640 medium containing the indicated FCS[1] concentration; 1-ml samples were then incubated as described in the legends to Figs. 1 and 2. P-815 cells were grown in culture in RPMI medium as previously described (6), resuspended in 1 ml of RPMI containing 10% FCS at a concentration of 2×10^6 cells/sample, and incubated in the presence or absence of the indicated valinomycin and ATP concentration for 6 h. Cells were lysed by addition to the culture medium of 5 × lysis buffer (2 M NaCl, 50 mM Tris/Cl, 10 mM NaEDTA, pH 8, 1% sodium dodecyl sulfate, 0.25 mg/ml proteinase K), and DNA was extracted by the salting-out method. Electrophoresis was carried out on 1 or 0.8% agarose gel containing 0.5 μg/ml ethidium bromide. The same amount of DNA, as assessed by spectrophotometric measurement, was loaded in each lane. Isolation of cell nuclei and mitochondria from mouse liver was performed according to standard procedures (8, 9). Hybridization with the ^{32}P-labeled mitochondrial DNA probe was carried out at 60 °C. The rat cDNA probe was kindly provided by Dr. Cecilia Saccone, University of Bari (10).

RESULTS AND DISCUSSION

We have induced apoptosis of mouse thymocytes by three procedures: (*a*) short-term *in vitro* culture in the absence of mitogens; (*b*) incubation in the presence of 1 μM dexamethasone; (*c*) incubation in the presence of high valinomycin concentrations. Both dexamethasone and incubation in the absence of mitogens ("spontaneous fragmentation") triggered extensive DNA degradation which was easily detected by agarose gel electrophoresis of cellular DNA (Fig. 1). Spontaneous DNA fragmentation occurred after a 4–6-h incubation and was prevented by increasing the serum concentration of the incubation medium (Fig. 1, *lane 6*). Valinomycin, a widely used and powerful DNA-fragmenting agent (11), was a rather weak stimulus for DNA degradation in mouse thymocytes under our experimental conditions (not shown).

DNA was transferred to nitrocellulose filters and hybridized at high stringency with a rat cDNA probe complementary to a mitochondrial DNA sequence of 3000 base pairs (10). This cDNA hybridized with purified mouse mitochondrial but not nuclear DNA, giving a major band of 21,000 base pairs and a minor band of apparent lower M_r, probably corresponding to supercoiled DNA (Fig. 2, *panel A*). However, as shown in Fig. 2, *panels B* and *C*, the cDNA probe did not hybridize with low M_r DNA fragments isolated from apoptotic thymocytes (*lanes 1* and *3* in *panel B*; *lanes 1* and *4* in *panel C*), suggesting that the ladder of DNA fragments contained no mitochondrial DNA. To prove the ability of the probe to specifically recognize the mitochondrial DNA in the total cellular DNA extract, we digested cellular DNA with three different restriction enzymes, *Pvu*II, *Pst*I, and *Hin*dIII. After digestion of cellular

* This work was supported by grants from the National Research Council (Target Project Biotechnology and Bioinstrumentation; Special Project Biology and Pathology of Calcium), Ministry of Scientific Research (MURST), and Associazione Italiana per la Ricerca sul Cancro (AIRC). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) 14848.

§ To whom correspondence should be addressed: Institute of General Pathology, University of Padova, via Trieste 75, I-35121 Padova, Italy. Tel.: 49-828-6573; Fax: 49-828-6576.

[1] The abbreviation used is: FCS, fetal calf serum.

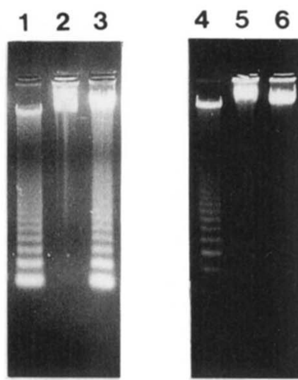

FIG. 1. **Agarose gel electrophoresis of total cellular DNA from murine thymocytes showing the pattern of DNA bands typical of apoptosis.** Cells were incubated at 37 °C in RPMI medium containing either 10% (*lanes 1–3*) or 20% (*lanes 4–6*) FCS. *Lanes 2* and *5*, DNA extracted from control thymocytes immediately after the isolation from the thymus; *lanes 1* and *4*, DNA extracted from thymocytes incubated for 6 h in the presence of 1 μM dexamethasone; *lanes 3* and *6*, DNA extracted from thymocytes after 6 h of incubation in the absence of dexamethasone (spontaneous fragmentation).

DNA, Southern hybridization with the mitochondrial DNA probe identified discrete bands corresponding to the known restriction map of the intact mitochondrial DNA (*lanes 2* and *4* in *panel B*, and *lanes 2, 3, 5*, and *6* in *panel C*). These results provide conclusive evidence that no fragmentation of the mitochondrial DNA occurred in the apoptotic cells. However, they do not rule out the possibility that failure of mitochondrial DNA to undergo fragmentation during apoptosis could be due to an intrinsic resistance of native mitochondrial DNA to intracellular nucleases. To exclude this possibility, we investigated the effect of valinomycin in a cell type, the P-815 mastocytoma cells, which is known to undergo massive DNA fragmentation when stimulated with this ionophore (11, 12). As shown in Fig. 3, *panel A,* valinomycin promoted extensive fragmentation of cellular DNA (*lane 1, left*); however, under these conditions mitochondrial DNA was also degraded, as shown by hybridization of the mitochondrial probe with the DNA (*panel A, lane 1, right*). It is likely that, at the high concentration used to induce DNA fragmentation, valinomycin also caused a generalized alteration of intracellular membranes, thus allowing degradation of mitochondrial DNA. On the contrary, external ATP, recently reported to be a DNA-fragmenting agent in P-815 and other cell types (6, 7), caused no mitochondrial DNA breakdown (Fig. 3, *panel B, lanes 1*). DNA fragmentation was specifically due to ATP in its fully dissociated (ATP^{4-}) form since addition of Mg^{2+} completely prevented the effect (*panel B, lanes 2*).

An increasing number of physiologic and pathologic events are known to involve cell death by apoptosis (13), but although this mechanism is thought to be active and to require protein synthesis and an intact energy metabolism, how this controlled suicide is triggered and regulated is still mysterious. A handful of surface molecules whose ligation triggers apoptosis is known, *e.g.* APO-1 (14), the TNF receptor (15), and a still ill defined membrane molecule bound by extracellular ATP (6, 7). In addition, the recent observation that overexpression of a protein located in the mitochondria, the product of the nuclear gene bcl-2, prevents lymphoid cells from undergoing apoptosis also suggested intriguing links between apoptosis and the mitochondria (16, 17). One of the most surprising aspects of apoptosis is the early degradation of cellular DNA that is considered to be the hallmark of this process. DNA degradation in apoptotic cells is highly reminiscent of DNA digestion by bacterial restriction enzymes, and this has

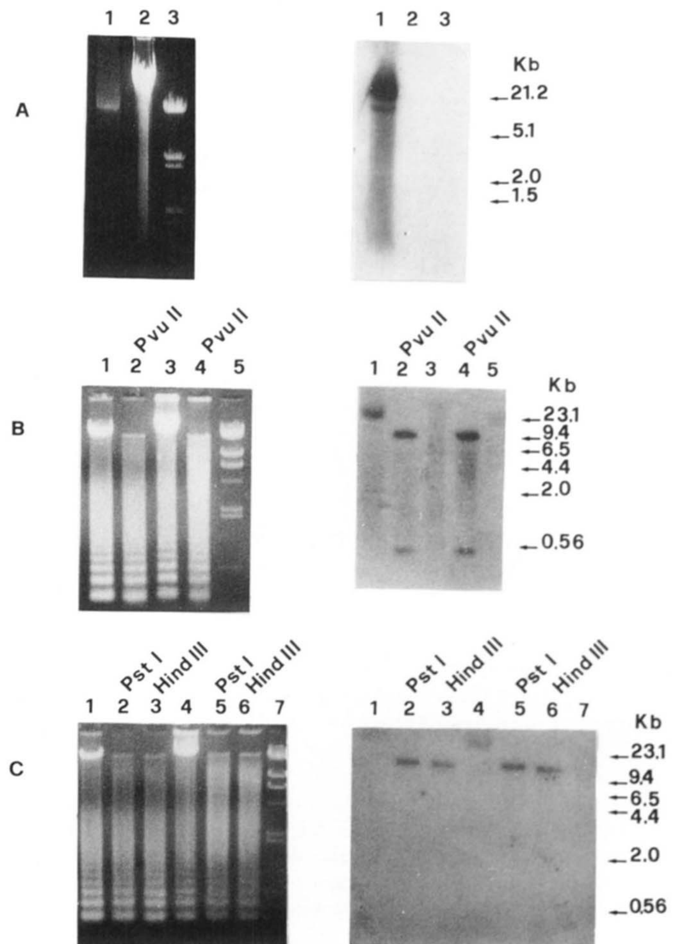

FIG. 2. **Southern blot analysis of total cellular DNA from apoptotic murine thymocytes by hybridization to a rat cDNA probe specific for mitochondrial DNA.** Agarose gel electrophoresis (*left*) and corresponding autoradiography (*right*) are shown in each *panel*. *Panel A*: *lanes 1*, purified mouse liver mitochondrial DNA specifically recognized by the probe; *lanes 2*, purified mouse liver nuclear DNA which did not react with the probe; *lanes 3*, molecular weight markers (λ-DNA digest of *Eco*RI and *Hin*dIII). *Panel B:* total cellular DNA from thymocytes treated with dexamethasone (*lanes 1* and *2*) and thymocytes that underwent spontaneous fragmentation (*lanes 3* and *4*); DNA in *lanes 2* and *4* was also digested with restriction endonuclease *Pvu*II, while DNA in *lanes 1* and *3* was undigested; *lanes 5*, molecular weight markers (λ-DNA digest of *Hin*dIII). *Panel C:* total cellular DNA from thymocytes treated with dexamethasone (*lanes 1–3*) and thymocytes that underwent spontaneous fragmentation (*lanes 4–6*); DNA digested with restriction endonuclease *Pst*I (*lanes 2* and *5*); DNA digested with restriction endonuclease *Hin*dIII (*lanes 3* and *6*); undigested DNA (*lanes 1* and *4*). Equal amounts of DNA from the various samples (5 and 2.5 μg in *panels B* and *C*, respectively) were digested with the indicated restriction enzyme. *Kb*, kilobase(s).

prompted speculation that specific endonucleases are activated in apoptotic cells. On the other hand, it is difficult to rule out that DNA is rather digested as a consequence of an early and generalized damage to intracellular membranes which could permit digestion not only of nuclear but also mitochondrial DNA. Furthermore, it has also been suggested that DNA fragmentation could be directly caused by free radicals which are generated during several processes leading to cell death (18). Were these suggestions true, we would expect to find fragmentation of mitochondrial as well as nuclear DNA, since collapse of intracellular membranes or generation of highly reactive oxygen intermediates would cause unrestricted DNA damage. On the contrary, the three

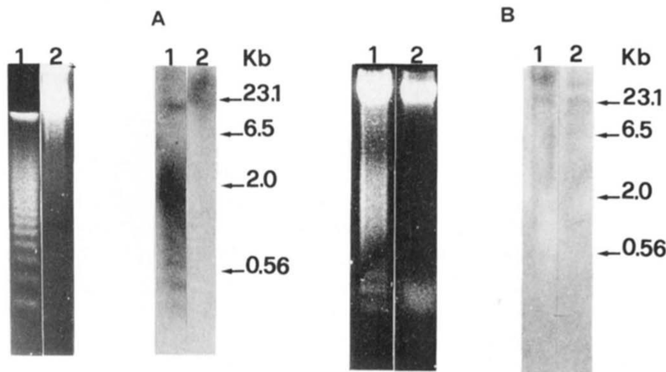

FIG. 3. **Effect of valinomycin and external ATP on mitochondrial DNA extracted from P-815 mastocytoma cells.** Agarose gel electrophoresis (*left*) and autoradiography (*right*) are shown in each *panel. Panel A: lanes 1,* DNA extracted from cells treated with valinomycin (15 μM); *lanes 2,* control cells. *Panel B: lanes 1,* DNA extracted from cells treated with extracellular ATP (5 mM); *lanes 2,* control cells treated with 5 mM ATP in the presence of 5 mM $MgCl_2$. *Kb,* kilobase(s).

apoptotic stimuli used in the present experiments left mitochondrial DNA remarkably intact, even in the presence of extensive nuclear DNA degradation. This observation lends support to the hypothesis that DNA cleavage during apoptosis is a specific nuclear event likely dependent on enzyme pathways which are exclusively located in the nucleus.

Acknowledgments—We wish to thank Prof. C. Saccone (University of Bari) for the kind gift of the mitochondrial cDNA probe and Prof. T. Pozzan (University of Padova) for helpful suggestions.

REFERENCES

1. Willye, A. H., Kerr, J. F. R. & Currie, A. R. (1980) *Int. Rev. Cytol.* **68,** 251–305
2. Duvalle, E. & Willye, A. H. (1986) *Immunol. Today* **7,** 115–119
3. Busch, W., Kleine, L. & Tenniswood, M. (1990) *Biochem. Cell Biol.* **68,** 1071–1074
4. Collins, R. J., Verschuer, L. A., Harmon, B. V., Prentice, R. L., Pope, J. H. & Kerr, J. F. (1989) *Br. J. Haematol.* **71,** 343–350
5. Ucker, D. S. (1987) *Nature* **327,** 62–64
6. Zanovello, P., Bronte, V., Rosato, A., Pizzo, P. & Di Virgilio, F. (1990) *J. Immunol.* **145,** 1545–1550
7. Zheng, L. M., Zychlinsky, A., Liu, C-C., Ojcius, D. M. & Young, J. D-E. (1991) *J. Cell Biol.* **112,** 279–288
8. Palva, T. K. & Palva, E. T. (1985) *FEBS Lett.* **192,** 267–270
9. Busch, H. (1967) *Methods Enzymol.* **12,** 421–434
10. Gadaleta, G., Pepe, G., De Candia, G., Quagliarello, C., Sbisá, E. & Saccone, C. (1989) *J. Mol. Evol.* **28,** 497–516
11. Martz, E. & Howell, D. M. (1989) *Immunol. Today* **10,** 79–86
12. Albritton, N. L., Verret, C. R., Wolley, R. C. & Eisen, H. N. (1988) *J. Exp. Med.* **167,** 514–527
13. Williams, G. T. (1991) *Cell* **65,** 1097–1098
14. Trauth, B. C., Klas, C., Peters, A. M., Matzku, S., Moller, P., Falk, W., Debatin, K-M. & Krammer, P. H. (1989) *Science* **245,** 301–305
15. Laster, S. M., Wood, J. G. & Gooding, L. R. (1988) *J. Immunol.* **141,** 2629–2634
16. Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Kieff, E. & Rickinson, A. (1991) *Cell* **65,** 1107–1115
17. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. & Korsmeyer, S. J. (1990) *Nature* **348,** 334–336
18. Halwell, B. & Aruoma, O. I. (1991) *FEBS Lett.* **281,** 9–19