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THE PROTEIN KINASE INHIBITOR STAUROSPORINE INDUCES MORPHOLOGICAL CHANGES TYPICAL OF APOPTOSIS IN MOLT-4 CELLS WITHOUT CONCOMITANT DNA FRAGMENTATION

Elisabetta Falcieri+°, Alberto M. Martelli^*, Renato Bareggi^, Amelia Cataldi+ and Lucio Cocco§

+ Istituto di Morfologia Umana Normale, via dei Vestini, 66100 Chieti, Italy

° Istituto di Citomorfologia Normale e Patologica del C.N.R., c/o I.O.R., via di Barbiano 1/10, Bologna, Italy

^ Istituto di Anatomia Umana Normale, via Manzoni 16, 34138 Trieste, Italy

§ Istituto di Anatomia Umana Normale, via Irnerio 48, 40126 Bologna, Italy

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The protein kinase inhibitor staurosporine induces ultrastructural changes typical of apoptotic cell death in MOLT-4 cells in a concentration range of 10-200 nM. The well known chromatin margination was indeed present, followed by characteristic nuclear protrusions. The formation of numerous homogeneously electron dense micronuclei was the final step of the process. Nevertheless we did not detect the distinctive internucleosomal DNA fragmentation which has been demonstrated to occur in a variety of cells exposed to agents causing apoptosis. Our results strengthen recent argument showing that DNA breakage cannot be considered the sole criterion for detection of apoptosis.

Apoptosis is a process of active cell death which plays a key role in tissue and organ development during embryogenesis as well as in adult tissues undergoing cell turnover and in selective removal of T cells during clonal selection in the thymus (1,2). Apoptosis can be triggered by a variety of chemical, pharmacological and physical agents (1,2). The main distinctive features of apoptosis are: i) endonuclease-mediated cleavage of internucleosomal DNA linker sections resulting in a "ladder-like" electrophoretic pattern of degraded DNA products; ii) striking nuclear morphological changes, such as chromatin condensation, disappearance of nucleoli, shrinkage and micronucleation; iii) cytoplasmic blebbing followed by micronuclei extrusion. Cytoplasmic organelles as well as membrane integrity are preserved during the early stages of apoptosis which usually ends in secondary necrosis (1). Recently, Bruno et al. (3) have reported that in human lymphocytic leukemia MOLT-4 cells staurosporine, a protein kinase inhibitor, induces marked changes in nuclear morphology such

*To whom correspondence should be addressed.

Abbreviation: DAPI = 4',6-diamidino-2-phenylindole.

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as micronucleation and extensive fragmentation . These changes are suggestive of apoptosis but the authors limited their analysis to the observation of DAPI-stained samples and neither did they perform any electron microscope investigation nor did they analyzed DNA. In this report we show that in MOLT-4 cells exposed to staurosporine the typical ultrastructural features are present without any concomitant fragmentation of DNA.

MATERIALS AND METHODS

Cell culture and drug treatment. MOLT-4 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at a density between 5 x 10^4/ml and 5 x 10^5/ml. All the experiments were performed on cells during the exponential growth phase (4). Staurosporine was obtained from Boehringer Mannheim (Germany) and was dissolved in ethanol at a concentration of 100 μg/ml. For drug treatment, cells were incubated in the presence of 10, 20 or 200 nM staurosporine for 6 or 24 hr (3). Control cultures received an equal volume of solvent.

DNA gel electrophoresis. DNA was routinely extracted as reported by Boe et al. (5) and analysed on a 1.8% agarose gel. In some cases cells were first lysed in 5 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100 and the lysate was then centrifuged for 20 min at 13,000 *g* to separate fragmented DNA (soluble) from intact chromatin (pellet) according to Bissonette et al. (6).Soluble DNA was then purified as described (6) and run on agarose gels as above. Molecular weight markers were from Boehringer Mannheim.

Transmission electron microscope analysis. Treated and control cells were immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). They were successively postfixed with 1% OsO_4 in veronal buffer, alcohol dehydrated and embedded in araldite. Toluidine blue stained sections allowed the identifications of apoptotic cells.

RESULTS AND DISCUSSION

In Fig. 1 we show agarose gel analysis of DNA prepared from MOLT-4 cells treated with various concentrations of staurosporine for different times. It is evident that most DNA was of high molecular weight (approximately 20 Kb) and that we did not detect the DNA ladder typical of apoptotic cell death in any sample, independently from the concentration of the drug or the length of the treatment. There was also some DNA smearing, conceivably caused by the extraction procedure . In this particular experiment we analyzed total cell DNA but the results were the same also when "soluble" DNA was prepared according to Bissonette et al. (6), i.e. a procedure which should produce a substantial enrichment in small DNA fragments (not shown).

Fig. 2 shows some early morphological features appearing in MOLT-4 cells treated with 10 nM staurosporine for 6 hr. Characteristic chromatin condensation close to the nuclear envelope was present in 10% of the cells. This aspect, clearly distinguishable from apparently unaffected (see Fig. 2a, right) or control cells, is commonly considered typical of cells undergoing apoptosis (7). It was frequently followed by the appearance of unusual nuclear

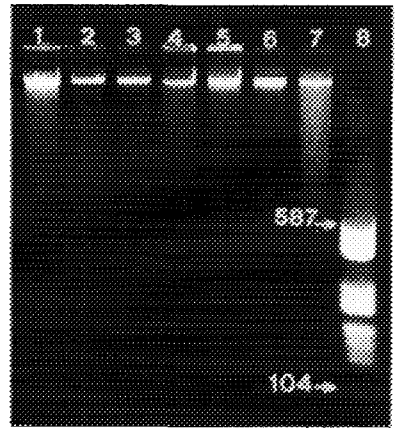

Fig. 1. Agarose gel analysis of DNA extracted from MOLT-4.
Lanes 1-3:cells exposed to staurosporine for 6 hr. Lanes 4-6:cells exposed to staurosporine for 24 hr. Lane 7: untreated cells. Lane 8: molecular weight markers (from 104 to 587 base pairs). Lanes 1 and 4: 10 nM staurosporine. Lanes 2 and 5: 20 nM staurosporine. Lanes 3 and 6: 200 nM staurosporine. Approximately 4 μg of DNA was loaded in each lane.

protrusions in which heterochromatin areas seemed to be segregated and extruded into the cytoplasm. This structure could represent initial micronuclear formation. A careful observation of the envelope surrounding these structures suggested a particular involvement of the nuclear membrane, which appeared multilaminated, probably because of the initial folding formation. Interestingly enough, nuclear pores which were absent in membrane delineating compact chromatin areas, were widely distributed, and in close contact with each other, in correspondence of decondensed nuclear areas. In a following stage, characteristic cap-shaped chromatin areas appeared in cells treated with higher staurosporine concentration (Fig. 3): highly electron dense micronuclei were also detected, containing a strongly compact chromatin. Until the advanced stages of the process, they always were surrounded by a well defined double membrane, the external side of which was uniformly covered with ribosomes, indicating the preservation of membrane integrity. The plasma membrane and cytoplasmic morphology looked nevertheless well preserved. Only after treatment with high staurosporine concentration for a longer time, nuclear fragmentation was followed by a slight cytoplasm hydration, probably preceding a final "secondary necrosis".

DNA fragmentation has been long considered to be the "hallmark" of apoptic cell death (8). However, it is becoming increasingly clear that some cell types can show morphological features typical of apoptosis even in the absence of internucleosomal DNA breakage. For example, PC 12 cells can undergo apoptotic death upon deprivation of trophic factors without DNA fragmentation (9) and the same holds true for P815 murine mastocytoma cells exposed to hyperthermia (8). Furthermore, it has been recently shown that zinc ions, despite completely inhibiting DNA fragmentation, cannot protect rat thymocytes from spontaneous or dexamethasone-induced apoptotic death (10). All these results point to the likelihood that DNA fragmentation cannot be considered anymore the universal "hallmark" of apoptosis and our

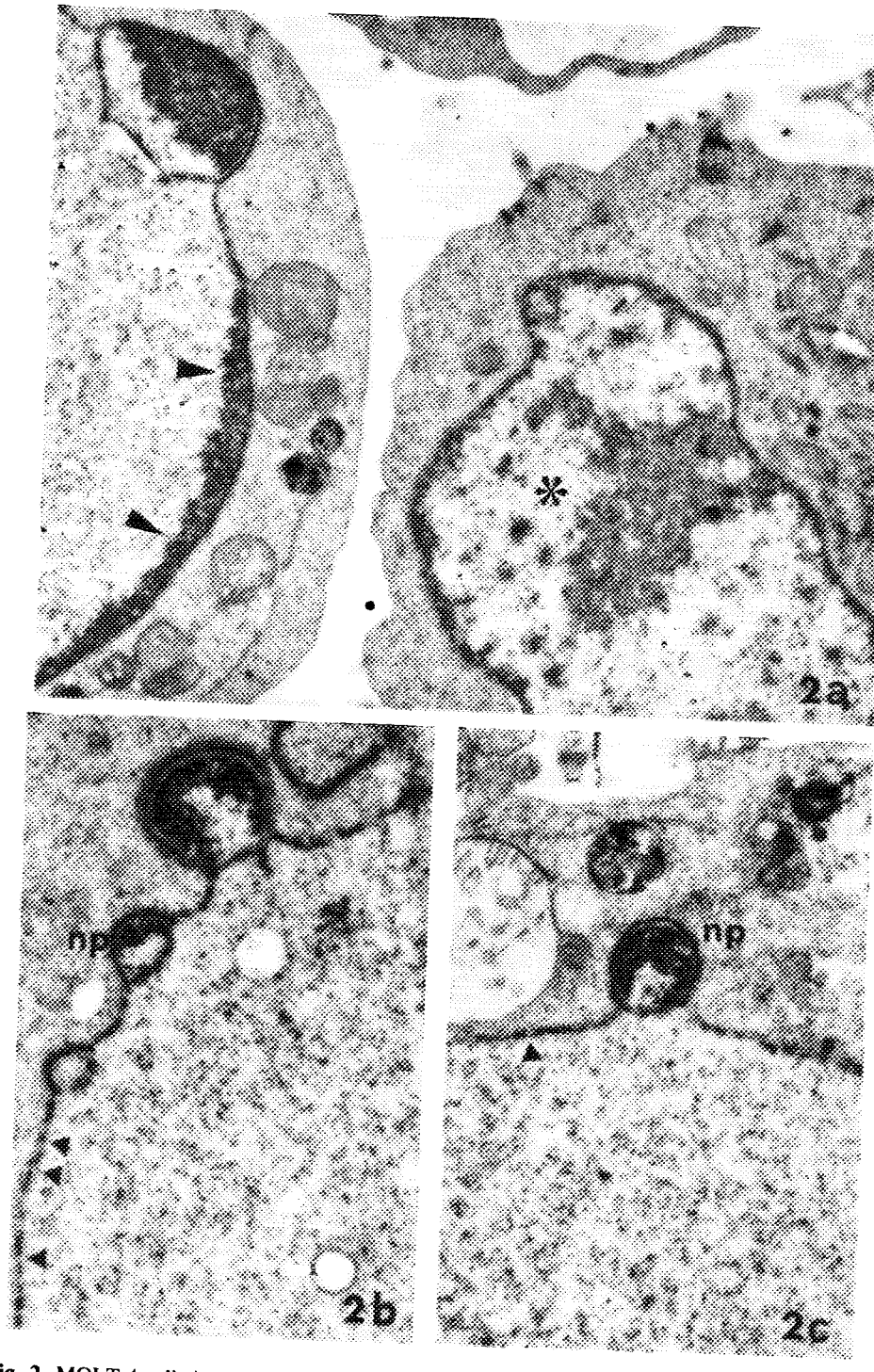

Fig. 2. MOLT-4 cells incubated with 10 nM staurosporine for 6 hr.
a: a typical chromatin margination can be observed in the cell on the left (➨), significantly different from nuclear arrangement displayed by the apparently unaffected cell on the right(✱). b,c: nuclear protrusions (np), characteristically involving nuclear envelope are also evident. Numerous clustered nuclear pores are also visible in this area (▶). a: x11700; b,c: x18000.

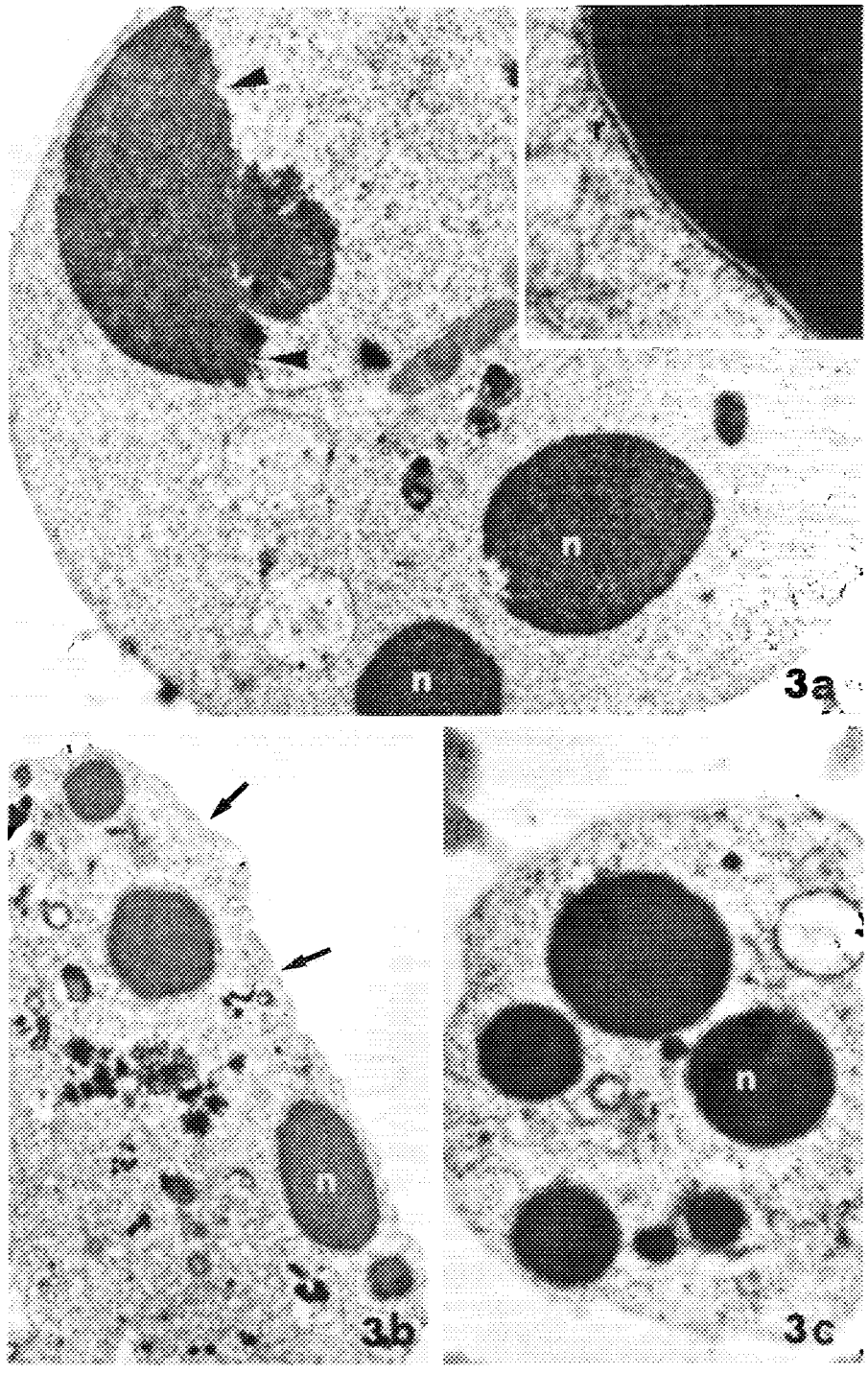

Fig. 3. MOLT-4 cells incubated with 200 nM staurosporine for different times.
a, 6 hr: cap-shaped chromatin margination (➨) is clearly present. Two homogeneously electron dense micronuclei (n) are shown, definitely surrounded by the nuclear envelope. The inset shows ribosomes (r), regularly distributed on the nuclear outer membrane. b, same as a: plasma membrane and organelles are well preserved. c, 24 hr: an increasing cytoplasm hydration can be noticed. a,b,c: x10800; inset: x36000.

data are in complete agreement with such an hypothesis. It could be argued that we did not detect DNA breakage because only a few cells underwent apoptosis upon exposure to staurosporine but we consider this hypothesis unlikely because it has been shown that in P815 cells incubated in an oxygen-depleted atmosphere as few as 4% apoptotic cells were sufficient to visualize by ethidium bromide staining the oligonucleosomal DNA in gel electrophoresis (8). Moreover, we loaded high amount of DNA in our gels so that we should have been able to detect even a limited amount of DNA breakage (9). Concerning the mechanism(s) by which staurosporine induces apoptotic cell death it is of interest to note that a previous report has shown that in rat thymocytes this drug actually inhibits apoptosis triggered by irradiation (11) whereas, in MOE-7 cells exposure to staurosporine results in apoptosis which is accompained by DNA fragmentation (12). Since at present our knowledge about the signalling pathways leading to apoptosis is very limited (13) it is conceivable that, depending on the cell type and on the triggering agent, staurosporine can either suppress or stimulate apoptosis and in the latter case DNA fragmentation can be present or not. Staurosporine is an inhibitor of several protein kinases (14). In this sense it is intriguing to note that apoptosis can be induced by c-myc proto-oncogene (6) and that c-myc encodes a nuclear DNA binding protein which is known to be phosphorylated by casein kinase II, even though the effect of phosphorylation on c-myc product have not been fully elucidated (15). Moreover, also the phosphatase inhibitor okadaic acid is capable of inducing apoptosis in a variety of cell types thus strengthening the hypothesis that protein phosphorylation/dephosphorylation plays a key role in modulating the biochemical events leading to apoptosis (5). Further investigations in this field should be helpful in clarifying such an issue.

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