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The detergent Triton X-100 induces a death pattern in human carcinoma cell lines that resembles cytotoxic lymphocyte-induced apoptosis

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Abstract The detergent Triton X-100 (TX100) was used with the intention to establish a model for necrotic cell death. However, TX100 was found to induce apoptotic and necrotic death in prostate and colon cancer cell lines. Apoptosis was characterized by the typical morphological features and internucleosomal DNA fragmentation. The rapid onset within 60 min and the lack of inhibition by cycloheximide indicated that apoptosis induced by TX100 was not dependent on protein synthesis. Removal of extracellular calcium blocked internucleosomal DNA fragmentation. This pattern of cell death shows a striking similarity to the effect of cytotoxic lymphocytes on their target cells.

Key words: Apoptosis; Human cancer cell line; Triton X-100; Cytotoxic lymphocyte

1. Introduction

A wide variety of physiological and pathological stimuli are inducing apoptosis, a distinctive form of cell death that is defined by characteristic morphological changes and a typical pattern of internucleosomal DNA fragmentation [1,2]. In contrast to passive necrotic death caused by severe environmental perturbations, apoptosis is thought to be an active process where the cell participates in its own demise [2,3]. This is evidenced by the critical dependence of apoptosis on macromolecular synthesis in various systems, its genetic regulation and need for energy [1,2,4].

We have previously shown that apoptosis can be induced in the human hormone-insensitive prostate carcinoma cell line PC-3 by 48–72 h exposure to various anticancer agents such as cisplatin, camptothecin, tenoposide, fluorouracil, vincristine and doxorubicin, and that low concentrations of the protein synthesis inhibitor cycloheximide abrogate this process (Borner et al., manuscript submitted). Since disturbance of membrane integrity and the subsequent loss of osmoregulation and cell lysis are typically involved in the pathogenesis of necrosis [2], we sought to establish a model for necrosis by exposing PC-3 cells to Triton X-100 (TX100), a non-ionic detergent which is used to lyse cells. However, we found TX100 to induce the typical hallmarks of apoptosis within less than one hour.

2. Materials and methods

2.1. Materials

Agarose, fetal bovine serum, saturated phenol, 10 × TAE buffer, and 100 bp DNA ladder marker were obtained from Gibco (Grand Island, NY). Proteinase K and glycogen were purchased from Boehringer Mannheim (Indianapolis, IN). Cisplatin, cycloheximide, DMSO, pancreas ribonuclease A were from Sigma Chemical Co. (St. Louis, MO) and Triton X-100 from RPI (Mount Prospect, IL). Tenoposide was a kind gift of Bristol-Myers Pharmaceutical Co. (Syracuse, NY). [*methyl*-^{3}H]Thymidine was from New England Nuclear (Wilmington, DE).

2.2. Cell culture and drug exposure

PC-3, SW-620, and HT-29 cells (all obtained from ATCC, Rockville, MD) were grown at 37°C in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine in an atmosphere containing 5% (v/v) CO_2. Cultures were regularly tested and found free of mycoplasma contamination. For experiments involving TX100, cells were grown to confluency in 100 × 20 mm style culture dishes and maintained confluent for 48 h before adding the respective agent. At this point each dish typically contained 5×10^6 (PC-3) or 1×10^7 (SW-620, HT-29) cells, respectively. Immediately before starting the experiments, the culture medium in all dishes was replaced by fresh prewarmed serum-free medium alone to prevent interaction of TX100 with serum proteins. Cisplatin and tenoposide were added to cells in logarithmic growth phase (1.5×10^6 cells). Stock solutions were prepared in DMSO with tenoposide (10 mM), in PBS with cycloheximide (1 mM), cisplatin (3.3 mM), and sterile water with TX100 (2%; w/v). The solvents alone had no cytotoxic effect at the concentrations used for the experiments.

2.3. Analysis of DNA integrity

To determine whether DNA fragmentation had occurred in a given sample, all cells were collected and the DNA extracted by a modification of the method originally described by Wyllie et al. [5]. Cells were detached from the culture dish by adding EDTA directly to the culture medium to a final concentration of 5 mM, collected by centrifugation and lysed in 5 mM Tris pH 7.4, 5 mM EDTA, and 0.5% Triton X-100 for 2 h on ice. The lysate was centrifuged at 27,000 × *g* for 20 min. The supernatant was incubated with 200 μg/ml proteinase K for 1 h at 50°C, extracted with phenol/chloroform, and the DNA precipitated overnight at −20°C in 2 volumes of ethanol and 0.13 M NaCl with 20 μg glycogen. After treatment with 1 mg/ml boiled bovine pancreatic RNase A for 1 h at 50°C the DNA was loaded onto a 2% (w/v) horizontal agarose gel containing 0.3 μg/ml ethidium bromide and run in 1 × TAE buffer at 2.5 V/cm. Gels were photographed under UV light with Polaroid 57 type film. Parallel samples were normalized according to the initial cell number. Representative films of at least 3 similar experiments are shown.

2.4. Measurement of intracellular calcium

PC-3 cells were grown on 2-well coverglass chambers (Nunc, Napaville, IL) and loaded for 1 h with 1 μM Fluo-3 (Molecular Probes, Eugene, OR) as previously described [6]. Cells were treated in Locke's solution and the intracellular calcium changes measured with ACAS 570 (Meridian, Okemos, MI) interactive laser cytometer by collecting data every 30 s [6].

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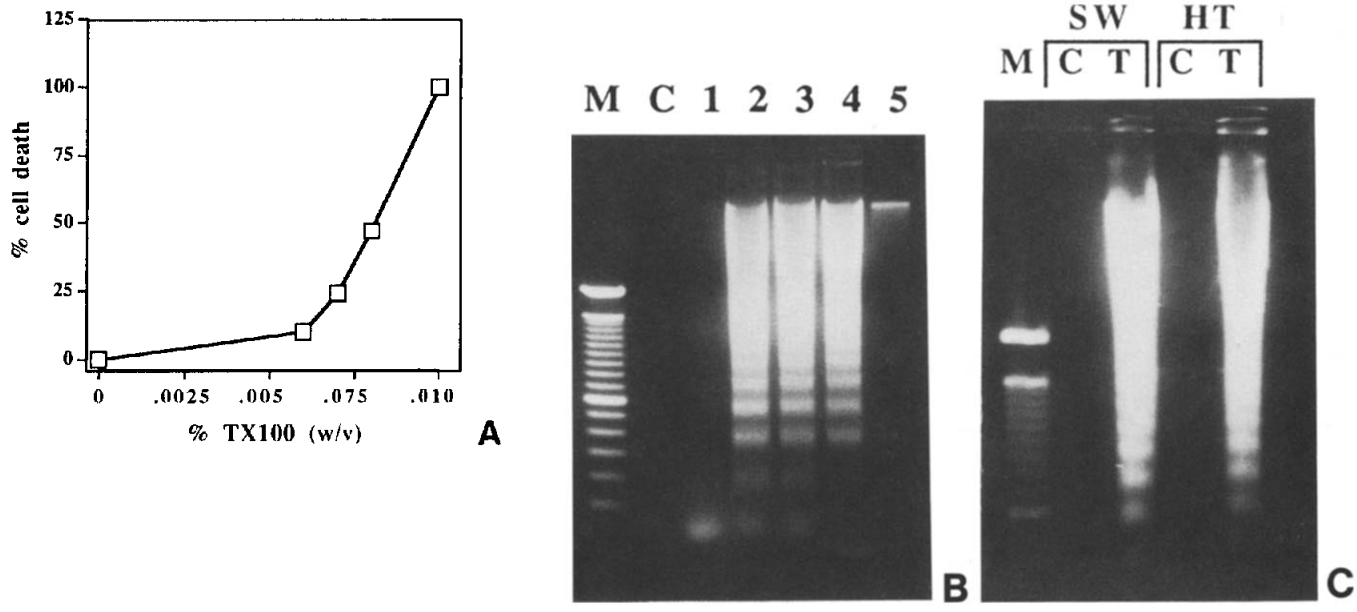

Fig. 1. Cytotoxic effect of TX100. (A) Confluent PC-3 cells were treated with various TX100 concentrations for 1 h. The percentage of dead cells was determined by counting trypan blue positive and negative cells. (B) DNA agarose gel electrophoresis of PC-3 cells treated as in A: 0.004% TX100 (w/v) (lane 1), 0.008% TX100 (lane 2), 0.009% (lane 3), 0.010% (lane 4), 0.016% (lane 5). (C) Agarose gel electrophoresis of confluent HT-29 and SW-620 cells treated with 0.01% TX100 for 90 min (lanes T). Untreated controls (lanes C), 100 bp size marker (lanes M).

3. Results

3.1. Induction of apoptosis by TX100

Fig. 1A illustrates the concentration dependent killing of PC-3 cells after one hour of exposure to various TX100 concentrations. A TX100 concentration of 0.01% (w/v) was enough to kill 100% of the cells. To determine the type of cell death, morphological alterations were assessed by electron microscopy and DNA integrity analysed by agarose gel electrophoresis. Treatment with 0.01% TX100 resulted in a mixture of typical apoptotic (10–15% of total cells) and necrotic cells (Fig. 2A) at 1 h, while untreated control cells did not display any apoptotic features such as condensation of chromatin or cytoplasm (Fig. 2B). Fig. 1B demonstrates that toxic concentrations of TX100 induced internucleosomal DNA fragmentation typical for apoptosis within 1 h. Labelling of the cellular DNA with 1 μCi [*methyl*-^{3}H]thymidine prior to the addition of TX100 showed that 21 ± 1.6% of total DNA was fragmented after 1 h treatment with 0.01% TX100 corresponding to the estimated proportion of apoptotic cells as assessed by electron microscopy. Similar results were also obtained in the human colon cancer cell lines SW-620 and HT-29 (Fig. 1C) and thus the induction of apoptosis by TX100 does not represent a specific characteristic of PC-3 cells.

3.2. Role of calcium and protein synthesis in TX100-induced apoptosis

Calcium has been reported to be important for the induction

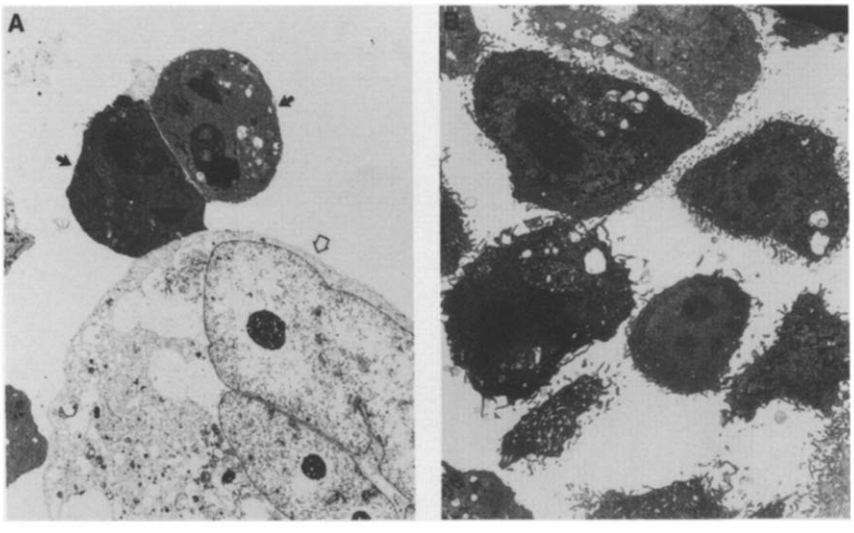

Fig. 2. Transmission electron microscopy. (A) PC-3 cells treated with 0.01% TX100 for 1 h. Solid arrow, apoptosis; open arrow, necrosis. (B) Untreated PC-3 cells. (Magnification × 1600).

of apoptosis in various systems [7–9]. The rapidity of the process allowed us to examine the role of calcium in TX100 induced apoptosis. Single cell calcium measurement by ACAS interactive laser cytometry showed that treatment with TX100 caused a more than 5-fold (541 ± 76%) increase of the average (± S.E.M.) intracellular calcium concentration (Fig. 3). A rapid increase was followed by a sharp drop in intracellular calcium concentration (Fig. 3), which was probably due to a leak of fluorescent dye through the damaged cell membrane. To test the dependence of TX100 induced apoptosis on extracellular calcium, the calcium chelating agents EDTA and EGTA were used to remove calcium from the culture medium (Fig. 4A). TX100 induced considerably less internucleosomal DNA fragmentation if calcium was removed from the culture medium (Fig. 4A). Thus, apoptotic cell death was dependent on extracellular calcium in this system.

To examine whether TX100 induced apoptosis requires protein synthesis, PC-3 cells were cotreated with 1 μM cycloheximide and TX100. Cycloheximide was added 5 h before TX100 since previous experiments have shown that protein synthesis inhibition was nearly maximal after 4 h in PC-3 cells (Borner et al., manuscript submitted). Fig. 4A shows that cycloheximid did not prevent TX100 induced DNA fragmentation but rather intensified the fragmentation pattern probably by further damaging the cells. However, cycloheximide reduced the induction of DNA laddering by the anticancer agent tenoposide and to a lesser extent by cisplatin (Fig. 4B).

4. Discussion

Apoptosis is defined by the occurrence of characteristic morphological changes such as chromatin and cytoplasm condensation, which are typically accompanied by internucleosomal DNA fragmentation [4]. These features and the participation of the cell in its own demise through the activation of a genetically programmed death machinery distinguish apoptosis from passive necrotic death [3,4]. However, despite intensive research the mechanisms by which the induction of apoptosis causes cell death are still poorly defined.

The requirement for macromolecular synthesis has been taken as evidence that apoptosis is an active process [3,10]. We have previously found that various anticancer agents induced apoptosis in the human prostate cancer cell line PC-3 after 48–72 h exposure and that the protein synthesis inhibitor cycloheximide was able to suppress this process. Here we show that the non-ionic detergent TX100 not only caused the expected ultrastructural changes of necrosis but also the distinct morphology and internucleosomal DNA fragmentation typical for apoptosis in the human prostatic cancer cell line PC-3. These features of apoptosis appeared within less than 1 h after TX100 treatment and were not inhibited by cycloheximide. This ex-

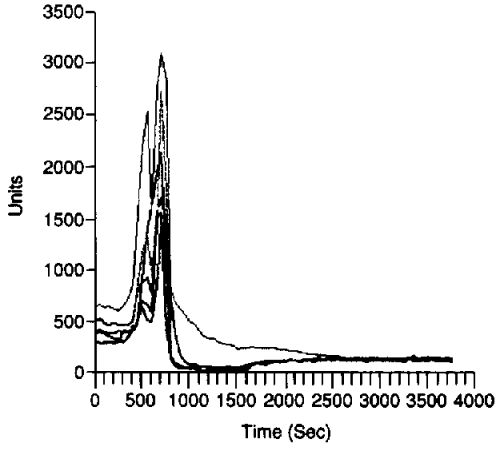

Fig. 3. Time course of relative intracellular calcium concentration determined by ACAS interactive laser cytometry. Cells were continuously exposed to 0.01% TX100. Each line represents a single cell. Calcium concentrations are given in relative units.

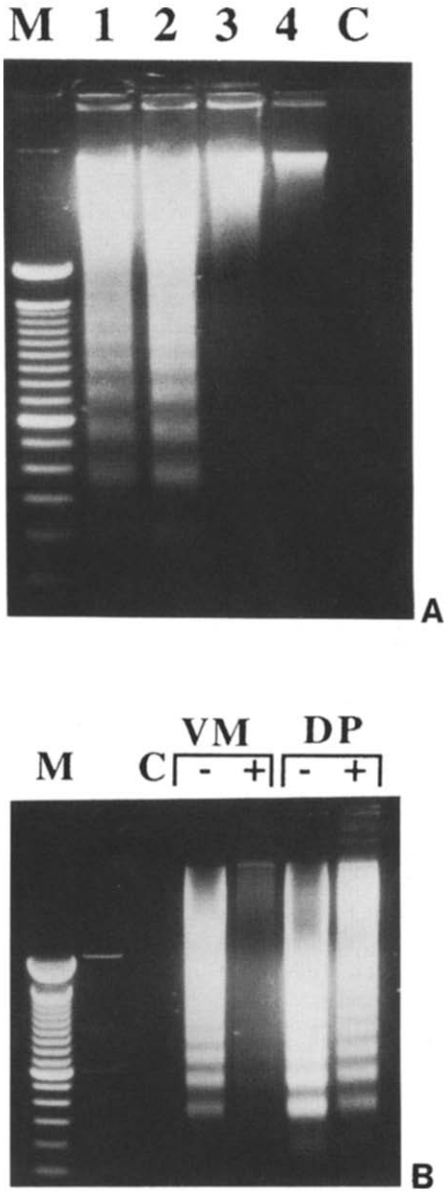

Fig. 4. Effect of extracellular calcium depletion or protein synthesis inhibition on internucleosomal DNA degradation in PC-3 cells. (A) DNA agarose gel electrophoresis. Confluent cells were treated for 1 h with 0.01% TX100 alone (lane 1) or in the presence of 1 μM cycloheximide (lane 2), 5 mM EGTA (lane 3), or 5 mM EDTA (lane 4). (B) DNA agarose gel electrophoresis. Cells in log growth phase were exposed to 10 μM tenoposide (VM) or 22 μM cisplatin (DP) for 72 h alone (−) or in the presence of 1 μM cycloheximide (+). Untreated controls (lanes C), 100 bp size marker (lanes M).

tremely fast time course makes it unlikely that active gene expression and protein synthesis were involved in cell death, apoptotic morphology and DNA degradation in this system. Rather, the cellular components required for apoptosis were already in place and were activated by TX100. These findings were not confined to PC-3 cells since TX100 induced apoptosis in two colorectal cancer cell lines within the same time-frame. Interestingly, the apoptotic response to TX100 was most pronounced in confluent cells and could be abolished by adding fresh serum 24 h before exposure to TX100 (data not shown). In contrast, anticancer drugs triggered apoptosis most efficiently in logarithmically growing cells. It is thus possible, that the depletion of serum factors primed the cells for apoptosis. A similar observation was recently described for the induction of apoptosis in thymoma cells by cold shock [11].

The observations that apoptosis was accompanied by a rise in intracellular calcium concentration in various systems and that calcium chelating agents such as EGTA or EDTA could prevent cell death and internucleosomal DNA fragmentation [7–9] have led to the hypothesis that a calcium dependent endonuclease is critically involved in apoptotic cell death [12–14]. This prompted us to test whether TX100 induced apoptosis by cell membrane disruption thus leading to calcium influx into the cells. We found that extracellular calcium depleting manoeuvres reduced TX100 triggered apoptosis supporting the dependence on calcium.

TX100 induced cell death shows striking similarities to the pattern of cell death induced by cytotoxic lymphocytes in target cells. In both systems apoptotic death is induced within less than 240 min [13,15–19] and is dependent on extracellular calcium [16,17] but is not or only partially dependent on protein synthesis [16,18]. The co-secretion of different cytotoxic lymphocyte granule proteins such as perforin (cytolysin) and serine proteases such as fragmentin or granzyme B has been taken as explanation for the induction of both necrotic and apoptotic cell death [13,16,18–20]. Most authors agree that the serine proteases are mediating apoptotic morphology and target DNA fragmentation while perforin is facilitating serine protease uptake by stimulating endocytosis or directly perforating the target cell membrane [13,15,16,19]. Perforin alone has been shown to induce necrotic cell lysis but not DNA fragmentation [21]. However, there is a conflicting report that perforin alone can also induce apoptosis if the cell can survive the initial attack [17]. The detergent TX100 is widely used to permeabilize cell membranes and it is hardly conceivable that this agent might have serine protease activity. One would expect that its mode of action has more similarity to perforin activity.

Thus, our finding that TX100 alone can induce both apoptosis and necrosis suggests various explanations. (1) The increase of cell membrane permeability by TX100 allows toxic metabolites, which are present in the culture medium to enter cells and to induce apoptosis. This hypothesis is supported by the fact that the triggering of apoptosis could be increased if cells were cultured at suboptimal conditions (confluence) thus favouring the formation of toxic metabolites. (2) Recent reports show that the target cells have to be in a 'state of commitment' for cytotoxic lymphocytes to induce apoptosis [22,23]. The proliferative status seems to be critical since quiescent cells were refractory to DNA fragmentation [22]. It is possible that in our system TX100 was triggering apoptosis only in cells escaping growth arrest caused by confluent culture conditions. Cell cycle analyses have shown that a small fraction of PC-3 cells are still cycling at these conditions (M. Borner, unpublished observation). (3) Finally, we cannot exclude the possibility that TX100 induces apoptosis by denaturating an inhibitor of an inherently active apoptotic pathway in these human carcinoma cell lines. The clarification of the mechanism of TX100 induced apoptosis will shed further light on the essential components of the apoptotic program and its regulation in cancer cells.

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