

Prevention of necrosis and activation of apoptosis in oxidatively injured human myeloid leukemia U937 cells

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Abstract A 3 h exposure to 1 mM H₂O₂ followed by 6 h post-challenge growth in peroxide-free medium induces necrosis in U937 cells. Addition of the poly(ADP-ribose)polymerase inhibitor 3-aminobenzamide during recovery prevents necrosis and triggers apoptosis, as shown by the appearance of apoptotic bodies, extensive blebbing and formation of multimeric DNA fragments as well as 50 kb double stranded DNA fragments. Thus, the same initial damage can be a triggering event for both apoptotic and necrotic cell death. Furthermore, necrosis does not appear to be a passive response to overwhelming damage.

Key words: Hydrogen peroxide; Poly(ADP-ribose)polymerase; Necrosis; Apoptosis; DNA fragmentation

1. Introduction

It is now well established that many cytotoxic drugs and physical agents induce apoptosis or necrosis, depending on the severity of the insult inflicted on the cells. The apoptotic program can be modulated, since apoptosis is the result of a complex series of events which can be activated by many diverging stimuli. Thus, the apoptotic process can be suppressed by inhibiting protein synthesis [1,2] or via enforced expression of bcl-2 [3,4], and accelerated [5,6] or reduced [7,8] by changing the level of protein phosphorylation. These are only a few examples, among many others, which point out that once the initial damage has been inflicted, the cell undergoes a series of events that can either be blocked at different levels or ultimately lead to apoptosis. As to necrosis, it would appear that this is a no-return path which cannot be modulated unless the extent of the insult given to the cells is directly or indirectly altered. This view stems from the general opinion that necrotic cell death occurs as a result of massive damage which overwhelms the defense and repair capacities of the cell.

The concept of the above-mentioned concentration-dependent induction of apoptotic or necrotic cell death applies very well to cells challenged with hydrogen peroxide. That H₂O₂ can trigger apoptotic cell death has been clearly demonstrated in a number of reports [9–12] and it is just as clear that the mode of cell death switches from apoptosis to necrosis when the concentration of the oxidant is increased.

In this report, we provide experimental evidence contrary to the widely held 'dogma' that necrosis will necessarily occur

once massive damage which overwhelms the defense and repair capacities of the cell has been provoked.

2. Materials and methods

2.1. Cell line and cell culture

U937 cells were cultured in suspension in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 µg/ml) (Sera-Lab, Ltd, Crawley Down, UK), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air–5% CO₂. Stock solutions of hydrogen peroxide were freshly prepared in double-distilled water. 3AB was dissolved directly in the culture medium. Because the cytotoxicity of hydrogen peroxide toward cultured cells is dependent on cell density [13], a constant density of 2.5 × 10⁵ cells/ml dish was used at the treatment stage.

2.2. Cytotoxicity assay

Cells were inoculated into 30 mm tissue culture dishes and exposed for 3 h to 1 mM H₂O₂. Cytotoxicity was determined using the trypan blue exclusion assay either immediately after treatments or after 6 h of post-incubation in fresh pre-warmed medium. Briefly, an aliquot of the cell suspension was diluted 1:1 with 0.4% trypan blue and the cells were counted with a hemocytometer. The level of cell death was expressed as percent ratio of stained vs. unstained cells. Cells unable to exclude trypan blue (stained) are referred to as dead cells.

2.3. DNA fragmentation analysis by the filter binding assay

Secondary DNA fragmentation was quantified using the filter binding assay developed by Bertrand et al. [14] with minor modifications. Briefly, cells were labelled overnight with [¹⁴C]thymidine (0.05 µCi/ml) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 µg/ml). After treatment, cells were sedimented at 1000 rpm for 5 min at 4°C and the pellet was then resuspended in saline A (0.14 M NaCl, 5 mM KCl, 4 mM NaHCO₃, 5 mM glucose) containing 5 mM EDTA (disodium salt), pH 8.3. 0.5 × 10⁶ cells were loaded onto protein adsorbing (polyvinylchloride) filters (25 mm, 2 µm pore) (Nuclepore, Pleasanton, CA, USA). Cells were then washed with 10 ml of saline A containing 5 mM EDTA (disodium salt), pH 8.3 and lysed with a solution (5 ml) containing 0.2% sarkosyl-2 M NaCl–0.04 M EDTA (pH 10.1). Lysates were rinsed with 7 ml of 0.02 M EDTA (pH 10.1). The filters were then removed and the filter holders were washed twice with 3 ml 0.4 N NaOH. Radioactivity was counted in the lysates, EDTA wash, filter and filter holder washes. DNA fragmentation was determined as the percentage of the ¹⁴C-labelled DNA in the lysate plus the EDTA wash of the total ¹⁴C-labelled DNA. Hydrogen peroxide is an efficient inducer of DNA single strand breaks which are then rapidly removed. These lesions cannot be detected by the filter binding assay (or by conventional or pulsed field gel electrophoresis, described below) even if these lesions were still present at the time in which the biochemical assays for apoptosis were being performed.

2.4. DNA fragmentation analysis by gel electrophoresis

Genomic DNA was isolated from samples of 3.5 × 10⁶ U937 cells recovered at different times after treatment. Cells were sedimented at 1300 rpm for 5 min at 4°C in a 15 ml conical tube and the pellet was washed three times with 5 ml prechilled PBS (0.121 M NaCl, 10 mM NaH₂PO₄, 1.5 mM Na₂HPO₄, 3 mM KCl) containing 5 mM EDTA, pH 8.3. Cells were lysed with 100 µl of buffer containing 10 mM

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Abbreviations: PBS, phosphate-buffered saline; PARP, poly(ADP-ribose)polymerase; 3AB, 3-aminobenzamide; FIGE, field inversion gel electrophoresis

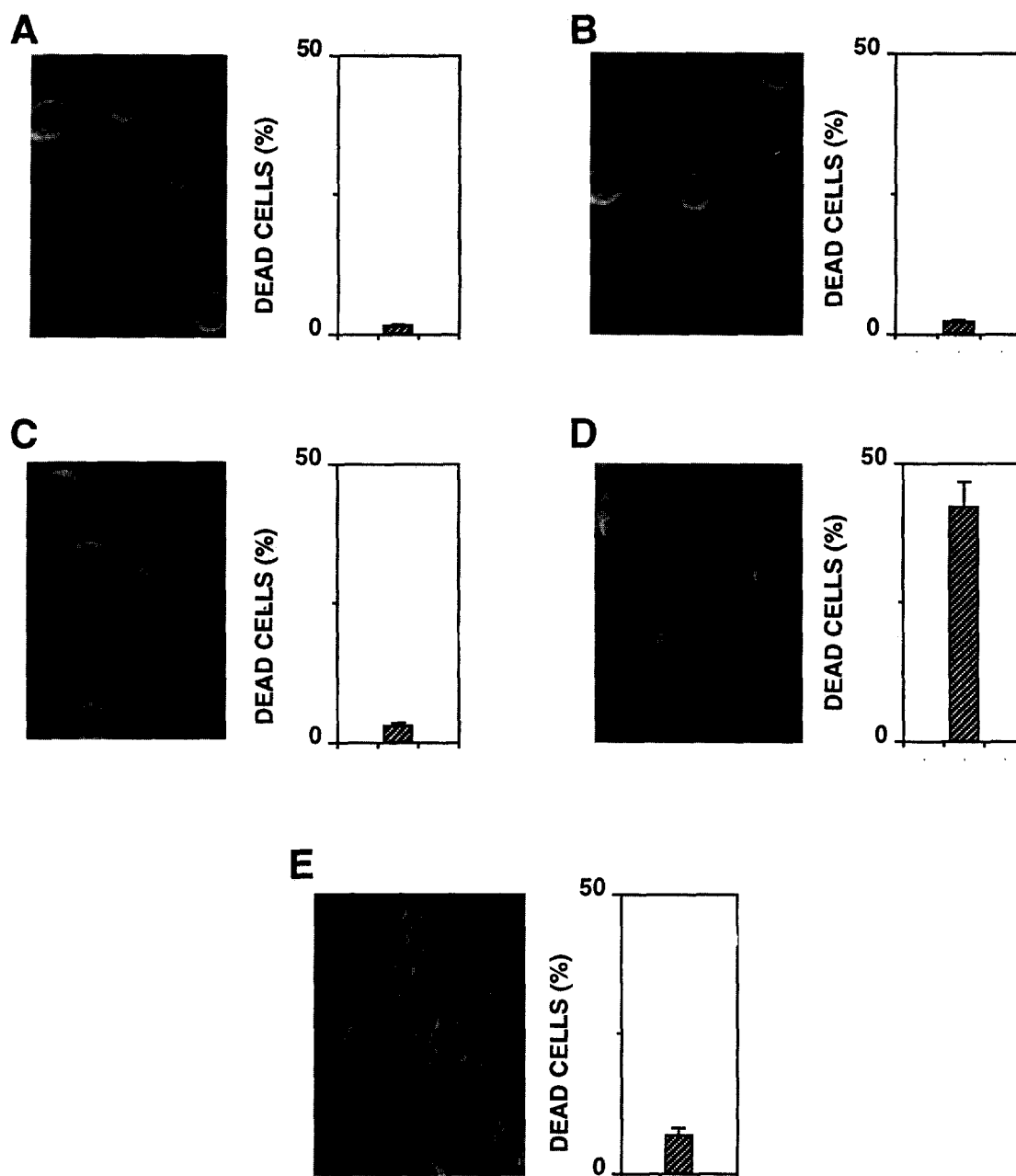


Fig. 1. Effect of 3-aminobenzamide on H₂O₂-induced U937 cell killing and changes in cell morphology. Cells were treated with 1 mM H₂O₂ for 3 h and then photographed or analyzed for viability either immediately (C) or after post-incubation for 6 h in fresh medium in the absence (D) or presence of 1 mM 3AB (E). Also shown are cells that were incubated in drug-free culture medium for 3 h and then grown for a further 6 h in the absence (A) or presence of 1 mM 3AB (B). Cell death was assessed with the trypan blue exclusion assay and photomicrographs were taken using an Olympus IX-70 inverted microscope. Each point is the mean $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. * $P < 0.001$ vs. H₂O₂ alone.

Tris-HCl, 25 mM EDTA, 100 mM NaCl, 0.2% SDS, 0.5 mg/ml proteinase K and the cell suspension was left in a 55°C water bath for 2 h. 2% agarose (low melt agarose, BioRad, Richmond, CA, USA) was melted in TBE buffer (89 mM Tris base, 89 mM sodium borate, 2 mM EDTA, pH 8.3) and 50 μ l was added to the cell lysate. The mixture was quickly dispensed into a plug former on ice. The plugs were analyzed by electrophoresis using a 1.5% agarose gel in TBE for 4 h at 50 V. The gel was incubated with DNase-free RNase (20 μ g/ml) at 37°C for 4 h before staining with ethidium bromide and then photographed under a UV transilluminator.

2.5. FIGE analysis of double stranded DNA fragments

Following treatments, cells were sedimented at 1000 rpm for 5 min

at 4°C and the pellet was resuspended in PBS containing 5 mM EDTA, pH 8.3. This procedure was repeated twice, and the final suspension in PBS was adjusted to give a density of 1×10^7 cells/ml. 300 μ l of this suspension was mixed with 300 μ l of melted agarose (low melting agarose, 1% w/v in PBS) in a 15 ml conical tube maintained at 45°C. The cell-agarose mixture was vortexed, transferred to a gel plug former on ice, and refrigerated for 15 min. The plugs (three to four per experimental point) were removed and incubated in 1 ml of lysis solution (0.5 M EDTA, 1% sarkosyl, 1 mg/ml proteinase K, pH 9) for 1–2 h at 4°C then at 45°C for 20 h. The plugs were washed three times for 1 h in sterile 0.5 $\times$ TE buffer (composition of the 0.5 $\times$ concentrated buffer: 5 mM Tris HCl, 0.5 mM Na₂EDTA [pH 8.0]) and stored at 4°C in 0.5 M EDTA (pH 8). FIGE was carried out

using a BioRad Pulsewave 760 Switcher connected to a BioRad 200/2.0 Power supply. Gels were cast using 1.5% w/v high strength agarose in 0.5×TBE (composition of the 0.5× concentrated buffer: 44.5 mM Tris base, 44.5 mM sodium borate, 0.5 mM Na₂EDTA [pH 8.0]) and stored at 4°C in 0.5 M EDTA (pH 8). The switch time was linearly ramped from 1 to 15 s with a forward/reverse ratio of 3/1 over 5 h of run time. The current was kept constant at 50 mA and the temperature of the running buffer (0.5×TBE) was maintained at 8°C. Gels were stained with ethidium bromide and photographed under a UV transilluminator.

3. Results and discussion

The results illustrated in Fig. 1 indicate that a 3 h exposure to 1 mM H₂O₂ neither causes gross morphological changes (C) in cultured U937 cells nor does it impair their ability to exclude trypan blue (C). Subsequent incubation of these cells in peroxide-free medium, however, leads to the appearance of dead cells as documented by the approximately 42% trypan blue positivity detected after 6 h of post-challenge growth (Fig. 1D). The morphological changes accompanying this process were mainly represented by swelling of the cells followed by loss of nuclear material (Fig. 1: compare panels A and D showing untreated and H₂O₂-treated cells, respectively), with no evidence of chromatin condensation or fragmentation (not shown). Conventional electrophoretic analysis of the DNA from cells treated under the same experimental conditions indicated the absence of internucleosomal DNA cleavage (Fig. 2B) and, consistently, no signs of DNA fragmentation were observed using the filter binding assay (Fig. 2A). Thus, under the experimental conditions utilized in this study, H₂O₂ induces necrotic cell death, a conclusion which finds further demonstration in the results illustrated in Fig. 2C. Indeed, FIGE analysis of the DNA from cells treated according to the above protocol did not reveal the presence of 50 kb DNA fragments which are currently considered to be a hallmark of apoptosis (Fig. 2C).

The results displayed in Figs. 1 and 2 demonstrate an important role of PARP in the above responses since addition of the PARP inhibitor 3AB was found to both abolish necrosis and trigger apoptosis in oxidatively injured cells. This conclusion finds experimental support in the observation that cells exposed for 3 h to 1 mM H₂O₂ and then incubated for 6 h in fresh medium containing 1 mM 3AB maintained their ability to exclude trypan blue (Fig. 1E) and underwent morphological alterations typical of apoptosis. Extensive cytoplasmic blebs and apoptotic bodies can be easily seen in Fig. 1E. Further demonstration that apoptosis was the mode of death of cells treated with H₂O₂ and then post-incubated in the presence of 3AB is given by the results shown in Fig. 2. The DNA from these cells was analyzed by the filter binding assay as well as by conventional and field inversion gel electrophoreses. These experiments indicated that 56.5% of the DNA was fragmented (Fig. 2A), revealed a distinct DNA ladder pattern (Fig. 2B) and that fragments of 50 kb were being generated (Fig. 2C). Importantly, a 6 h treatment with 3AB did not affect cell morphology or viability (Fig. 1B) and did not cause secondary DNA fragmentation, as measured by the techniques utilized in Fig. 2A–C (not shown).

Taken together, the above results provide morphological and biochemical evidence suggesting that a 3 h exposure of U937 cells to 1 mM H₂O₂, followed by a 6 h growth in drug-free medium, elicits necrotic cell death and that this response

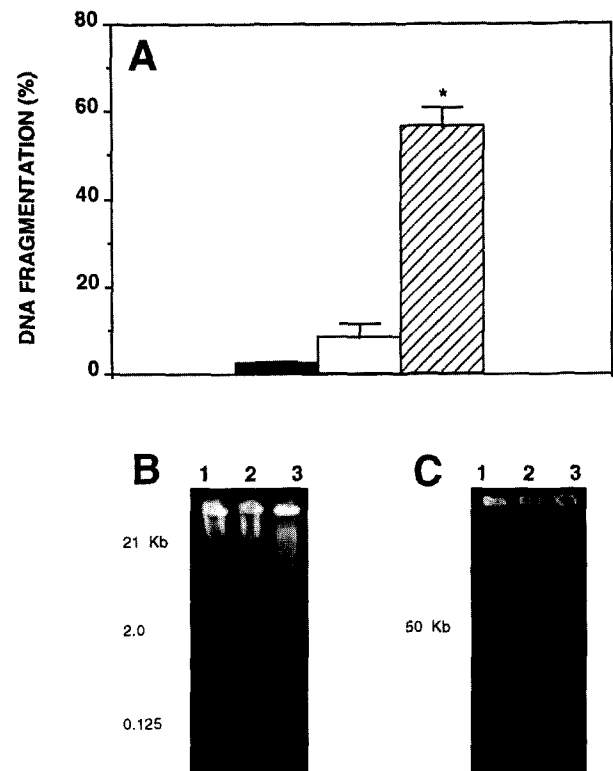


Fig. 2. Effect of 3-aminobenzamide on H₂O₂-induced secondary DNA fragmentation. Cells were treated as detailed below and DNA fragmentation was estimated by the filter binding assay (A), agarose gel electrophoresis (B) or FIGE (C), as described in Section 2. The results illustrated in A are the means ± S.E.M. of three separate experiments, each performed in duplicate. **P* < 0.001 (Student's *t*-test). The gels shown in B and C are representative of three experiments with similar outcomes. Key: Lane 1 or closed bar: DNA from untreated cells. Lane 2 or open bar: DNA from cells that were exposed for 3 h to 1 mM H₂O₂ and then grown for a further 6 h in drug-free culture medium. Lane 3 or striped bar: DNA from cells that were exposed for 3 h to 1 mM H₂O₂ and then grown for a further 6 h in the presence of 1 mM 3AB.

can be inhibited by post-treatment addition of 3AB. Under the latter experimental condition, however, U937 cells undergo apoptosis.

These results provide experimental evidence against the 'dogma' that cellular disintegration is the only possible consequence of exposure to a concentration of a toxic agent causing extensive damage. In particular, our results lead to the univocal conclusion that 3AB prevents necrosis and triggers apoptosis without changing the extent and the nature of the initial damage induced by H₂O₂. Hence, the same type of damage can be a triggering event for both necrotic and apoptotic cell death.

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