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Low levels of hydrogen peroxide and L-histidine induce DNA double-strand breakage and apoptosis

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Abstract

The results presented in this study demonstrate that L-histidine triggers a lethal response in U937 cells exposed to nontoxic, albeit growth-inhibitory, levels of H_2O_2. Treatment for 1 h with the cocktail H_2O_2/L-histidine promotes the formation of a low level of DNA double-strand breaks that are rapidly rejoined, and this process is followed by secondary DNA fragmentation at about 7 h of post-treatment incubation, at which time cells are still viable. The appearance of oligonucleosomal DNA fragments associated with the detection of morphological changes typical of apoptosis strongly suggests that a portion of the cells was undergoing an apoptotic process. The relative level of cells with fragmented chromatin never exceeded 15–20% throughout the 20 h post-treatment incubation. Treatment with high concentrations of H_2O_2 in the presence of L-histidine was found to trigger necrotic cell death. The results presented in this paper provide further experimental evidence in support of the notion that DNA double-strand breaks mediate the lethal effects of the cocktail H_2O_2/L-histidine and suggest that this type of DNA lesion can promote both apoptotic and necrotic cell death, depending on the concentration of the oxidant.

Keywords: Hydrogen peroxide; L-Histidine; DNA double-strand breaks; Pulsed field gel electrophoresis; Apoptosis; Necrosis

1. Introduction

The amino acid L-histidine has the ability to enhance a number of deleterious effects induced by hydrogen peroxide in cultured mammalian cells (Tachon and Giacomoni, 1988, 1989; Link, 1988; Oya and Yamamoto, 1988; Tachon, 1990; Shacter et al., 1990). Recent studies performed in our laboratory have demonstrated that, in the presence of this amino acid, H_2O_2 produces DNA double-strand breakage (Brandi et al., 1992; Cantoni et al., 1992, 1994a,b; Sestili et al., 1992), a type of lesion that is not produced by the oxidant alone and which might mediate the enhanced cytotoxic response. This conclusion is supported by a number of reported observations, including the existence of a linear relationship between the level of DNA double-strand breaks and the cytotoxic response which can be obtained using different experimental conditions (e.g., treatment for different lengths of time with various concentrations of H_2O_2 and/or L-histidine, Sestili et al., 1995a, and treatment of L-histidine pre-loaded cells with the oxidant at 37°C or 4°C, Sestili et al., 1995b). Furthermore, the same relationship was observed using clones characterized by a high degree of resistance to the oxidant and their parental sensitive cell line (Sestili et al., 1996) as well as cell lines derived from different species and characterized by markedly different sensitivity to the oxidant alone (Cantoni et al., 1994a). In these studies, however, cytotoxicity was measured using a conventional cloning efficiency assay, which has the great advantage of being sensitive but also the important and, to some extent, critical limitation of providing information only about the number of cells which can still reproduce after exposure to a toxin. In other words, these toxicity measurements are based on the assumption that a cell which loses the ability to form colonies has also lost its viability. Although this might be true, this approach does not allow us to distinguish between apoptotic and necrotic cell death.

The studies described herein were undertaken to gain a greater understanding of the mechanism whereby L-histi-

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dine enhances the toxicity of hydrogen peroxide. In particular, we investigated the mode of death promoted by the oxidant alone and associated with L-histidine. The U937 cell line was utilized in this study since it has the great advantage of being responsive to diverse apoptotic stimuli and is thus widely utilized in investigating the mode of cell death triggered by different toxins. It was found that nontoxic concentrations of the oxidant, in the presence of the amino acid, elicit apoptotic cell death and that this event is preceded by the formation of a low level of DNA double-strand breaks. In addition, high concentrations of H_2O_2 and L-histidine produce a very high level of DNA double-strand breaks and, under these conditions, the markedly enhanced cell killing was mediated by a necrotic process.

2. Materials and methods

2.1. Cell line and cell culture

U937 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sera-Lab, Crawley Down, UK), penicillin (50 U/ml), and streptomycin (50 μg/ml), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air/5% CO_2. Stock solutions of hydrogen peroxide and L-histidine were freshly prepared in saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose). Because the cytotoxicity of hydrogen peroxide for cultured cells is dependent on cell density (Cantoni et al., 1986), a constant density of 1×10^6 cells/ml was used at the hydrogen peroxide treatment stage. Treatments (1 h) were performed in saline A.

2.2. Cytotoxicity assay

After treatments, the cells were rinsed with saline A and then plated in triplicate onto 60 mm tissue culture dishes and incubated at 37°C for 4, 7 or 20 h. Cytotoxicity was determined by using the trypan blue exclusion assay. Viable cells were defined as those that excluded Trypan blue.

2.3. Measurement of double-strand breaks

Cells were labelled overnight with [^{14}C]thymidine (0.05 μCi/ml) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 μg/ml). The pulsed field gel electrophoresis (PFGE) assay was performed essentially as described by Blocher et al. (1989); Blocher and Kunhi (1990). To prepare agarose plugs, cells were sedimented at 1000 rpm for 5 min at 4°C and the pellet was resuspended in phosphate-buffered saline (PBS) containing 5 mM EDTA, pH 8.3. This procedure was repeated three times, and the final suspension in PBS was adjusted to give a density of 3.64×10^6 cells/ml. 300 μl of this suspension was mixed with 300 μl of melted agarose (low melt agarose, Bio-Rad, Richmond, CA, USA; 1% (w/v) solution 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 (four per experimental point) were removed and incubated in 1 ml of lysis buffer (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 five times for 1 h in sterile 0.5× Tris/EDTA buffer (a 1:1 (v/v) dilution of Tris/EDTA buffer: 10 mM Tris-HCl, 2 mM Na_2EDTA, pH 8.0) and stored at 4°C in 0.5 M EDTA (pH 8.0). The DNA in the plugs was separated by PFGE in a 0.5% agarose gel (chromosomal grade, Bio-Rad) in 0.5× Tris/borate/EDTA (TBE) buffer (a 1:1 (v/v) dilution of TBE buffer: 89 mM Tris base, 89 mM sodium borate, 2 mM Na_2EDTA, pH 8.2). The gel was run for 20 h on a CHEF-DR II system (Bio-Rad) operating at 1.21 V/cm with a switch time of 75 min. The buffer (0.5× TBE) was maintained at 25°C. The gel was stained with ethidium bromide, viewed under a UV transilluminator and photographed. The distribution of radioactivity in the gel was determined by cutting portions of the gel containing the DNA. These portions were then melted in glass scintillation vials in the presence of 50 μl of 1 M HCl and processed for liquid scintillation counting.

2.4. DNA fragmentation analysis by gel electrophoresis

Genomic DNA was isolated from samples of 3.5×10^6 U937 cells recovered at different times after treatment. Cells were sedimented at 1000 rpm for 5 min at 4°C in a 15 ml conical tube and the pellet was washed three times with 5 ml of prechilled PBS containing 5 mM EDTA, pH 8.3. Cells were lysed with 100 μl of buffer containing 100 mM Tris/HCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate, 0.5 mg/ml proteinase K and the cell suspension was left in a 48°C water bath overnight. Low melt agarose (2%) was melted in TBE and 50 μl were added to the cell lysate and the mixture was quickly dispensed into the plug former on ice. The plugs were analysed by electrophoresis using a 1.5% agarose gel in TBE for 3 h at 50 V. The gel was incubated with DNase-free RNase (20 μg/ml) at 37°C for 4 h before staining with ethidium bromide and then photographed under a UV transilluminator.

2.5. DNA fragmentation analysis by the filter-binding assay

Secondary DNA fragmentation was quantified using the filter binding assay developed by Bertrand et al. (1991) with minor modifications. Briefly, cells were labelled overnight with [^{14}C]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

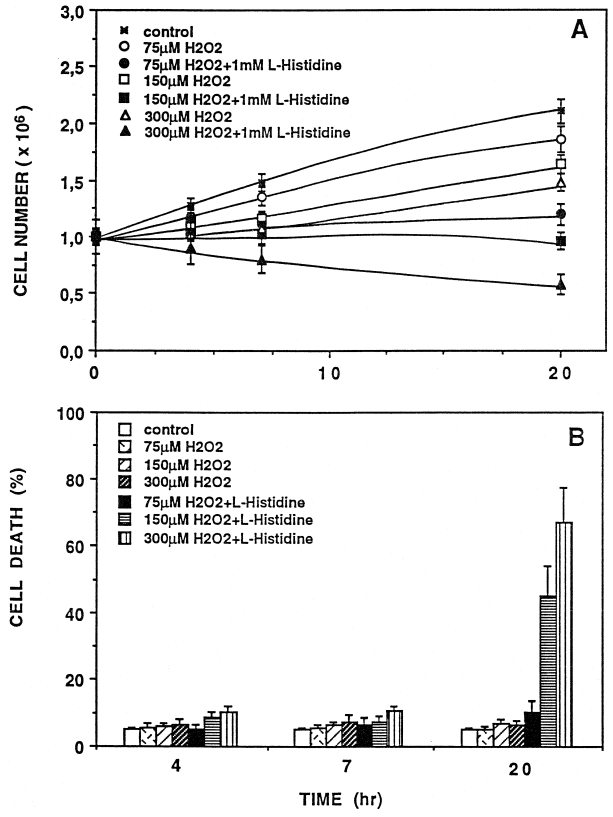

Fig. 1. Cytotoxic response of U937 cells challenged with hydrogen peroxide in the absence or presence of L-histidine. The number of viable cells and the percentage of dead cells after treatment for 1 h with different concentrations of H_2O_2 with (closed symbols) or without (open symbols) 1 mM L-histidine, followed by post-challenge growth for increasing time intervals, are shown in panels A and B, respectively. Under these experimental conditions, L-histidine was neither growth-inhibitory nor toxic for the cells. Results are the mean ± S.E.M. calculated from three separate experiments, each performed in duplicate.

then resuspended in saline A containing 5 mM EDTA, pH 8.3. Cells (0.5×10^6) were loaded onto 2 μm pore-size polyvinylchloride filters (Nuclepore, Pleasanton, CA, USA), washed with 10 ml of saline A containing 5 mM EDTA 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 of 0.4 M NaOH. Radioactivity was counted in the lysates, EDTA wash, filter and filter holder washes. DNA fragmentation was determined as the percentage of the ^{14}C-labelled DNA in the lysate plus the EDTA wash of the total [^{14}C]thymidine DNA.

2.6. Microscopic analysis of chromatin fragmentation

The hematoxylin/eosin stain was used to detect chromatin fragmentation. U937 cells, recovered after treatment as described above, were sedimented at 1000 rpm for 10 min at 4°C in an Eppendorf tube and the pellet was resuspended in PBS/paraformaldehyde (1:1) to give 4.0×10^6 cells/ml. 50 μl of this suspension were stained with haematoxylin/eosin and observed with an Olympus BH-2 microscope. Quantitative analysis of apoptotic cells was performed by counting 200 cells at 400× magnification.

3. Results

3.1. Effect of L-histidine on the cytotoxic response of U937 cells challenged with H_2O_2

The effect of L-histidine on the toxicity of hydrogen peroxide in U937 cells was investigated. As shown in Fig. 1, the oxidant was growth-inhibitory when given alone to the cultures in a concentration range of 75–300 μM (Fig. 1A) and no cell death was observed under these conditions over the 20 h of post-treatment incubation (Fig. 1B). A significant level of cell killing was observed after exposure to millimolar levels of H_2O_2 (not shown). In marked

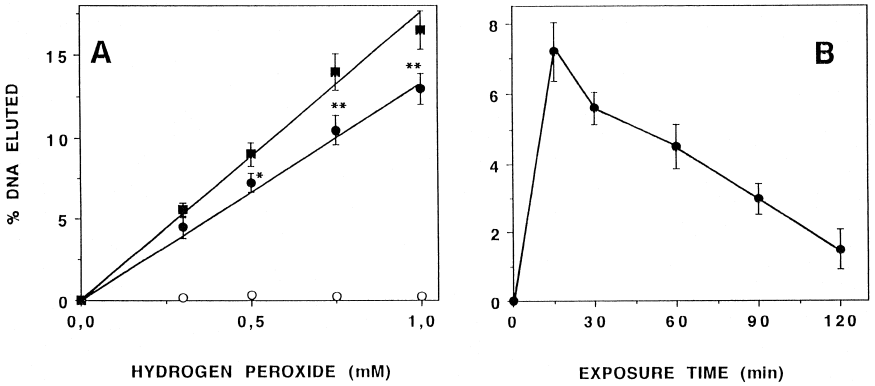

Fig. 2. PFGE analysis of the DNA from U937 cells treated with hydrogen peroxide in the absence or presence of L-histidine. (A) Measurement of DNA double strand breaks in cells treated for 30 (closed squares) or 60 min (closed circles) with increasing concentrations of H_2O_2/1 mM L-histidine. The open circles indicate the level of DNA double-strand breaks in cells treated with the oxidant in the absence of L-histidine. Results are the mean ± S.E.M. calculated from three separate experiments, each performed in duplicate. (B) DNA double-strand breaks in U937 cells treated for increasing time intervals with 300 μM H_2O_2/1 mM L-histidine. *, $P < 0.05$; **, $P < 0.02$ compared with DNA strand scission caused by treatment for 30 min.

contrast, treatment with micromolar levels of hydrogen peroxide in the presence of L-histidine (1 mM) produced a remarkable cell killing which was however detected only at the 20th h of post-treatment incubation (Fig. 1B). Importantly, under the experimental conditions utilized in this study L-histidine was neither growth-inhibitory nor toxic for the cells (not shown).

Taken together, these results indicate that sublethal, albeit growth-inhibitory, levels of H_2O_2 become strongly cytotoxic when exposure to the oxidant is performed in the presence of L-histidine.

3.2. Effect of L-histidine on the level of DNA double-strand breaks in U937 cells challenged with hydrogen peroxide

Previous results from our laboratory demonstrated that the enhanced cytotoxicity of hydrogen peroxide in the presence of L-histidine was accompanied by the induction of DNA double-strand breakage in various cell lines, including U937 cells (Cantoni et al., 1994a). The results illustrated in Fig. 2A are in general agreement with those previously obtained (Cantoni et al., 1994a) and indicate that whereas no DNA double-strand breaks are produced in cells exposed to concentrations of hydrogen peroxide up to 1 mM, treatment with the oxidant in the presence of L-histidine (1 mM) induces a significant level of this lesion over the same concentration range used in toxicity studies. It is important to note that in these experiments U937 cells were used at a higher density than that used previously (Cantoni et al., 1994a) and therefore it is not surprising that lower levels of double-strand breaks were detected. Indeed, the extent of the deleterious effects of hydrogen peroxide given as a bolus to cultured cells is an inverse function of the number of cells that are being challenged (Cantoni et al., 1986). The results illustrated in Fig. 2A also show that the level of DNA double-strand breaks is lower after 60 min of incubation than at the 30 min time point, thus suggesting that DNA double-strand breaks are rapidly generated and that the level of these lesions present at a fixed time is not simply a function of the net amount of double-strand breaks that have been produced but also depends on the proportion of those breaks that have been rejoined during treatment. The influence of double-strand break-rejoining on the steady-state level of double-strand breaks can be appreciated from the data illustrated in Fig. 2B, which indicate a rapid accumulation of DNA double-strand breaks during the first 15 min of exposure to 300 μM H_2O_2/1 mM L-histidine, followed by a marked decrease in the level of these lesions at longer times. It is important to stress that the levels of DNA double-strand breaks detected under these conditions are very low and therefore these data, while suggesting that DNA double-strand breaks are being rapidly produced and removed, certainly do not allow an accurate estimation of the kinetics of DNA double-strand break-repair. In order to do so we investigated the migration of U937 cell DNA, analysed either immediately after a 30 min exposure to 1 mM H_2O_2/1 mM L-histidine (which induces a considerable amount of DNA double-strand breaks; see Fig. 2A) or after increasing time intervals of post-treatment incubation. The data obtained after quantitative analysis of the repair kinetics lead to the estimation of a $t_{1/2}$ value of approximately 90 min (not shown).

Taken together, these results indicate that growth-inhibitory levels of H_2O_2 do not produce DNA double-strand breaks in U937 cells; this type of DNA lesion, however, becomes apparent when the cells are exposed to the oxidant in the presence of L-histidine, a condition which leads to cell death.

3.3. Effect of L-histidine on secondary DNA fragmentation in U937 cells treated with hydrogen peroxide

The DNA obtained from cells treated with increasing concentrations of H_2O_2 in the absence or presence of

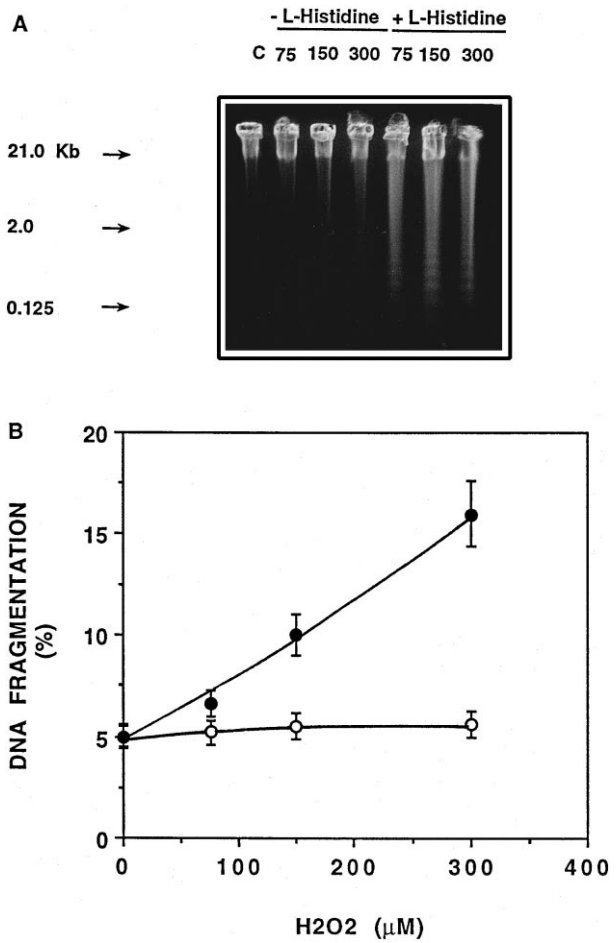

Fig. 3. DNA fragmentation in U937 cells exposed to H_2O_2 in the absence or presence of L-histidine. The cells were treated with the indicated amounts of H_2O_2 alone or associated with 1 mM L-histidine for 1 h and incubated for a further 7 h. The formation of oligonucleosomal fragments was determined by agarose gel electrophoresis (A) or by the filter binding assay (B), as detailed in Section 2. The results in A are from a representative study of three with comparable outcomes. The results in B are the mean ± S.E.M. calculated from 3–4 separate experiments.

L-histidine was also analysed by conventional agarose gel electrophoresis. As illustrated in Fig. 3A and B, the DNA from control cells (or cells exposed to L-histidine alone, not shown), as well as that from cells treated with 75–300 μM H_2O_2 alone and post-incubated in drug-free medium for 7 h, did not show signs of fragmentation. Similar results were obtained after 20 h and marginal DNA fragmentation was observed only in cells that had been exposed to a very high concentration of the oxidant (5 mM) (Fig. 4B). In contrast, treatment with the cocktail H_2O_2/L-histidine revealed a distinct DNA ladder pattern even at the lowest oxidant level tested in this study, both at the 7th (Fig. 3A) and 20th (Fig. 4B) h of post-challenge incubation. Interestingly, oligonucleosomal DNA fragments were found to progressively decrease and then disappear when the oxidant concentration was increased from 150 μM to 5 mM (Fig. 4B). DNA fragmentation at the 7th h of post-treatment incubation was also measured using a recently developed filter binding assay (Bertrand et al., 1991), with results (Fig. 3B) consistent with those obtained using conventional gel electrophoresis (Fig. 3A). These data demonstrate that 15% of the DNA was fragmented 7 h after treatment with 300 μM H_2O_2/1 mM L-histidine.

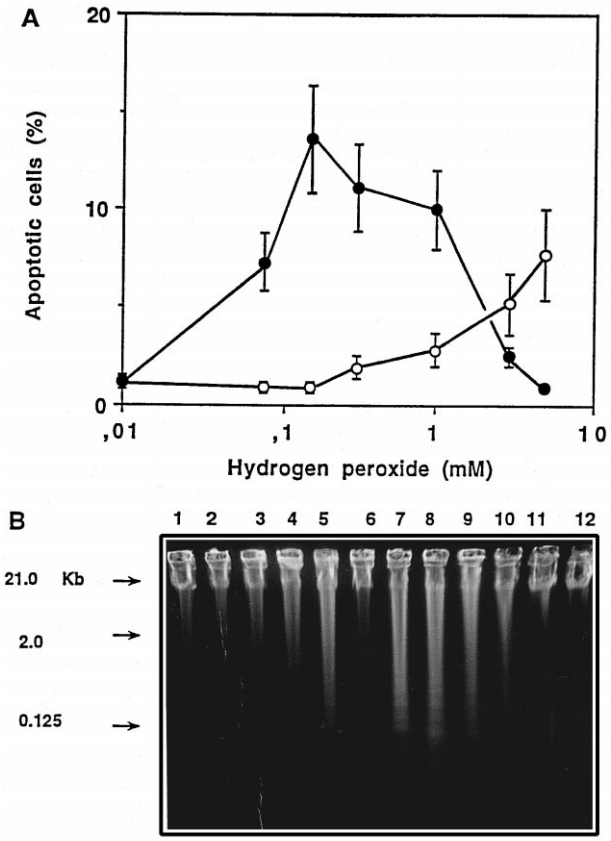

Fig. 4. Morphological and biochemical evidence of apoptosis in U937 cells exposed to H_2O_2 in the absence or presence of L-histidine. (A) Cells were treated with the indicated amounts of H_2O_2 alone (open circles) or associated with 1 mM L-histidine (closed circles) for 1 h and incubated for a further 20 h. The percentage of apoptotic cells was assayed by the haematoxylin-eosin stain as described in Section 2. (B) Agarose gel electrophoresis of DNA isolated from U937 cells treated with H_2O_2 in the absence or presence of L-histidine (1 h) and then post-incubated in drug-free medium for additional 20 h. The lanes are designated as follows: lane 1, control; lane 2, 300 μM H_2O_2; lane 3, 1 mM H_2O_2; lane 4, 3 mM H_2O_2; lane 5, 5 mM H_2O_2; lane 6, 1 mM L-histidine; lane 7, 75 μM H_2O_2 /1 mM L-histidine; lane 8, 150 μM H_2O_2 /1 mM L-histidine; lane 9, 300 μM H_2O_2 /1 mM L-histidine; lane 10, 1 mM of H_2O_2 /1 mM L-histidine; lane11, 3 mM H_2O_2 / 1 mM L-histidine; lane 12, 5 mM of H_2O_2 /1 mM histidine.

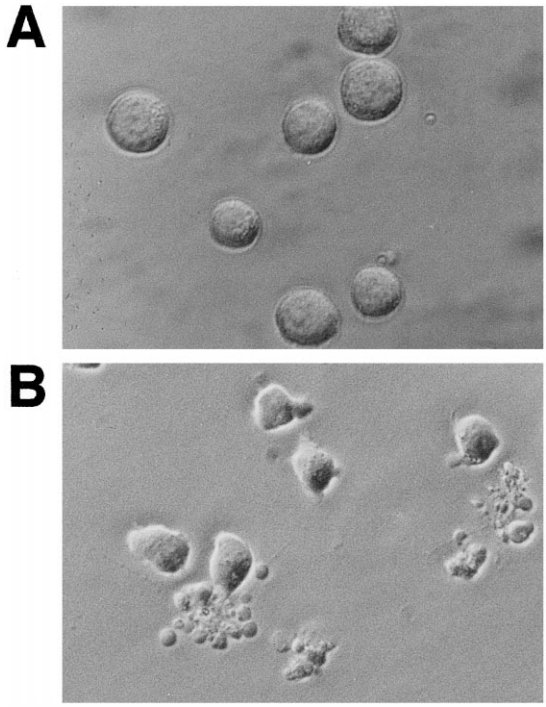

Fig. 5. Morphological features of U937 cells: (A) untreated; (B) cells exposed for 1 h to 300 μM H_2O_2 /1 mM L-histidine and grown for an additional 7 h in fresh drug-free medium.

The above results provide biochemical evidence suggesting that L-histidine triggers apoptosis in U937 cells exposed to micromolar levels of H_2O_2 and, as shown in Fig. 5, this hypothesis is reinforced by the results obtained from the morphological analysis. Untreated U937 cells are shown in Fig. 5A. Importantly, L-histidine alone did not affect their morphology (not shown). Exposure for 1 h to the cocktail H_2O_2/L-histidine followed by growth for 7 h in drug-free medium produced some of the characteristic features of apoptosis in the majority of the cells, including cell shrinkage and membrane blebbing (Fig. 5B). However, evidence of other changes typically associated with apoptosis such as fragmentation of nuclear chromatin were observed in a fraction of cells which never exceeded 15–20%, although this proportion was maintained throughout the 20 h of post-treatment growth (not shown), a time at which a large number of cells was trypan blue positive (Fig. 1B). The results illustrated in Fig. 4A show that the relative number of apoptotic cells observed 20 h after challenge with H_2O_2/L-histidine correlated well with the

degree of DNA fragmentation shown in Fig. 4B. Indeed, morphological analysis confirmed that the maximal level of apoptotic cells was achieved using 150 μM H_2O_2 and increasing the oxidant concentration progressively decreased the percentage of apoptotic cells (Fig. 4A) while enhancing the number of trypan blue positive cells (not shown).

Thus, U937 cells undergo apoptosis under conditions in which H_2O_2/L-histidine generate a low level of DNA double-strand breaks whereas, following treatment with higher concentrations producing a vast amount of these lesions, the majority of the cells seems to die via a necrotic process. Treatment with moderate levels of H_2O_2 does not cause cell death but, rather, transient inhibition of cell proliferation. On the other hand, consistent with previous findings (Nosseri et al., 1994; Ghibelli et al., 1995; Coppola et al., 1995), apoptotic cell death can be elicited by exposure to higher levels of the oxidant in U937 cells.

4. Discussion

A large body of evidence suggests that the formation of DNA double-strand breaks is responsible for the toxicity elicited by the cocktail H_2O_2 /L-histidine in CHO cells (Cantoni et al., 1992, 1994b; Sestili et al., 1992, 1995a,b,1996) as well as in other cell lines (Cantoni et al., 1994a). In this study we performed a more detailed characterization of the events leading to cell death, and found that a low level of DNA double-strand breaks is transiently detectable in U937 cells treated with H_2O_2/L-histidine (Fig. 2B) and that, under these conditions, both biochemical (Fig. 3 and 4B) and morphological signs (Fig. 4A and Fig. 5) of apoptosis develop in these cells. Importantly, the oxidant was used at concentrations which, when given alone to the cultures, did not elicit cell death but rather a transient delay in cell proliferation (Fig. 1). Indeed, H_2O_2 over a concentration range of 75–300 μM did not affect cell viability and did not cause significant biochemical and morphological changes. When associated with L-histidine, however, the oxidant triggered events typical of apoptosis that were readily detectable after 7 h of post-treatment incubation. The photomicrograph presented in Fig. 5 clearly shows that these cells displayed extensive membrane blebbing and that apoptotic bodies were being formed, possibly as a consequence of bleb detachment. These events were associated with the appearance of double-stranded DNA fragments (Fig. 3B) which form a characteristic DNA ladder pattern when analysed by conventional gel electrophoresis (Fig. 3A). It may therefore be concluded that sublethal concentrations of H_2O_2 trigger apoptosis in the presence of L-histidine and that, under these conditions, the oxidant also generates a low level of DNA double-strand breaks.

In this study we also utilized concentrations of H_2O_2 higher than 300 μM and found that, at millimolar levels, the oxidant itself leads a small proportion of the cells into apoptosis. This conclusion finds experimental support in the results of the analyses of nuclear fragmentation and DNA ladder formation illustrated in Fig. 4A and B, respectively. It is unclear why H_2O_2 is such a poor inducer of apoptosis under our experimental conditions. Indeed, the oxidant has been reported to efficiently induce apoptosis in a number of different cell lines (Lennon et al., 1992; Ueda and Shah, 1992), including U937 cells (Nosseri et al., 1994; Ghibelli et al., 1995; Coppola et al., 1995). Since in this study the treatments were performed in saline A, whereas in the above studies exposure to the oxidant was in complete culture medium, we suspect that specific factors present in the culture medium are essential for the apoptotic response. Experiments in progress in our laboratory confirm this possibility since, consistent with the results obtained by other investigators using U937 cells (Nosseri et al., 1994; Ghibelli et al., 1995; Coppola et al., 1995), we found that exposure to H_2O_2 in FBS-containing RPMI medium leads a large proportion of these cells into apoptosis. It will be of interest to determine the identities of the factors or components of the culture medium that are responsible for the observed differences. The fact that in the presence of L-histidine low levels of the oxidant also induce apoptosis upon treatment in saline A might suggest that the amino acid is one of these components.

Treatment with high concentrations of H_2O_2 in the presence of L-histidine promotes the formation of extensive DNA double-strand breakage (Fig. 2A) which is also repaired upon post-incubation in drug-free medium (not shown). These events, however, are followed by a progressive concentration-dependent decline in the apoptotic response (Fig. 4) and the induction of necrotic cell death.

We therefore conclude that L-histidine promotes an apoptotic response in oxidatively injured U937 cells and that DNA double-strand breaks produced by H_2O_2/L-histidine are potential triggering factors for apoptosis. High levels of this lesion, however, cause necrotic cell death.

Acknowledgements

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