Induction of Cell Differentiation Potentiates Apoptosis Triggered by Prior Exposure to DNA-damaging Drugs

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Abstract

At the end of their life span, differentiated cells die by apoptosis. Subsets of cells also die, in some cell systems, shortly after exposure to differentiating agents. This suggests that early during differentiation the cells may undergo “priming,” during which synthesis and/or activation and accumulation of effectors of apoptosis occurs. The objective of the present study was to test the hypothesis that the signal for apoptosis provided by DNA-damaging drugs given prior to induction of differentiation will be more effective in triggering apoptosis than when given following induction of differentiation. Human promyelocytic HL-60 cells were treated with the topoisomerase I inhibitor camptothecin, the alkylating agent nitrogen mustard, or 5'-azacytidine, an antimetabolite affecting predominantly RNA metabolism. Following drug removal, the cells were postincubated with n-butyrate, which induces differentiation of HL-60 cells along the monocytic pathway, or with all-trans-retinoic acid, which triggers myelocytic differentiation. Multiparameter flow cytometry using two different methods of analysis of apoptosis-associated DNA breakage in situ, as well as evaluation of cell morphology and DNA gel electrophoresis, were used to ascertain the mode of cell death. Increases of 100–200% in the percentage of apoptotic cells were seen when cells were first treated with camptothecin or nitrogen mustard, followed by n-butyrate or retinoic acid, compared to the combined percentage of apoptotic cells when these agents were used individually. In contrast to the DNA-damaging agents camptothecin and nitrogen mustard, no enhancement of apoptosis was observed when n-butyrate or retinoic acid was added after cell preexposure to 5'-azacytidine. The data suggest that although the sensitivity of the DNA damage detection and/or the apoptosis trigger mechanism may be higher in proliferating cells, the execution of apoptosis is potentiated in cells undergoing either monocytic or myelocytic differentiation. The data also support the antitumor strategy of using differentiating agents subsequent to DNA-damaging drugs or radiation.

Introduction

The inherent propensity of tumor cells to trigger the cascade of events leading to cell suicide denoted as apoptosis, or programmed cell death (1–4), is believed to be predictive of the sensitivity of the cell to antitumor drugs of different classes in vitro, as well as of the clinical response of patients to treatment with chemotherapeutic agents in vivo (5–9). Various strategies, therefore, are being considered to enhance tumor cell responsiveness by apoptosis, as well as to decrease the responsiveness of nontumor cells, that would lead to improved treatment efficacy and lower drug toxicity (5–9).

Apoptosis is the mode of death common to differentiated cells at the end of their life span (1–4). There are many observations that subsets of differentiating cells undergo apoptosis relatively early after induction of cell differentiation (10–13). This often manifests as the appearance of DNA strand breaks in situ, reflecting activation of the apoptosis-associated nuclease(s). These observations suggested that early during differentiation the cells may undergo “priming” (4), i.e., develop a readiness to undergo apoptosis. Attempts have been made, therefore, to develop strategies for enhancement of the response of a tumor cell to antitumor drugs by apoptosis via induction of cell differentiation (14–18). Suppression rather than enhancement of the cytotoxicity of antitumor drugs, however, was generally observed when the cells were initially exposed to inducers of differentiation and subsequently to cytotoxic agents (14, 16–19).

It was noticed by Waxman et al. (20) that the cytotoxicity of 5-fluorouracil toward the Friend virus murine erythroleukemia cells was enhanced by the posttreatment of the cells with the differentiating agents DMSO, HMBA, or N-methylformamide. Based on this observation, the authors have proposed sequential cytotoxic-differentiation antitumor therapy. It also was observed by Studzinski et al. (21) that the cytotoxicity of ara-C was potentiated by the subsequent exposure of human promyelocytic HL-60 cells to vitamin D3, an agent that causes their differentiation along the monocytic/macrophage pathway. In none of these early reports was the mechanism of cell death studied. We have noticed recently, however, that apoptosis of HL-60 cells induced by CAM was potentiated by posttreatment with DMSO (18). Also Huang and Waxman (22) have recently provided evidence that the cytotoxicity of 5-fluorouracil on Friend erythroleukemia cells, potentiated by HMBA, involves induction of apoptosis.

Although a useful in vitro model of cell differentiation, the Friend virus induced murine erythroleukemia is unique in many respects and has several features that make it

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3 The abbreviations used are: HMBA, hexamethylene bisacetamide; AZA, 5-azacytidine; CAM, camptothecin; dUTP, digoxigenin-conjugated dUTP; RA, all-trans-retinoic acid; NM, nitrogen mustard; TdT, terminal deoxynucleotidyl transferase.
different from human leukemic lines. One of the aims of the present study, therefore, was to extend the observation of Waxman et al. (20) and Huang and Waxman (22) to the human promyelocytic cell line, HL-60. This widely studied differentiating human leukemic line overexpresses c-myc, is null for wild-type p53, and is well characterized with respect to other molecular markers related to proliferation, differentiation, and apoptosis. Another aim of this study was to screen less toxic inducers of differentiation than the polar compounds DMSO, HMBR, or N-methylformamide for their ability to enhance apoptosis. Toward this end, we have explored the activity of a simple chemical, n-butyrate, which triggers differentiation of HL-60 cells along the monocytic pathway, and the clinically relevant agent RA (23), which induces myelocytic differentiation. Still another aim was to investigate cytotoxic agents with different mechanisms of action. Namely, DNA-damaging agents such as the topoisomerase I inhibitor CAM and the alkylating agent NM were compared with the antimetabolite AZA, the target of which (in terms of induction of cytotoxicity) appears to be RNA metabolism (24). Based on our present findings that differentiating agents potentiate apoptosis induced by the DNA-damaging drugs CAM and NM but have no measurable effect following AZA treatment, and from our previous observation on the effect of DMSO on apoptosis induced by CAM (18), we propose a novel explanation of this phenomenon. This testable hypothesis assumes that although cell differentiation lowers the sensitivity of mechanisms responsible for detection of DNA damage and/or the triggering of apoptosis, it may, at the same time, potentiate the execution of apoptosis, perhaps by enhancing the expression of apoptotic effectors.

Results

Fig. 1 presents DNA content frequency histograms of HL-60 cells, untreated, treated with CAM or n-butyrate alone, as well as treated with CAM for 4 h and then incubated in the presence of n-butyrate. Apoptotic cells can be distinguished in these histograms by their fractional DNA content (25, 26), while the nonapoptotic cells can be classified as in G1, S, and G2+M phases of the cell cycle. It is quite evident that exposure of HL-60 cells to 60 nM CAM for 4 h, followed by 24 h cell incubation in the absence of the drug, had a rather modest effect on cell viability because there were only 8% apoptotic cells after this treatment. The increase in the proportion of S phase cells, from 37 to 51%, however, was apparent, as was the predominance of cells in early S.

Much different results, however, were obtained when, following a 4-h exposure to CAM, instead of growth in drug-free medium, the cells were incubated in the presence of 1 mM n-butyrate. Such treatment resulted in a nearly 5-fold higher proportion of apoptotic cells, compared to incubation with CAM alone. Also, over a 10-fold lower proportion of cells in S phase was observed. Incubation of HL-60 cells with n-butyrate alone for 24 h resulted in the appearance of 5% apoptotic cells and a decrease in the proportion of S phase cells, from 37% (seen in the control culture) to 15%, concomitant with an increase in the proportion of G1 and G2 + M cells (Fig. 1).

The results of many experiments, such as the one shown in Fig. 1, are summarized in Fig. 2 and Table 1. The data presented in Fig. 2 clearly indicate that n-butyrate and RA, included into cultures after cell exposure to CAM or NM, significantly potentiated apoptosis, regardless of the concentration of the DNA-damaging drugs. Thus, for example, following cell exposure to 15 nM CAM, incubation with n-butyrate resulted in a nearly 5-fold higher percentage of apoptotic cells. Over a 2-fold higher proportion of apoptotic cells was observed when NM-treated cells were subsequently incubated with n-butyrate or RA.

The results were also expressed as the percentage of increase in the proportion of apoptotic cells during the sequential treatment with CAM, NM, or AZA and the differentiating agents, compared to the cumulative percentage of apoptotic cells in both the culture treated with a particular drug and the differentiating agent alone (Fig. 2, Inset). A nearly 200% increase in the percentage of apoptotic cells was observed when the cells were pretreated with 15 nM CAM and then grown in the presence of n-butyrate, while the increase in apoptotic cells was within the range of 100 to 160% when the cells were treated at a higher concentration of CAM (60 nM) or NM, followed by n-butyrate or RA. In contrast to CAM or NM, no potentiation of AZA cytotoxicity was observed following posttreatment with either n-butyrate or RA (Fig. 2).
Potentiation of apoptosis of HL-60 cells by n-butyrate (BUT) or RA by prior triggering with CAM and NM but not AZA. The cells were pretreated with CAM, NM, or AZA for 4 h at the indicated concentrations, washed, and incubated in the absence or presence of 1 mM n-butyrate or 1 μM RA for an additional 24 h. Left panel, the percentage of apoptotic cells was determined by flow cytometry as shown in Fig. 1. The data represent the mean from several independent experiments reported in Table 1; error bars, SEM, are shown for three or more determinations. Inset, potentiation of apoptosis by n-butyrate or RA. The percentage of potentiation in cultures posttreated with the differentiating agents was calculated as the increase above the sum of the percentage of apoptotic cells in cultures treated with the respective drug and n-butyrate or RA alone: a, 15 nM CAM; b, 60 nM CAM; c, 0.6 μM NM; d, 4 μM AZA; e, 8 μM AZA. Right panel, viable cells were determined by the trypan blue exclusion assay and are expressed as a percentage of total cells. The data represent the mean (n = 3); bars, SEM.

Table 1 Percentage of cells in the respective phases of the cell cycle in cultures of HL-60 cells treated with CAM (60 nM), NM (0.6 μM), and AZA (4 μM) and/or posttreated with n-butyrate or RA

<table>
<thead>
<tr>
<th>Drug</th>
<th>n-Butyrate</th>
<th>RA</th>
<th>n</th>
<th>Cell cycle distributions*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>None</td>
<td>–</td>
<td>–</td>
<td>8</td>
<td>44.9 ± 3.0</td>
</tr>
<tr>
<td>CAM</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>28.8 ± 3.0</td>
</tr>
<tr>
<td>NM</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>20.7 ± 5.4</td>
</tr>
<tr>
<td>AZA</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>48.4 ± 6.3</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>–</td>
<td>7</td>
<td>66.1 ± 2.5</td>
</tr>
<tr>
<td>CAM</td>
<td>+</td>
<td>–</td>
<td>6</td>
<td>73.6 ± 2.9</td>
</tr>
<tr>
<td>NM</td>
<td>+</td>
<td>–</td>
<td>3</td>
<td>29.6 ± 4.1</td>
</tr>
<tr>
<td>AZA</td>
<td>+</td>
<td>–</td>
<td>5</td>
<td>77.0 ± 4.5</td>
</tr>
<tr>
<td>None</td>
<td>–</td>
<td>+</td>
<td>4</td>
<td>48.9 ± 3.9</td>
</tr>
<tr>
<td>CAM</td>
<td>–</td>
<td>+</td>
<td>4</td>
<td>36.8 ± 5.3</td>
</tr>
<tr>
<td>NM</td>
<td>–</td>
<td>+</td>
<td>2</td>
<td>25.9</td>
</tr>
</tbody>
</table>

* The cell cycle distributions were determined as described in the text and Fig. 1.

b Number of independent experiments, mean ± SEM.

accumulation of typical mono- and oligonucleosomal-sized fragments forming a "ladder" during electrophoresis, was observed in all treated samples. Furthermore, the presence of excessive DNA breakage in situ (27, 28), detected by labeling free 3' OH termini with d-dUTP in the reaction catalyzed by exogenous TdT, was also detected in all samples (Fig. 3A). Changes in cell morphology typical of apoptosis, showing overall cell shrinkage, condensation of chromatin, nuclear fragmentation, and detachment of apoptotic bodies, were also observed in all treated samples (Fig. 4).

Changes in the cell cycle distribution of the nonapoptotic cell population during cell treatment with the drugs are presented in Table 1. As is evident, exposure of cells to CAM alone led to their arrest in S and G2 + M; NM alone caused cell arrest in G2 + M, whereas AZA alone arrested...
cells in S phase of the cell cycle. An increase in the proportion of G1 cells was observed in the cultures treated with n-butyrate, but no significant changes were evident in cultures treated with RA alone. Posttreatment of the cells exposed to CAM with n-butyrate resulted in marked decrease in the proportion of S phase cells, whereas no such effect was seen in the case of RA. Posttreatment of the cells exposed to NM with n-butyrate or RA led to a decrease in the proportion of S and G1 phase cells.

Discussion
In the present study, we compared the effect of AZA, an antimetabolite the target of which (in terms of cytotoxicity) appears to be RNA (24), with drugs that induce well-defined lesions in DNA, i.e., the alkylating agent NM and the DNA topoisomerase I inhibitor CAM. These drugs also show differences in cell cycle phase specificity in the induction of apoptosis of HL-60 cells. Namely, AZA, at low concentrations similar to those used in the present study, preferentially triggers apoptosis of G1 cells (29). Although NM also shows a preference for G1 cells, it is less discriminating compared to AZA (29). DNA topoisomerase inhibitors, on the other hand, induce DNA cleavage and cause formation of DNA-protein “cleavable complexes” (30). It has been postulated that collision of the advancing DNA replication forks with these complexes triggers cell death (30). In accordance with this postulate, it has been observed that CAM, indeed, selectively triggers apoptosis of S phase cells,
Fig. 4. Morphology of HL-60 cells untreated (a), treated with 60 μM CAM for 4 h followed by 24 h incubation in the drug-free medium (b), incubated for 24 h in the presence of 1 mM n-butyrate (c), and treated for 4 h with 60 μM CAM, followed by 24 h exposure to 1 mM n-butyrate (d). The cells were stained with DNA-specific fluorochrome 4,6-diamidino-2-phenylindole and photographed under UV light microscope (Nikon: × 40). Note chromatin condensation and nuclear fragmentation typical of apoptosis, predominant in (b) and (d).

having no immediate effect on cells in G₁ or G₂ + M phases (29). Our present data, thus, indicate that potentiation of apoptosis by differentiating agents occurs following induction of DNA damage by the agents causing very different DNA lesions and having different cell cycle phase specificities. Furthermore, both induction of cell differentiation along the monocytic (n-butyrate) and myelocytic (RA) pathways enhance apoptosis triggered by NM and CAM. The observation of Studzinski et al. (21) of the enhancement of cytotoxicity of 1-β-D-arabinofuranosylcytosine by vitamin D₃ suggests that incorporation of this nucleoside analogue into DNA (which may cause DNA breaks) is recognized by the cell as still another type of DNA lesion capable of triggering apoptosis following subsequent induction of cell differentiation.

In contrast to NM or CAM, we did not observe the potentiation of apoptosis triggered by AZA. In light of the evidence that the primary target of both 5-fluorouracil and AZA is RNA metabolism (24, 31), it is difficult to reconcile this observation with the data of Waxman et al. (20) and
Huang and Waxman (22), who noticed a potentiation of the cytotoxic effect of 5-fluorouracil on Friend virus murine erythroleukemia cells. These differences may stem from the differences in cell types used in the respective studies.

There is no clear explanation as to why induction of cell differentiation may have opposite effects, depending on the sequence of administration of inducing agent vis-à-vis administration of a cytotoxic drug, as presently observed for HL-60 cells. We do advance a hypothesis, however, that this phenomenon may be due to the inherent differences in the sensitivity of the DNA damage detection and/or apoptosis trigger mechanisms versus the efficiency (abundance) of apoptosis effectors between proliferating and differentiating cells (Fig. 5). It is possible that proliferating cells have a very sensitive mechanism for DNA damage detection that is coupled with the signal for cytostasis and damage repair, or alternatively, if the damage is extensive or repair unsuccessful, the signal for apoptosis (as a default system). Evidence that overexpression of c-myc, the oncogene which drives the cell toward proliferation, potentiates the response of the cell by apoptosis (32, 33) may be an indication of the increased sensitivity of the apoptosis-triggering mechanism. High sensitivity of the apoptotic trigger is essential for proliferating cells, especially stem cells, because survival of even a single cell with unrepair DNA may have potentially fatal consequences for the organism, e.g., in the case of DNA damage (mutation), which may involve an oncogene or a tumor suppressor gene. On the other hand, DNA damage to a differentiated cell is expected to have minor consequences to the organism, and therefore, the mechanism of its detection coupled with the activation of an apoptotic response need not be as effective. The change in sensitivity of the apoptosis triggering mechanism may be associated with down-regulation of c-myc, as observed during differentiation of HL-60 cells (34). We presently observed that cell treatment with n-butylate did, indeed, down-regulate expression of c-myc, regardless of whether cells were preincubated or not with CAM (data not shown). Thus, induction of cell differentiation, as measured in terms of down-regulation of c-myc, was not affected by their preexposure to CAM. Because HL-60 cells lack wild-type p53, the observed potentiation of cytotoxicity is unrelated to expression of this gene.

A caution should be exercised, however, in generalizing the conclusions. It should be pointed out, for example, that induction of differentiation of M1 myeloid leukemic cells by interleukin 6 or granulocyte-colony-stimulating factor increases their apoptotic response to Adriamycin, 1-β-D-arabinofuranosylcytosine, heat shock, or cyclohexamide (35). This effect is just opposite to that seen for HL-60 cells treated with DMSO (18) or vitamin D3 (21). This may be due to either the cell lineage or reflect differences in the mechanism of induction of differentiation by particular inducers.

The enhancement of apoptosis by triggering differentiation of the cells with damaged DNA, as presently seen, may reflect activation or synthesis and accumulation of apoptotic effector molecules. Indeed, it has been observed that a Ca2+/Mg2+-dependent endonuclease, the enzyme suspected to be essential during apoptosis (1-4), is induced at early stages of murine erythroleukemic cell differentiation (36). The presence of cell subsets with extensive DNA strand breakage, typical of apoptosis, was also observed at early stages of apoptosis (10, 12). The apoptotic execution machinery, dependent on the presence and abundance of effectors, therefore, appears to be more efficient in differentiating than in proliferating cells.

HL-60 cells have certain unique features, such as lack of the tumor suppressor gene p53 and high levels of expression of bcl-2, and are easily triggered to undergo apoptosis. The present findings, therefore, to be generalized, have to be reproduced in other cell types. We have obtained evidence recently, however, that the apoptotic response to DNA-damaging drugs of the myeloblastic cell line ML-1, which expresses wild-type p53 (37), is also markedly potentiated by posttreatment with a variety of differentiating agents.

Several differentiating agents have been introduced to the clinic as antitumor drugs, and their use already has a long history. The most effective of them is RA. Our present data suggest that the effectiveness of these agents may be increased when they are combined with DNA-damaging drugs and administered shortly after the latter. The strategy of modulating cell differentiation or the cell cycle to enhance apoptosis induced by antitumor drugs has been recently discussed by us in more detail elsewhere (38).

Materials and Methods

Cell Culture. The human promyelocytic leukemic cell line HL-60 was kindly provided by Dr. Harry A. Crissman (Los Alamos National Laboratory, Los Alamos, NM). The cells were maintained in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (GIBCO).

Reagents. CAM, NM, AZA, and RA (all-trans) were purchased from Sigma Chemical Co. (St. Louis, MO). n-Butyrate was purchased from Pfaltz & Bauer, Inc. (Stamford, CT). A stock solution of CAM was prepared in DMSO at a concentration of 0.6 mM. Solutions of NM and AZA were freshly prepared in distilled water at 30 mM concentrations and further diluted in PBS. n-Butyrate was prepared in PBS

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at a 1 M concentration and added directly to the growth medium. Stock solutions of RA were prepared at 2 mM concentration in ethanol and stored at −80°C for up to 2 weeks. RA-treated cultures were kept shielded from the light.

**Drug Treatments.** Exponentially growing cultures were exposed to either CAM, NM, or AZA at the indicated concentrations for 4 h, washed free of drug, and incubated in medium in the presence of either n-butyrate or RA for an additional 24 h. Following treatment, cells were fixed in suspension in 70% ethanol and stored at −20°C for cell cycle analysis.

**DNA Content Analysis.** Following fixation, cells were rehydrated in HBSS, centrifuged, and resuspended in 40 µl of phosphate-citric acid buffer containing 192 parts of 0.2 M Na₂HPO₄ and 8 parts of 0.1 M citric acid (pH 7.8) at room temperature for 30 min to facilitate removal of low molecular weight DNA from apoptotic cells, as described (25). The cells were then stained with 1 µg/ml diamidino-2-phenylindole and 10 µg/ml sulforhodamine 101 (Eastman Kodak, Rochester, NY) as described previously (25, 26). The blue (DNA) and red (protein) fluorescence of individual cells was measured using an ICP-22 flow cytometer (Ortho Diagnostics, Westwood, MA). Data were collected, stored, analyzed, and displayed using Phoenix Flow Systems (San Diego, CA) software.

**TdT Assay for Labeling DNA Strand Breaks.** Following drug treatment, the cells were fixed in 1% formaldehyde in PBS for 15 min on ice, washed with PBS, postfixed in 70% ethanol, and stored at −20°C prior to analysis, up to 4 days. Cells were then centrifuged and resuspended in PBS. DNA strand breaks were labeled with d-dUTP in the reaction catalyzed by exogenous TdT (27), followed by staining with fluorescent antibody to digoxigenin (27). This was accomplished using the ApopTag kit kindly provided by ONCOR, Inc. (Gaithersburg, MD), as described recently (28).

**DNA Gel Electrophoresis.** Cells were fixed in 70% ethanol, then centrifuged and incubated in phosphate-citric acid buffer to extract low molecular weight DNA from apoptotic cells, as described above. DNA extracted with this buffer was concentrated and then subjected to electrophoresis on 1.2% agarose gel at 2 V/cm for 16 h. The gels were stained with ethidium bromide. Other details are presented elsewhere (25).

**References**


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