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Apoptosis in Antitumor Strategies: Modulation of Cell Cycle or Differentiation

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Abstract There is a strong evidence that administration of antitumor drugs triggers apoptotic death of target cells. A characteristic feature of apoptosis is active participation of the affected cell in its demise. Attempts have been made, therefore, to potentiate the cytotoxicity of a variety of agents by modulating the propensity of cells to respond by apoptosis. Several strategies to enhance apoptosis that involve modulation of the cell cycle or differentiation are discussed. Loss of control of the G_1 checkpoint in tumor cells allows one to design treatments that arrest normal cells at the checkpoint and attempt to selectively kill tumor cells with S phase specific drugs. The possibility of a restoration of the apoptosis triggering function of the tumor suppressor gene p53 when the G_1 checkpoint function is abolished is expected to increase tumor cells' sensitivity to S phase poisons. Because induction of apoptosis by many antitumor drugs is cell cycle phase specific, drug combinations that preferentially trigger apoptosis at different phases of the cycle, or recruitment of cells to the sensitive phase, offer another antitumor strategy. There is also evidence that apoptosis is potentiated when cell differentiation is triggered following DNA damage. This observation suggests that strategies which combine DNA damaging and differentiating drugs, under conditions where the latter are administered following DNA damage caused by the former, may be successful. © 1995 Wiley-Liss, Inc.

Key words: programmed cell death, DNA topoisomerase inhibitors, DNA damage, DNA repair, tumor suppressor gene p53, oncogene c-*myc*, cell cycle checkpoint, G_1 phase

INDUCTION OF APOPTOSIS BY ANTITUMOR DRUGS: APOPTOSIS AND OTHER MODES OF CELL DEATH

The most characteristic feature of apoptosis, which frequently is called "cell suicide," is active participation of the affected cell in its demise. In response to a variety of environmental inducers, or often triggered by a combination of intrinsic factors, the cell activates a preprogrammed cascade of metabolic events that culminate in its disintegration. The prominent events of the apoptotic mode of cell death involve rapid cell dehydration, increase in free Ca^{2+} concentration, activation of an endonuclease which has affinity to internucleosomal DNA sections, and activation of transglutaminase activity [reviews: Arends et al., 1980; Wyllie et al., 1980; Ellis et al., 1992; Wylie, 1992]. The endonucleolytic process, which involves the sequential DNA cleavage to the size of approximately 300 kb, 50 kb, and, eventually, 200 bp [Oberhammer et al., 1993], appears to be coupled or preceded by activation of serine proteases [Gorczyca et al., 1992].

These biochemical events are paralleled by changes in cell morphology. The loss of intracellular water is reflected, very early, by cell shrinkage and condensation of the cytoplasm. This is accompanied by condensation of chromatin, which starts from the nuclear periphery and is followed by fragmentation of the nucleus. Regions of cytoplasm containing intact and still functionally active organelles (mitochondria, lysosomes) together with fragments of nuclei, all wrapped in fragments of the plasma membrane, detach from the cell as apoptotic bodies, in the process of a characteristic undulation of the membrane ("blebbing"). Apoptotic bodies are phagocytized in the tissue by the neighboring cells. The integrity of the plasma membrane, including active transport, is preserved until the late stages of apoptosis. Because no leakage of the cytoplasmic contents into the intercellular space occurs, even extensive apoptosis does not lead to tissue inflammation or scarring.

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Some cell types or lines are "primed" to undergo apoptosis [Wyllie, 1992]. Such cells appear to have all the necessary effector molecules, and thus, without the need for new gene activation, can execute the full program of apoptosis that has all the characteristic biochemical and morphological features described above. Most cell types of hemopoietic lineage, normal or tumor transformed, belong to this category. In many instances, however, induction of apoptosis requires activation of new genes.

In contrast to apoptosis, the alternative mode of cell death, necrosis, is a passive, catabolic, and degenerative process. It is characterized by swelling of mitochondria and early rupture of the plasma membrane. Necrosis generally occurs in response to very high doses of toxic factors.

It should be stressed that in many instances, cell death is atypical, either showing only some features of apoptosis, or having mixed features of apoptosis and necrosis [e.g., Cohen et al., 1992; Collins et al., 1992]. It is possible that in these instances the factors triggering cell death (e.g., inhibitors of protein kinases or proteases, certain drugs) also block particular metabolic pathways of the apoptotic cascade. As a result, the pattern of apoptosis is incomplete, lacking the attributes of the affected pathways. In other instances, cells cannot execute all the metabolic events of apoptosis, because they may lack one or more effectors needed to carry out these processes. For example, regardless of the nature of the agent triggering death, the cells of T-lymphoblastic leukemia lines, such as MOLT-4 or CEM, die in a manner which does not resemble apoptosis [Akagi et al., 1993]. These cells appear to lack a Mg^{2+} dependent endonuclease and their fusion with cells that contain the enzyme, "primes" them, restoring their ability to respond by apoptosis [Matsubara et al., 1994].

The term "mitotic-" or "delayed reproductive-cell death" is frequently used to denote cell death which occurs after exposure to relatively low doses of drugs or radiation: following the exposure, the cells are able to progress through the cycle but they die during the subsequent cycle. This is in contrast to "interphase cell death," when following the insult, the cells die prior to the first mitosis. Although in many respects mitotic cell death resembles apotosis, it frequently lacks all the classical features of the latter [Shinohara and Nakano, 1993]. This may be due to secondary changes in cell metabolism induced by prolonged perturbation of cell cycle progression (e.g., unbalanced growth), occurring prior to cell death [Kung et al., 1990]. One may discriminate, therefore, between "immediate" apoptosis, which generally has all the classical features of apoptosis, most often does not require new genes activation, and is not complicated by secondary changes due to prolonged cell growth without division (growth imbalance), and "delayed" apoptosis, which may manifest cellular changes quite different from those observed during classical apoptosis.

It is still unclear which events, among the plethora of changes observed during "classical apoptosis" or "atypical" cell death, represent the active processes of self annihilation of the cell and which are passive, post-mortem alterations [Farber, 1994]. Identification of the active processes is necessary to develop strategies that can enhance the cell's capability for committing suicide. As will be discussed later in this article, the possibility of induction, or amplification, of the effectors of apoptosis ("cell priming"), by triggering cell differentiation, may be associated with enhancement of the active processes and pathways of apoptosis.

The evidence that antitumor drugs or radiation trigger either classical apoptosis, or atypical death which still has elements of active participation of the involved cell in the death process (and in this respect resembles apoptosis), is undisputable. A multitude of reports providing examples of apoptotic death of tumor cells following their in vitro exposure to pharmacological concentrations of drugs of different classes, including biological response modifiers, have been published in recent years [e.g., Kaufmann, 1989; Evans and Dive, 1993; Ormerod et al., 1994]. Although observations in vivo are less frequent and mostly limited to hematological tumors, the evidence that cell death in tumors of patients undergoing routine chemo- or radiotherapy has all the features of apoptosis, is also convincing [e.g., Li et al., 1994]. These observations, thus, provide a rationale for development of antitumor strategies that rely on modulation of the cell propensity to respond by apoptosis. Discussion of the strategies involving drugs that induce DNA damage and the role of the cell cycle and cell differentiation are the subject of this article.

CELL CYCLE AND APOPTOSIS

The Strategies Based on the Presence of the G_1 Checkpoint in Normal Cells

Most antitumor agents, (e.g., ionizing radiation, alkylators, *cis*-platin, nitroso-compounds, mitomycin C, bleomycin) induce direct damage to DNA of the target cell. Inhibitors of DNA topoisomerases I and II also cause lesions in DNA by causing strand breaks and stabilizing otherwise transient covalent complexes of topoisomerases with DNA. Figure 1 schematically illustrates various types of response of the cell to such agents. Generally, at very low drug concentrations no significant changes in cell cycle progression or cell viability are apparent, although some observations suggest that minor DNA damage (e.g., by some DNA topoisomerase inhibitors) may trigger cell differentiation [Aller et al., 1992]. Apparently, the rate at which the cell is capable of repairing DNA damage caused by such low drug concentrations exceeds the rate of induction of DNA damage, and there is no need for mobilization of cytostatic or cytotoxic mechanisms.

Two distinct types of cell response to higher drugs concentrations (in terms of perturbation of the cell cycle progression), are generally observed. The difference in response depends on the status of the cell cycle checkpoint in G_1 regulated by tumor suppressor gene, wild type (wt) p53. Among many functions, this gene activates the transcription of the cyclin dependent kinase inhibitor Cip1 (Waf-1, p21), which is the key component of the quaternary complex consisting, in addition to Cip1, of cyclin dependent kinase CDK4, cyclin D1, and proliferating cell nuclear antigen (PCNA) [reviews: Weinert and Lydall, 1993; Zhang et al., 1993; Pines, 1994]. The function of this complex is to recognize the damage and arrest the cell at the G_1 checkpoint until DNA repair is completed, or as a *default* mechanism, to direct the cell towards the apoptotic pathway. Participation of Cip1 in the complex is essential in arresting the cell and preventing its entrance to S. The duration of cell arrest at the checkpoint is proportional to the extent of DNA damage and the rate of DNA repair. It should be stressed, however, that with drugs that do not induce DNA damage (e.g., steroid hormones, ligands to certain cell surface receptors), the presence of wt p53 is not needed for apoptosis [Clarke et al., 1993].

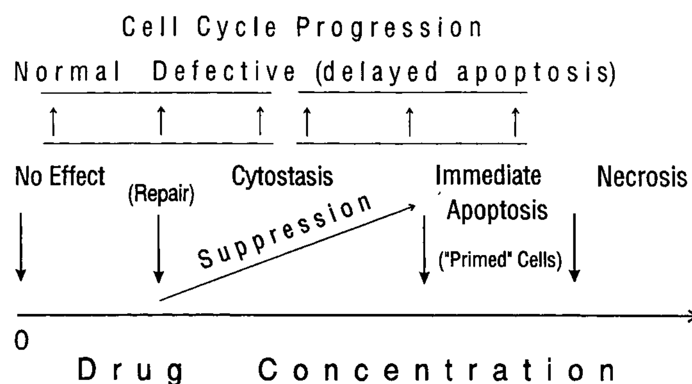

Fig. 1. Generalized scheme illustrating the effects of increasing concentrations of DNA damaging antitumor drugs, on cell cycle progression and apopotsis. Exposure of cells to very low drug concentrations has generally no, or minimal, effect on their viability or cell cycle, most likely due to the fact that the rate of DNA repair exceeds the rate of accumulation of the lesions. At higher drug concentrations, depending on the presence or absence of the G_1 checkpoint (which is associated with expression of tumor suppressor gene p53) two types of responses occur: a) In the presence of a functioning checkpoint, cell progression through G_1 is halted until the lesion is repaired. Alternatively, apoptosis is triggered when the damage is extensive (high drug concentration) or repair unsuccessful. b) If the G_1 checkpoint is malfunctioning (e.g., as in the case of of mutation of p53) the cells do enter S, but the rate of progression (rate of DNA replication) is suppressed proportionally to the drug concentration. In the case of cells "primed" to apoptosis, apoptosis generally occurs very rapidly (3–6 h, "immediate apoptosis") at the threshold drug concentration, slightly above that which completely halts their progression through S [Del Bino et al., 1991]. Cell priming to apoptosis may be associated with, among other factors, constitutive expression of *c-myc*. In the case of "nonprimed cells," prolonged suppression of cell cycle progression by the drug ("defective progression") leads to growth imbalance, secondary changes, their subsequent "priming" (development of effectors), and delayed apoptosis. Delayed apoptosis may often have atypical features, complicated by growth imbalance and secondary changes in cell metabolism [Kung et al., 1990]. Necrosis is seen at still higher drug concentration, generally above its pharmacological level.

A second type of response is observed in cells that have a malfunctioning G_1 checkpoint. This is generally the case when p53 is mutated, which occurs in over 50% of cancers. Inactivation of p53 function, however, also may occur as a result of wt p53 sequestration by certain viral proteins or an MDM2 oncogene [Chen et al., 1994]. As a consequence of a loss of wt p53 function, there is a lack of the inhibitor of kinases PIC1, and induction of DNA damage in such cells does not arrest them at the checkpoint. Instead, they enter S phase, but their progression through S is generally slowed, in proportion to the drug concentration (i.e., the extent of DNA damage), up to a certain threshold [Del Bino et al., 1991]. In primed cells, immediate apoptosis is triggered when the drug is administered at a concentration above this critical threshold. It appears, therefore, that the signal to undergo apoptosis is given when DNA damage is beyond the cell's capacity to repair it. The mechanism which senses the extent of the damage and directs the cell either towards DNA repair, which is associated with a slowdown in progression through S, or to apoptosis, is unknown. The survival or reproductive capacity of these cells depends very much on the nature (e.g., ss vs. ds DNA breaks) and extent of DNA damage. The time and mode of death of the affected cells also depends on the cell type (Fig. 1 legend).

The kinetic difference between cells exercising the G_1 checkpoint (nontumor cells) and the cells that have this checkpoint compromised (e.g., the cells with mutated p53), following DNA damage [Kuerbitz et al., 1992], offers possibilities for treatments that may be selectively cytotoxic to the latter cells. Some strategies based on this difference, have been recently discussed by Kerr et al. [1994], Kohn et al. [1994], and Fisher [1994]. One of the approaches involves cell treatment with relatively low doses of DNA damaging agent followed by high doses of a drug that is selective to DNA replicating cells, preferably the drug that is incorporated into DNA and whose incorporation generates a lethal lesion. Under these conditions the normal cells, being transiently arrested at the checkpoint, are expected to resist the drug that is selectively toxic to DNA replicating cells. In contrast, the cells with a defect at the checkpoint, do enter S phase and therefore are vulnerable to the drug.

It should be pointed out, however, that because the expression of wt p53 primes cells to respond by apoptosis to DNA damaging agents [Wang et al., 1993], the cells expressing the mutated protein may be more resistant to induction of immediate apoptosis. It is unknown, therefore, to what extent the advantage of cells with malfunctioning p53, in terms of their increased sensitivity to S phase poisons, as discussed above, is counterbalanced by the decreased propensity to respond by apoptosis. The poor prognosis observed in many human neoplasms characterized by mutated p53 may be an indication that loss of wt p53 function associated with triggering apoptosis outweighs the benefit of a loss of the checkpoint, the latter expected to increase their sensitivity to S phase poisons. A strategy, however, can be designed to dissociate the dual functions of p53, the apoptosis triggering from the one arresting the cell at the checkpoint. Cells in which the checkpoint is abolished while the apoptosis triggering function is preserved are expected to be very sensitive to S phase poisons. Such an approach, perhaps, will be possible, once the molecular mechanisms responsible for this dual function of p53 are elucidated. Elucidation of the duality of p53 function will also enable us to understand mechanisms responsible for tissue (cell-type)-specificity of the p53 dependent apoptosis [Slichenmyer et al., 1993].

The success of many empirically developed clinical protocols, which combine DNA damaging agents with drugs affecting DNA replication, may reflect mechanisms related to G_1 checkpoint discussed above. Likewise, the effectiveness of many cell cycle specific antitumor drugs, in particular DNA topoisomerase inhibitors, when used alone, also may be due to the differences between normal vs. tumor cells in the status of their G_1 checkpoint. Namely, while normal cells when treated with these drugs do arrest at the checkpoint to repair their DNA, the tumor cells enter the replicative phase with unrepaired DNA. The collision of the replication fork with DNA lesions (e.g., the "cleavable complexes" generated by DNA topoisomerase inhibitors and representing a stabilized complex of DNA and DNA topoisomerase) is lethal to the cells [D'Arpa et al., 1990], triggering their apoptosis. Clearly, the difference in rate of cell proliferation, which is frequently much higher in normal tissue (e.g., bone marrow, colon) than in tumor, does not explain the antitumor potency of cell cycle specific drugs.

Considering the role of the G_1 checkpoint in the kinetic response of the cell to antitumor drugs discussed above, one may expect that tumors having a functionally active G_1 checkpoint will have different drug sensitivities compared to tumors with impaired regulation of checkpoint regulation (e.g., mutated p53). Thus, the functional status of the G_1 checkpoint may be an independent prognostic marker, predictive of the tumor's sensitivity to the drugs that cause DNA damage and are toxic to S phase cells e.g. such as DNA topoisomerase inhibitors. Consequently, chemotherapy may be customized to individual tumors which differ in the status of their G_1 checkpoint, even if their histology and other features used for classification, are similar.

Another strategy that is based on the differences in status of the G_1 checkpoint between normal and tumor cells involves the application of an inhibitor of a protein kinase(s), whose activity is essential for the maintenance of the checkpoint in the active state. Crissman et al. [1991] and Bruno et al. [1992] have independently observed that the nonspecific protein kinase inhibitor staurosporine at low concentrations (10 nM), while arresting normal cells in G_1, has no effect on many tumor cell lines. The point of cell arrest by staurosporine appears to be associated with the G_1 checkpoint, because it involves expression of cyclin E [Gong et al., 1994b], the regulatory subunit of CDK2. The inability of staurosporine to arrest tumor cells may depend on unscheduled ("ectopic") expression of other cyclins and their association with their respective CDKs, which allows the cell to bypass the G_1 checkpoint [Gong et al., 1994a]. Regardless of the cause, however, the kinetic difference between normal and most tumor cell types, in response to a kinase inhibitor, such as staurosporine, can be exploited in tumor therapy. Namely, administration of the inhibitor simultaneous with an S phase poison, is expected to be selectively toxic to tumor cells [Crissman et al., 1991; Bruno et al., 1992]. Normal cells, under these conditions, being prevented from entering S phase by the inhibitor, may escape the drug's cytotoxicity.

Malfunction of cell cycle checkpoints resulting in an escape of the cell from its regulatory mechanisms, appears to be the defect that is the most common in any type of cancer. It also is apparent that cell progression through the cycle and apoptosis are both regulated at the G_1 checkpoint. The explosive progress in research in the area of apoptosis and cell cycle control is expected, in the near future, to reveal the molecular interactions at the checkpoints, that direct the cell either towards progression through the cycle or to apoptosis. We will be in a much better position, then, to develop new antitumor strategies and new drugs with entirely different mechanisms of action, compared to chemotherapeutics used today in the clinic.

Combination of Drugs Triggering Apoptosis in Different Phases of the Cell Cycle

It has long been recognized [e.g., reviews: Darzynkiewicz, 1986; Bhuyan and Groppi, 1989] that combinations of drugs with specificities for different phases of the cell cycle provide an attractive antitumor strategy. The rationale for developing drug combinations which may increase the efficiency of cell kill are predicated on a) combination of drugs with different phase-specific cytotoxicities; b) sequential combinations, in which the first drug synchronizes cells in a particular phase, while the drug given subsequently is lethal to cells in the phase to which they advance after the synchronization; c) combinations of drugs in which one drug blocks progression at a particular phase and the second is cytotoxic for cells in that phase. With the realization that apoptosis is the predominant mode of death of cells treated with antitumor drugs, the strategies listed above can be reevaluated in light of our knowledge on the cell cycle phase specificity of apoptosis.

We have recently developed a flow cytometric method that allows one to identify apoptotic cells and relate their position in the cell cycle [Gorczyca et al., 1992]. The method is based on labeling DNA strand breaks in apoptotic cells with fluorescent nucleotides in a reaction catalysed by terminal deoxynucleotidyl transferase, followed by bivariate analysis of the labeled cells with respect to their DNA content. This method, as well as the less direct approach based on analysis of the cell cycle distribution of the non-apoptotic cells, was used to evaluate the cell cycle phase specificity of a variety of a common antitumor drugs in terms of induction of apoptosis [Gorczyca et al., 1993] (Table I).

As is evident from Table I, cells progressing through S phase are selectively susceptible to apoptosis when treated with the DNA topoisomerase I inhibitor camtothecin, topoisomerase II inhibitors teniposide, *m*-AMSA (amsacrine) and

TABLE I. The Cell Cycle Phase Specificity of Various Antitumor Agents in Terms of Induction of Apoptosis [Gorczyca et al., 1993]

Cell cycle phase specificity	Drug
G_1	5-Azacytidine (low conc.), nitrogen mustard, hyperthermia
S	Camptothecin, teniposide, *m*-AMSA, Mitoxantrone, ara-C, 5-azacytidine (high conc.)
$G_2 + M$	Radiation, H7 (serine/threonine kinase inhibitor)
None	Fostriecin, cisplatin, cycloheximide, genistein

Mitoxantrone, DNA replication inhibitor hydroxyurea, antimetabolite ara-C, and the serine/threonine kinase inhibitor H7. Cells in G_2 +M preferentially undergo apoptosis when treated with ionizing radiation and H7, while G_1 cells appear to be preferentially affected by the antimetabolite 5-azacytidine, the alkylating agent nitrogen mustard and hyperthermia. No significant cell cycle specificity was observed in the case of the DNA topoisomerase II inhibitor fostriecin, the presumed tyrosine kinase inhibitor genistein, cycloheximide or cisplatin. It should be pointed out that unlike *m*-AMSA or mitoxantrone, inhibition of DNA topoisomerase II by fostriecin does not involve formation of "cleavable complexes," which may explain the differences between these drugs in terms of their cell cycle phase preference.

The cell cycle phase related differences in cell susceptibility to apoptosis induced by the drugs, as shown in Table I, most likely reflect the severity of the lesion induced by a given drug as well as the ability of the cell to repair such damage. Both can vary depending on the cell cycle phase. It was shown, for example, that toxicity of DNA topoisomerase I and II inhibitors, is due to a collision between the replication fork and the lesion (the cleavable complex), and that inhibition of DNA replication protects cells from these drugs [D'Arpa et al., 1990]. Thus, the same lesion may be lethal if it is present in a cell which replicates DNA, and nonlethal and perhaps repairable if it occurs during G_1, G_2, or even S phase, provided that DNA replication is halted.

The cell cycle phase differences in sensitivity to particular drugs (in terms of the cell's response by apoptosis) can be exploited in designing drug combinations for maximal efficiency. Thus, optimal drug combinations applied to histologically similar tumors may be quite different if the cell cycle kinetics of the tumor cell populations differ. It is expected, for instance, that camptothecin, teniposide, m-AMSA, or ara-C will be more effective for tumors with high S phase fractions, while fostriecin, 5-azacytidine, nitrogen mustard, or hyperthermia may be preferred to treat tumors with a low fraction of S phase cells. As mentioned above, combinations of drugs with different cell cycle phase specificities (Table I), are expected to have at least an additive effect and such combinations should be explored in the clinic.

The sequential treatment of HL-60 cells with radiation and camptothecin, enhancing the cytotoxicity of camptothecin, represents an example of a recruitment of cells to an apoptosis sensitive phase [Del Bino et al., 1992]. It was observed, in these studies, that apoptosis induced by camptothecin was markedly potentiated if the cells were pretreated with low doses of γ radiation. This potentiation was explained by transient cell arrest and recruitment to S phase (as a result of their irradiation), resulting in a higher proportion of cells sensitive to lethal effects of the subsequently administered camptothecin.

Conflicting Signals for Growth Stimulation and Suppression

There is a growing body of evidence that a combination of mitogenic signals and simultaneous suppression of cell growth, e.g., by depletion of growth factors or nutrients can trigger apoptosis of tumor cells. This is in contrast to normal cells, which in the absence of growth factors or nutrients re-enter the quiescent state (G0), where they remain viable for extended periods of time. The mitogenic signals can be provided from outside of the cell, through the transduction pathway, e.g., as hormonal stimulation [Colombel et al., 1992], or may be intrinsic, as in the case of induction of the constitutive expression of c-*myc* [Evan et al., 1992].

Selective potentiation of the cytotoxicity of tumor necrosis factor α (TNF α) against the tumor transformed cells, by depletion of external glucose [Volland et al., 1992], or inhibition of glycolysis by 2-deoxy-D-glucose (submitted for publication) may be an example of the mechanism discussed above. It is quite likely that TNF α provides a mitogenic signal, which under the conditions when glycolysis is suppressed, triggers apoptosis of tumor cells [Volland et al.,

1992]. The antitumor strategy employing simultaneous administration of mitogenic cytokines and growth suppressing agents, by virtue of low toxicity of these agents, has a potential to be tested in the clinic as an adjuvant to standard chemotherapy.

MODULATION OF CELL DIFFERENTIATION

There is extensive evidence in the literature that death of differentiated cells, at the end of their lifespan, occurs by apoptosis [e.g., Counis et al., 1989]. It has been observed that synthesis of a Ca^{2+}, Mg^{2+} dependent endonuclease (the enzyme suspected to be involved in apoptosis), is induced at early stages of cell differentiation [Modak and Beard 1980; McMahon et al., 1984]. It has also been noticed that shortly after induction of cell differentiation, extensive DNA breakage, typical of apoptosis, occurs in many cells [Farzaneh et al., 1982; Gunji et al., 1992]. This evidence suggested that differentiating cells may be more sensitive to the induction of apoptosis when triggered with antitumor drugs. Attempts have been made, therefore, to increase cell responsiveness by apoptosis by induction of their differentiation. It was observed, however, in most of these studies, that when the cells were first treated with differentiation inducing agents, and subsequently with antitumor drugs, their response by apoptosis to the former was decreased rather than increased [Xu et al., 1993; Solary et al., 1993; Del Bino et al., 1994]. The increased resistance of the differentiated cells appears to be unrelated to expression of bcl-2, an oncogene which protects cells against apoptosis [Xu et al., 1993]. Likewise, simultaneous administration of antitumor drugs and differentiating agents have failed to result in the potentiation of apoptosis.

Interestingly, enhancement of apoptosis is observed when the sequence of treatment is reversed, namely when cells are first treated with antitumor drugs and subsequently with the differentiating agents. We had noticed, for example that apoptosis was potentiated when HL-60 cells were exposed to very low concentrations of camptothecin and posttreated with dimethyl sulfoxide [Del Bino et al., 1994]. These observations were recently extended in our laboratory to other drugs and other differentiation agents (submitted for publication). In these studies, we observed that pre-exposure of HL-60 cells to subtoxic concentrations of nitrogen mustard or camptothecin triggered apoptosis only when it was followed by induction of cell differentiation by retinoic acid or *n*-butyrate. Similar findings were also reported by Studzinski et al. [1986], who noticed that the cytotoxic effect of ara-C on HL-60 cells was enhanced by the posttreatment with another inducer of cell differentiation, vitamin D_3; the mode of cell death, however, was not explored in their study. It appears therefore, that induction of cell differentiation potentiates apoptosis triggered by prior cell exposure to various antitumor drugs.

An explanation as to why induction of cell differentiation may have opposite effects depending on the sequence of induction vis-a-vis administration of the cytotoxic drug was proposed by us recently [Del Bino et al., 1994; submitted for publication]. Namely, we advanced the hypothesis that this phenomenon may be due to the inherent differences between proliferating and differentiating cells, in the sensitivity of the DNA damage detection and/or apoptosis trigger mechanisms vs. the efficiency (abundance) of apoptosis effectors (Table II). Proliferating cells may have a very sensitive mechanism for DNA damage detection, coupled with a signal for halting cell cycle progression and damage repair. If the damage is extensive or repair unsuccessful, apoptosis is then triggered. The proliferative status of the cell and high sensitivity of the mechanism triggering apoptosis may be associated with an activation of c-*myc*, the oncogene shown to precondition cells to respond by apoptosis [Evan et al., 1992]. Such high sensitivity of the apoptotic triggering mechanism is essential for proliferating cells, especially stem cells: survival of a single cell with unrepaired DNA, having, e.g., DNA damage (mutation) which involves an oncogene or tumor suppressor gene, may be fatal for the whole organism. Clearly, a

TABLE II. Possible Differences in Sensitivity and Efficiency of the DNA Damage and Apoptosis Triggering vs. Apoptosis Execution Mechanisms Between Proliferating and Differentiating Cells

Cell feature	Proliferating (stem) cells	Differentiating cells
Damage detection/ apoptosis triggering mechanisms	Increased	Decreased
Apoptosis execution mechanisms (effectors)	Decreased	Increased

sensitive mechanism recognizing and eliminating defective cells had to evolve for normal stem cells, and such a mechanism may also be present in proliferating tumor cells, known to have many features of stem cells.

In comparison with proliferating cells, DNA damage in cells that have entered the differentiation pathway and will not divide is potentially of lesser consequence to the organism. Therefore, mechanisms of detection of DNA damage and triggering of apoptosis, in response to the damage, need not be as effective. The decrease in *sensitivity* of the apoptosis triggering mechanism may be associated with downregulation of c-*myc,* as observed in differentiating HL-60 cells [Cayre et al., 1987]. On the other hand, because, as mentioned, induction of differentiation appears to activate the synthesis and accumulation of apoptotic effectors, the apoptotic execution machinery is expected to be more efficient in differentiating than in proliferating cells. Considering the above, the optimal moment to *trigger* apoptosis is the time of cell proliferation, while the subsequent induction of cell differentiation is expected to potentiate the process of *execution* of apoptosis.

Several agents which induce cell differentiation have already been tried in the clinic, and they are generally of low toxicity. Their effectiveness, however, when used alone, is relatively poor. The data discussed above suggest that the effectiveness of these agents may be improved if they are used in combination with DNA damaging agents, under conditions when induction of differentiation is subsequent to DNA damage.

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