Paclitaxel-Induced Apoptosis in HeLa Cells is Serum Dependent

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ABSTRACT: Exposure of HeLa cells to different concentrations of the antineoplastic drug paclitaxel resulted in a loss of cell viability that was dependent on the concentration and time of exposure to the drug. This phenomenon was associated with the appearance of nuclear morphology typical of apoptosis and DNA breakage into a “ladder” pattern of discrete fragments of nucleosomal size. The induction of cell death was dependent on the serum concentration of the culture media, repressed by pretreatment with a cAMP-dependent protein kinase (PKA) inhibitor, and enhanced by increasing the cell proliferation with previous exposure to a cAMP-analog and a protein kinase-C (PKC) inducer. The proliferative index modifies the effect of taxol on HeLa cells, probably by means of a more rapid accumulation of cells in the G2/M cycle blockade point, although a direct participation of PKA and PKC should not be excluded. © 1997 John Wiley & Sons, Inc.

KEYWORDS: Paclitaxel, Apoptosis, HeLa cells, Cervical Cancer, PKC, PKA.

INTRODUCTION

Apoptosis is an active form of physiological cell death that represents a mechanism of cell clearance in many physiological and physiopathological situations. Distinctive morphological and biochemical changes take place during this process. Apoptotic cells are typified by the highly condensed and fragmented state of their nuclei with breakage of the nuclear chromatin at internucleosomal sites as a result of activation of a specific endogenous endonuclease and, in several cases, the formation of multiple vesicles (apoptotic bodies). Physiological stimuli regulate this type of cell death, which is present in many species and tissues. Many chemotherapeutic agents cause dramatic changes in cellular gene expression and, in several cell types, induce apoptotic cell death (1–3).

Paclitaxel (taxol), a diterpenoid originally isolated from the stem bark of the Western yew, Taxus brevifolia (4), has a novel chemical structure and an unusual mechanism of action. Paclitaxel binds to the NH₂-terminal 31 aminoacids of the β-tubulin subunit of microtubules, with a maximum stoichiometry of approximately 1 mol of taxol/mol of tubulin. The drug increases the rate of microtubules assembly, reduces the critical concentration of microtubule protein required for this, and assembles tubulin under conditions in which polymerization would not normally occur, for example, in absence of GTP or microtubule-associated proteins (5,6). Taxol suppresses microtubule dynamic instability differently, depending upon the stoichiometry of taxol binding to the microtubules. As a consequence of a reorganization of the microtubule cytoskeleton, the interaction of taxol with cells results in the formation of discrete bundles of stable microtubules, a process that requires ATP (7–10).

Paclitaxel is a potent inhibitor of eukaryotic cell replication, blocking cells in the late G2 mitotic phase of the cell cycle (11). Antineoplastic activity has now been documented in lung cancer (both nonsmall and small cell), head and neck cancer, and particularly refractory ovarian and breast carcinomas (12–16).

In human myeloid leukemia cells, clinically relevant concentrations of paclitaxel induce internucleosomal DNA fragmentation and the characteristic morphologic changes associated with apoptosis. These events are temporally related to the repression of bcl-2 and c-myc oncogenes (17,18). Liu et al. (19) have shown that taxol induces apoptosis in a human ovarian tumor cell line. In this case, the process required adequate intracellular ATP levels and the presence of protein tyrosine kinase activity. Paclitaxel-induced apoptosis has not been investigated thoroughly in human solid tumor cells.
In the past few years, new activities have been assigned to taxol, which are related to the ability of the drug to mimic the effects of bacterial lipopolysaccharides (LPS). Like LPS, taxol induces the expression of the genes for the cytokines TNF-α, interleukin 1-β, and the early LPS-inducible genes (IP-10, D3, D7, D8) in macrophages (20). In addition, the drug causes a reduction in the surface expression of TNF-α receptors, induces tyrosine phosphorylation of MAP kinases in mammalian cells, and induces the expression of GM-CSF in B cells (19–21). These results suggest that its actions are more pleiotropic than previously appreciated.

There is little known about the effect of taxol in cervical cancer. Schiff et al. (6) demonstrated that taxol inhibits the division of exponentially growing cervical cancer cells (HeLa) at low concentrations (0.25 μM), with no significant effects on macromolecule synthesis during the first 4 hours after treatment, blocking the cell cycle in late G2 and M at 20 hours. Therefore, we decided to analyze if taxol could induce apoptosis in HeLa cells, as well as the effect of agents capable of modifying the cellular proliferation state.

**MATERIALS AND METHODS**

**Cell Culture**

Human HeLa cells were maintained as a monolayer in Dulbecco’s modified Eagle’s medium containing 1, 6, 8, or 12% (v/v) fetal bovine serum and incubated at 37°C in a humidified atmosphere with 5% (v/v) CO₂ in air. Cells at 70% confluence were treated with different concentrations of the drugs. Paclitaxel and PMA were dissolved in dimethylsulfoxide (DMSO); under the conditions used, the concentration of DMSO did not exceed 0.1% (v/v) in culture medium; this level did not affect growth or viability. cAMP-dependent protein kinase inhibitor (22) and membrane-permeable cAMP analog, cyclic chlorophenylthio-adenosine-3'5'-monophosphate were dissolved in water. Dulbecco’s modified Eagle’s media and fetal bovine sera were obtained from GIBCO, chlorophenylthio-cAMP was obtained from Boehringer Manheim, Germany; all other chemicals were obtained from Sigma, St. Louis, MO.

**Cytologic Examination**

Cells were fixed in ethanol at –70°C, pretreated with RNase, incubated for 5 minutes with ethidium bromide (10 mg/mL) in PBS, and extensively washed. The cells were visualized with a Zeiss microscope, using epifluorescence with the appropriate filter and photographed in a Kodak Plus X-Pan film.

**Cellular Viability**

Cells seeded in 96-chamber multiwell dishes at different fetal bovine serum concentrations were treated with mentioned drugs or vehicle. At the times described, viability was assessed by MTT assay as in Reference 23. Where indicated, statistical analysis was performed using a paired Students t-test.

**Analysis of Low-Molecular-Weight DNA**

Cells (approximately 1 × 10⁶) were scraped from culture dishes, pelleted by centrifugation for 1 minute at 5000 × g, resuspended in 0.25 mL of lysis buffer (10 mM tris-HCl pH 7.5, 1 mM EDTA, and 0.2% triton X-100), and incubated for 60 minutes at 50°C with 50 μg/mL RNase and 60 minutes at 37°C with 0.5 mg/mL of proteinase K. Two volumes of absolute ethanol at −20°C were added, and high-molecular-weight DNA was eliminated with a pasteur pipette. The low-molecular-weight DNA was obtained by centrifugation at 15,000 × g for 20 minutes. The pellet was resuspended in tris 10 mM, EDTA 1 mM and analyzed by conventional agarose gel electrophoresis.

**RESULTS**

Exposure of HeLa cells to different concentrations of paclitaxel (100, 250, and 500 nM) resulted in a loss of cell viability as compared with paclitaxel-free control cells (Figure 1), in concordance with previous re-
TABLE 1. Effect of Different Serum Concentrations on Paclitaxel-Induced Toxicity in HeLa Cells

<table>
<thead>
<tr>
<th>Paclitaxel (nM)</th>
<th>1% Serum</th>
<th>8% Serum</th>
<th>12% Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>71.22 ± 0.2</td>
<td>59.55 ± 0.2</td>
<td>36.31 ± 0.1</td>
</tr>
<tr>
<td>250</td>
<td>58.24 ± 0.2</td>
<td>48.89 ± 0.2</td>
<td>29.87 ± 0.2</td>
</tr>
<tr>
<td>500</td>
<td>31.22 ± 0.1</td>
<td>29.44 ± 0.2</td>
<td>21.51 ± 0.1</td>
</tr>
</tbody>
</table>

*Cells were exposed to different drug and serum concentrations. The cell viability was determined at the fifth day of exposure by the MTT assay (see Materials and Methods). The results shown are the arithmetic media and standard deviations from three independent experiments, each performed in triplicate, expressed as relative percentage from untreated cells.

FIGURE 2. Ethidium bromide-stained nuclei of HeLa cells. (A) Control cells. (B) Cells treated for 48 hours with a 500 nM concentration of paclitaxel as stated in Material and Methods.

FIGURE 3. Agarose gel electrophoresis of low-molecular-weight DNA of HeLa cells treated with paclitaxel (500 nM) for different times. (M) Size marker; the size in base pairs is shown above.

FIGURE 4. Effect of serum on HeLa cells proliferation. HeLa cells were cultivated with different serum concentrations for the times shown, and the viability was assessed by the MTT procedure (see Materials and Methods). (—○—) 1% serum; (—△—) 8% serum; (—□—) 12% serum concentration.

One of the hallmarks of the apoptotic process is the specific DNA fragmentation in internucleosomal-size fragments (1–3). In a temporal course, agarose gel electrophoresis of DNA extracted from paclitaxel-treated cells showed that the DNA was cleaved into a ‘ladder’ pattern, in concordance with the apoptotic morphologic features appearance (Figure 3).

We analyzed the effect of different serum concentration on the apoptosis induced by taxol, since there is evidence for the involvement of tyrosine phosphorylation in the paclitaxel-induced apoptosis (19), and it is well known that growth factors elicit their response by tyrosine phosphorylation-based events. Increasing concentrations of fetal bovine serum in the culture medium induced a concordant decrease of the time required for the cells to reach confluence, indicating the dependence between the growth factor concentration and the cellular proliferation state (Figure 4). When different concentrations of the drug were added to the cells, we found that the toxicity was enhanced with increasing serum concentrations (Table 1). It is noteworthy that the toxicity of a 500 nM concentration of paclitaxel at a low serum condition (1%) is comparable to the toxicity obtained with a very low dose of the drug (100 nM) with higher serum concentration (12%). This effect is not due to differential stability of the compound in varying serum concentration, since the drug has the same activity over HeLa cells in preincubated medium at 1% or 10% serum concentration (Figure 5). In this experiment, there was a decrease approximately...
FIGURE 5. Effect of preincubation in Paclitaxel activity at different serum concentrations. Paclitaxel was preincubated in DMEM at 10% or 1% serum concentrations for 18 hours at 37°C. The serum concentration was adjusted to 10% and added to HeLa cells; the viability was determined by the MTT assay as described in Materials and Methods 48 hours later. The results represent the arithmetic mean and standard deviations from three independent experiments performed in triplicate. *p > 0.05. NP: paclitaxel not preincubated; 10%: paclitaxel preincubated in medium containing 10% fetal bovine serum; 1%: paclitaxel preincubated in medium containing 1% fetal bovine serum.

of 20% in both serum concentrations, in accordance to a previous report (24). Hence, the paclitaxel-induced apoptosis in HeLa cells depends on the serum concentration of the culture media.

Since several cell types can be stimulated to proliferate in culture when treated with protein C-kinase activators, such as phorbol esters, we decided to mimic the effect of serum with PMA. As shown in Figure 6A, pretreatment of HeLa cells cultivated at low serum concentrations (1%) with different concentrations of PMA presented faster growth rates, as compared with nontreated cells. Addition of paclitaxel (500 nM) to PMA-treated cultures resulted in a loss of cell viability that was dependent on the PMA concentration, and comparable to that obtained with higher serum concentrations (Figure 6B). The morphology of the cells treated with PMA and paclitaxel was clearly apoptotic (data not shown).

It has been shown that cAMP-dependent protein kinase activity (PKA) can affect cell growth (25). We tested the effect of the Walsh PKA inhibitor (PKI), a 20 residue peptide that corresponds to the active site of this protein (22). Although PKI is a high-molecular-weight molecule and hence has a poor cellular penetration, addition of the peptide to the culture slightly inhibited the cell proliferation, with doses ranging from 1 to 10 μM (Figure 7A) with no associated cellular death or morphologic alterations. Paclitaxel toxicity was reversed when the HeLa cells were pretreated with PKI. The cultures treated with both drugs showed less apoptotic cells as compared with cultures treated with paclitaxel alone (results not shown). To support this result, we also treated HeLa cells with a membrane-permeable cAMP-analog, chlorophenylthio-cAMP (26). As expected, there was a potentiation of the paclitaxel effect (Figure 7B).

DISCUSSION

In this article, we show that paclitaxel exposure of HeLa cells causes a loss in cell viability due to apoptosis, a result supported by previous results of Schiff et al. (6). In their article, they showed that this drug induces a G2/M blockage, inhibiting completely the division of exponentially growing HeLa cells. It is noteworthy the delay between paclitaxel exposure and the death of the cells, as compared with other antineoplastic drugs that also block cell-cycle progression at the G2/M checkpoint (27). The appearance of the morphologic features and internucleosomal fragmentation is an early event, since most of the cells die two days after (Figures 1–3). This delay supports the idea of a protective mechanism(s) that avoids chromosomal damage during the mixing of nuclear and cytoplasmic compartments during the G2/M point (28).

The onset of the apoptosis in these cells was affected by the serum concentration in the culture media (Table 1). This could be explained by the higher proliferation index of the cells, with more cells entering the G2/M checkpoint, and, thus, suitable for the blockage. On the other hand, it has been proposed that a conflict between mitotic stimulus and signals for cell-cycle blockage can lead to apoptosis (29).

To get more insight on this, we mimicked the effect of high serum concentration with PMA, a phorbol ester that activates PKC and promotes proliferation (Figure 6). In this regard, it has been reported that HeLa cells present a delay in the G2/M progression during the cell cycle by similar concentrations of PMA (30). Since this delay occurs during the first four hours after exposure to the phorbol ester and it is transitory, our results, which analyze longer times, could be reflecting a secondary effect of PMA over HeLa cells proliferation. By addition of PMA, the toxicity is enhanced and resembles the death obtained with higher serum concentrations, supporting the previous data, although a direct PKC participation during the apoptotic process cannot be excluded.

Next, we used a 20 residue peptide (PKI) that inhibits PKA activity. Although a poor entrance into the cell can be expected, we found that the peptide inhibits slightly the cell growth (Figure 7A). The effect was not
dose dependent. This could be due to a constant rate of transport, a saturation of the binding sites, or a small PKA participation in the proliferation promotion, which could be completely avoided with small PKI amounts. Surprisingly, the cell death was inhibited almost completely, in contrast to the taxol-exposed cells cultivated with 1% serum (Table 1). This fact could be explained if PKA participates directly as a specific intracellular signal that drives the cells into the apoptotic process. This result is supported by the potentiating effect of the membrane-permeable cAMP-analog, chlorophenylthio-cAMP, over the proliferation and paclitaxel toxicity in HeLa cells (Figure 7B). Indeed, a previous report (31) showed that CREB, a PKA-responsive transcription factor, is increased during the onset of apoptotic cell death in differentiated PC12 cells after nerve growth factor removal.

Experiments designed to test the participation of both enzymes in this process should help to clarify the data presented here and to get more insight into the signals that conduce a cell into the apoptotic process pathway.

FIGURE 6. Effect of PMA on HeLa cell proliferation and paclitaxel toxicity. (A) Left bars: cells treated with vehicle. Right bars: cells treated with PMA (100 nM) for the times indicated. *p < 0.05. **p < 0.01. (B) Cells pretreated with 1, 50, and 100 nM of PMA for 24 hours and exposed to paclitaxel (500 nM) for 48 hours. The serum concentration used was 1%. PTX: paclitaxel. Each point represents the arithmetic mean and standard deviations of three independent experiments, each performed in triplicate. Procedures as stated in Materials and Methods.

FIGURE 7. Effect of PKA modulators on paclitaxel toxicity in HeLa cells after 48 hours of exposure. (A) Cells pretreated with 1, 5, and 10 μM of PKI [Walsh inhibitor (22)] for 24 hours. The serum concentration used was 12%. (B) Cells pretreated with 1 mM of the cAMP analog, cyclic chlorophenylthio-cAMP for 4 hours. The serum concentration used was 1%. PTX: paclitaxel. cAMP: cyclic chlorophenylthio-cAMP. Each point represents the arithmetic mean and standard deviations of three independent experiments, each performed in triplicate. *p < 0.05. Procedures as stated in Materials and Methods.
ACKNOWLEDGMENTS

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REFERENCES