Appearance of Aberrant Mitosis in Murine Leukemia Cells upon Combined Treatment with Low Concentrations of Cisplatin and Teniposide

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The combination of *cis*-diamminedichloroplatinum (II) (DDP, cisplatin) and topoisomerase II inhibitor teniposide (VM-26) has been shown to exert a synergistic effect in the clinical treatment of cancer. In this study, the combined effect of DDP and VM-26 on the growth and induction of apoptosis in synchronized murine erythroleukemia (MEL) cells, treated at the beginning or in the middle of S-phase of cell cycle, was examined. MEL cells, clone F4 N, were synchronized by a double thymidine block leading to accumulation of 70% of cells at the G_1/S boundary. The growth-inhibitory effect of DDP and VM-26 applied alone were stronger in the middle of the S-phase than at the beginning. Morphological analysis showed that the majority of the cells revealed typical signs of apoptosis: nuclei fragmentation and appearance of apoptotic bodies. The combination of both agents at low concentrations had a synergistic effect on cytotoxicity. At higher concentrations the effect was additive. The remainder of the cells were characterized by unbalanced growth, aberrant mitosis and appearance of multinucleated cells. These processes led to delayed cell death. The appearance of aberrant mitosis was more expressed after treatment in the middle of the S-phase. It is likely that as a result of the combined action of cisplatin and VM-26, cells become supersensitive to the ability of topoisomerase II inhibitor to influence mitosis, and this increased sensitivity may contribute to the observed synergism.

Introduction

The combination of cisplatin (c*is*-diamminedichloroplatinum (II), DDP) and topoisomerase II inhibitors has been worldwide successfully used in the treatment of lung cancer, malignant lymphoma and testicular cancer. The combined use of DDP with etoposide (VP-16) or teniposide (VM-26) resulted in a synergistic effect against small cell lung cancer (SCLC) in clinical trials (Evans *et al*., 1985; Splinter *et al.*, 1996). The mechanism of this synergy is not well understood. A hypothesis was proposed that synergy between cisplatin and etoposide is caused not by biochemical modulation at the cellular level but by pharmacokinetic changes at the organism level (Tsai *et al*., 1989; Kondo *et al*., 1994). *In vitro* experiments with this combination using different SCLC cell lines showed confusing results, leading in most cases to additive or supra-additive effects (Haller *et al*., 1993; Minagawa *et al*., 1997).

It is known that cisplatin as well as VM-26 are able to induce cell death in a number of cell types through apoptosis (Minagawa *et al*., 1997; Del Bino *et al.,* 1992; Lee *et al*., 1997). Moreover, induction of apoptosis was recently thought to be one of the main mechanisms of the antitumor effects of these agents (Barry *et al*., 1990; Solary *et al*., 1996; Dubrez *et al*., 1995). The cytotoxic effects of cisplatin and VM-26 alone are concentration and cell-cycle dependent: S-phase has been shown to be most sensitive to their action (Gorczyca *et al*., 1993; Del Bino *et al*., 1992). However, the dependence of the combined action of DDP and VM-26 on the cell cycle is not yet clear.

In the present study, we examined the combined effect of DDP and VM-26 on growth and induction of apoptosis in synchronized murine erythroleukemia cells, treated at the beginning or in the middle of the S-phase of the cell cycle.

Materials and Methods

Cell culture and synchronization

Murine erythroleukemia cells, clone F4 N (virus-transformed erythroid precursor cells)

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(Dube *et al.*, 1975), were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) (Dulbecco and Freeman, 1959) supplemented with 10% calf serum, under 5% CO_2 atmosphere at 37° C, and passed every day at a concentration of 5×10^5 cells/ml. Cells were synchronized at G_1/S boundary by a double thymidine block as described by Russev and Boulikas (1992). Briefly, exponentially growing cells were treated twice for 12-h with 2 mM thymidine, with a 9-h interval in thymidine-free medium in between.

To assess cell synchronization, at different times after releasing from the block cells were labeled in duplicate for 15 min at 37° C with 0.004 MBq/ml [^{3}H]thymidine to monitor precursor incorporation into DNA. Aliquots of cell suspension were pipetted onto Whatman filter paper disks, the acid-soluble radioactivity was extracted with cold trichloroacetic acid (5%), and the incorporated activity determined by scintillation counting. The average value of quadruplicate determinations in three independent experiments ±SD was calculated.

Incubation with drugs and cytotoxicity evaluation

Synchronized cells (0.5×10^6 cells/ml) were incubated with cisplatin (prepared according to Spassovska *et al.* (1981)) and VM-26 (4'-demethylepipodophyllotoxin-9-(4,6-O-thenylidene -β-D-glucopyranoside) (teniposide) (Bristol-Mayers, Syracuse, NY) in 24-well or 96-well microtiter plates immediately (0 h) or 4 h after releasing from the block. After 72 h of drug treatment, the cells were counted hemocytometrically and analyzed by fluorescent microscopy. The number of dead cells was determined by staining with trypan blue. The mean of triplicate determinations of three independent experiments ±SD was calculated. The 50% inhibitory dose (IC_{50}) was defined as drug concentration that reduced the number of living cells by 50%.

Fluorescence microscopy

The cells were fixed with methanol-acetic acid (3:1, v/v), stained with propidium iodide (6 μg/ml, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and viewed using a Leitz orthoplane epifluorescence microscope.

Results and Discussion

In this study, mouse erythorleukemia cells, clone F4 N, were used in which cisplatin has been shown to readily trigger apoptosis (Vodenicharov *et al.*, 1996). To study the dependence of the combined action of cisplatin and teniposide on cell cycle phases, the cells were synchronized at the G_1/S boundary by a double thymidine block. To assess the level of cell synchronization, DNA biosynthesis rate after release from the block was used. The cells showed a typical well-expressed wave of thymidine uptake (Fig. 1) which indicated that over 70% of the cells were synchronized. A maximal DNA synthesis rate was observed 4 h after releasing from the block showing that at this time the majority of cells were in the middle of the S-phase.

First, we examined the growth-inhibitory effect of incubation of F4 N cells with cis-DDP or teniposide alone, added at G_1/S boundary or in the middle of S-phase (0 h or 4 h after release from the block, respectively). The cells proved to be more sensitive to cisplatin in the middle rather than at the beginning of S-phase (IC_{50} of 0.40 μM or 0.6 μM, respectively). Trans-DDP exerted in both cases no effect on cell growth (IC_{50} more than 80 μM). The percentage of dead (trypan blue positive) cells upon drug treatment showed that

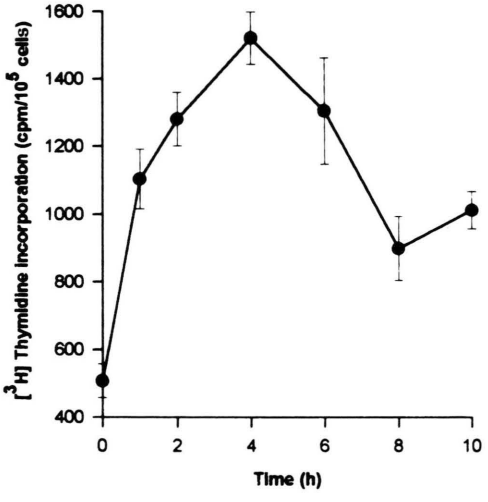

Fig. 1. Time course of [^{3}H]thymidine incorporation into macromolecular material of F4 N cells after releasing from a double thymidine block. Values are mean ± SD (N = 3 with 4 replicates per experiment).

the toxic effect of cisplatin was also most expressed in the middle the S-phase (data not shown).

With topoisomerase II inhibitor VM-26, a less expressed dependence of the growth-inhibitory effect on cell cycle phases was observed. Thus, treatment with this agent at 0 h or 4 h after release from the block resulted in IC_{50} of 0.04 μM and 0.02 μM, respectively. It is worth noting that a cell-cycle dependence of the growth-inhibitory as well as of the toxic effects of VM-26 (Fig. 2) were registered only in a limited interval of relatively low concentrations (< 0.05 μM).

To study the combined action of both drugs, the following cisplatin concentrations were used: 0.25 μM which did practically not influence cell growth when added at 0 h; 0.5 μM and 1 μM causing about 30% or 70% growth inhibition, respectively, as well as 2 μM causing 100% growth inhibition (a cytostatic concentration). The dependence of cell growth on VM-26 concentration in the presence of the above amounts of cisplatin is illustrated in Fig. 2. The drugs were added immediately after release from the block (Fig. 2 A) or at the middle of S-phase (Fig. 2 B). The enhancement of the growth-inhibitory effect of VM-26 by cisplatin was more expressed at low concentrations of both drugs and at middle S-phase rather than at G_1/S boundary. This was clearly observed at a non-effective concentration of VM-26 (0.0125 μM) in combination with growth inhibiting concentrations of cisplatin (0.5 μM and 1 μM) leading to a complete block of cell growth after treatment in the middle of S-phase. A super-additive effect was observed showing a synergistic action of these drugs. Similar data have been published with Chinese hamster ovary cells in which the combination of etoposide and DDP resulted in cytotoxicity that was greater than additive at low DDP concentrations (Eder *et al.*, 1990).

To gain more detailed insight into the cell death process, a morphological analysis of treated F4 N cells was performed using the fluorescent dye propidium iodide. Using this method as well as by comet assay, we have previously shown that cisplatin caused death of F4 N cells mainly by apoptosis (Kushev *et al.*, 1999). The apoptotic cells exhibited fragmented condensed nuclei. At a later stage, these cells lost their DNA and only cell debris and few apoptotic bodies (small bright bodies) were observed.

Representative results are shown in Fig. 3. Untreated exponentially growing cells had round nuclei of uniform size (panel a). For comparison, in panel **b** are presented cells treated with azide at concentration (30 mM) causing necrosis in about 70% of cell population: necrotic cells appeared to have one small condensed nucleus.

The morphology of cisplatin or VM-26 treated cells depended on drug concentration. No morphological changes were observed in cells treated either at G_1/S boundary or in the middle S-phase at concentrations which caused only growth retardation but did not block cell division (up to 0.5 μM

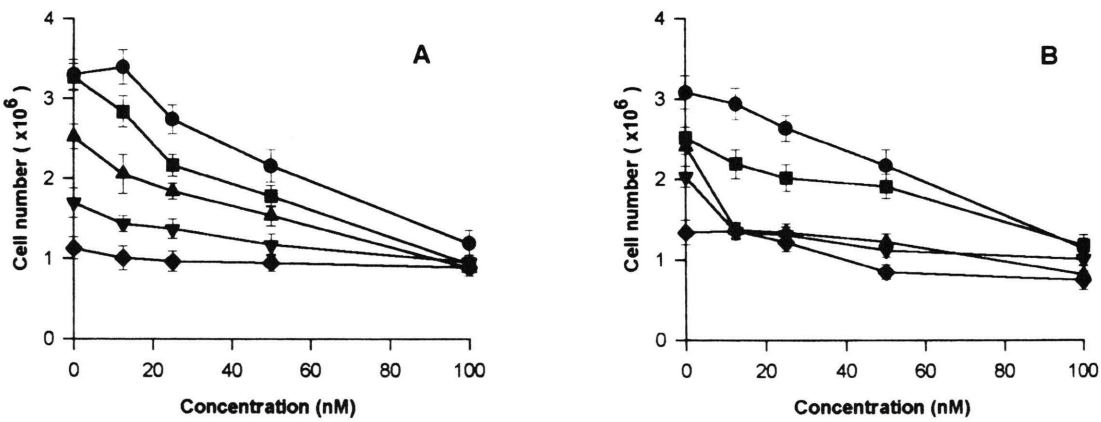

Fig. 2. The dependence of the amount of F4 N cells (10^6 cells/ml) on VM-26 concentration in the presence of various concentrations of cisplatin: (●) control, (■) 0.25 μM, (▲) 0.5 μM, (▼) 1 μM, and (◆) 2 μM. The drugs were added immediately (0 h) (**A**) or 4 h (**B**) after releasing of cells from a double thymidine block. Values are mean ± SD (N = 3 with 3 replicates per experiment).

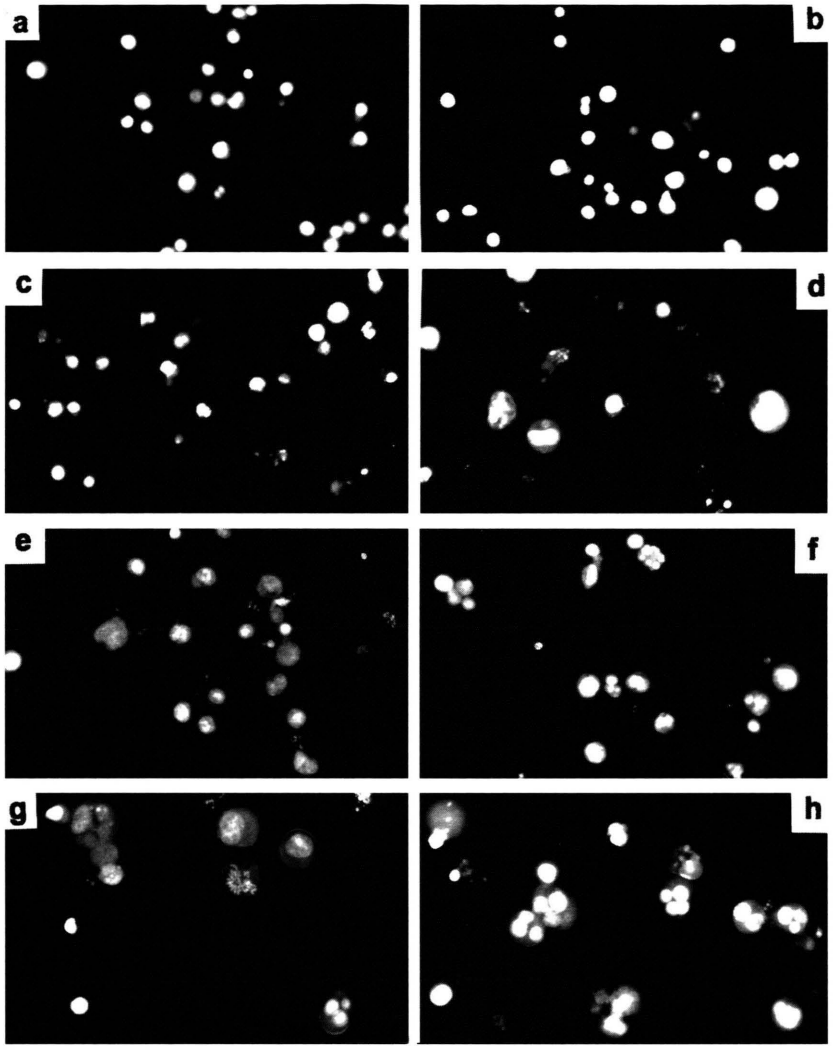

Fig. 3. Morphological analysis of treated F4 N cells. Cells were incubated for 72 h with (**b**) 30 mM azide, (**c,d**) 0.1 μM VM-26, (**e,f**) 1 μM cisplatin, and (**g,h**) 1 μM cisplatin plus 0.0125 μM VM-26; (**a**) control cells. The drugs were added immediately (0 h) (**a,c,e,g**) or 4 h (**b,d,f,h**) after releasing of cells from the double thymidine block. Cells were fixed and stained with propidium iodide (6 μg/ml) for 20 min.

for DDP, or 0.05 μM for VM-26). The treatment of cells with 0.1 μM VM-26 led to significant morphological changes, depending on the phase of the cell cycle. Thus, after treatment at the beginning of S-phase (panel **c**) about 20% of cells exhibited a morphology similar to that of control cells or were slightly enlarged. A minor part of the dead cells appeared to have small condensed nuclei pointing to a cell death through necrosis, similar to that upon azide treatment (panel **b**). In the majority amount of dead cells, however, fragmented nuclei were apparent, which is a characteristics of apoptosis. The small dimensions of the apoptotic cells indicated that these cells have developed apoptosis before entering into mitosis. On the other hand, when VM-26 was added in the middle of the S-phase, nearly all dead cells revealed signs of apoptosis (panel **d**). The remaining living cells proved to have increased diameters and in some of them a non-regular mitosis (karyokinesis) was observed: division of the nucleus without division of the cell (without cytokinesis).

After treatment of F4 N cells with 1 μM DDP, the majority of dead cells showed signs of apoptosis (fragmented nuclei). Following treatment at G_1/S, the viable cells (panel **e**) became larger and appeared to contain a homogeneously distributed chromatin. Most probably these cells are arrested in G_2-phase, as known for the effect of cisplatin (Gorczyca *et al.*, 1993; Vaisman *et al.*, 1997). A mitotic morphology was not detected. On the contrary, DDP addition in the middle of the S-phase (panel **f**) resulted in appearance of cells entering mitosis. In a few cells, mitosis proceeded without cytokinesis leading to the appearance of binuclear cells. Some of the cells, however, contained multiple partial nuclei of different size. Similar effects have been reported for cisplatin-treated Chinese hamster ovary cells (Rodilla, 1993).

A different pattern of cell morphologies was observed when cells were treated with 1 μM DDP in the presence of a very low concentration of VM-26 (0.0125 μM) (Fig. 3, panels **g** and **h**). At this concentration, VM-26 alone was not cytotoxic exerting only a weak growth-retardation effect (when added in the middle of S-phase), or having no influence on cell growth (when added at G_1/S boundary); in both cases no changes in cell morphology were observed. The combined treatment did not lead to changes in the morphology and in the amount of dead cells in comparison with those upon DDP addition only. However, a decrease in the number of viable cells and a drastic change of their morphology were observed. Many of the viable cells were greatly enlarged containing several nuclei (mostly 4). Some of them underwent aberrant/abortive mitosis. This effect was more expressed after treatment in the middle of the S-phase. Multinucleated cells are not viable, cannot propagate and subsequently die. The morphology of these cells differed from that of apoptotic cells which exhibited nuclei fragmented to small apoptotic bodies with a bright highly condensed chromatin.

It is known that topoisomerase II inhibitor VM-26, besides its ability to induce apoptosis via DNA breaks, induces polyploidization by preventing chromatid separation and uncoupling chromosome dynamics from other cell cycle events (Downes *et al.*, 1991; Clarke *et al.*, 1993). We observed the latter effect at concentrations toxic to VM-26. Interesting to note that we were able to register such activity at 10-fold lower VM-26 concentration when combined with cisplatin. There are no published data on the effect of cisplatin on chromosome segregation or mitosis. Most likely as a result of the combined action of cisplatin and VM-26, cells become supersensitive to the ability of topoisomerase II inhibitor to influence mitosis, and this sensitivity could be one of the mechanisms of synergism.

It seems that the cell suspension that we used is heterogeneous with respect to its sensitivity to cisplatin. Some of the cells became apoptotic after 72 h incubation in the presence of 1 μM cisplatin and contained apoptotic bodies, or became cell ghosts. The surviving cells appeared to exhibit a certain resistance to the induction of apoptosis by cisplatin, revealing at the same time super-sensitivity toward the ability of VM-26 to disturb mitosis. In agreement with this hypothesis, Minagawa *et al.* (1997) have recently reported synergism between DDP and VP-16 in KFr cells, a DDP-resistant subline of the KF epithelial ovarian carcinoma cell line, but not in the parent KF cells.

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