Alteration in p53 pathway and defect in apoptosis contribute independently to cisplatin-resistance

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Abstract

The accumulation of molecular genetic defects selected during the adaptation process in the development of cisplatin-resistance was studied using progressive cisplatin-resistant variants (L1210/DDP₂, L1210/DDP₅, L1210/DDP₁₀) derived from a murine leukemia cell line (L1210/0). Of these cell lines, only the most resistant L1210/DDP₁₀ was cross-resistant to etoposide and deficient in apoptosis induced by these two drugs, indicating that resistance to DNA-damaging agents correlates with a defect in apoptosis. This defect was tightly associated with the loss of a Ca²⁺/Mg²⁺-dependent nuclear endonuclease activity present in the less cisplatin-resistant cells. Evidence is presented that p53-dependent function (a) is lost not only in the apoptosis defective L1210/DDP₁₀ cells, but also in the apoptosis susceptible L1210/DDP₅ cells; (b) is unrelated to drug-induced cell cycle perturbations. These results suggest that deficiency in the p53 pathway and resistance to DNA-damaging agents due to a defect in apoptosis are independent events.

Keywords: cisplatin-resistance; apoptosis; endonuclease; p53; p21; L1210 leukemia cells

Abbreviations: cisplatin, cDDP, *cis*-diamminedichloroplatinum(II); VP16, etoposide; IC₅₀, concentration giving 50% cell survival; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; Ab, antibody; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; PI, propidium iodide; FITC, fluorescein isothiocyanate

Introduction

Although cisplatin is an important drug in the treatment of a variety of cancers (Loehrer and Einhorn, 1984), the rapid development of resistance to this drug frequently limits its clinical effectiveness. Cisplatin resistance is due to several

mechanisms (reviewed in Andrews and Howell, 1990) that all contribute to prevent the accumulation of cellular damage, i.e., decreased drug accumulation (Richon *et al*, 1987; Gately and Howell, 1993), increased intracellular thiols such as glutathione and metallothioneins (Richon *et al*, 1987; Hamilton *et al*, 1993), increased thymidilate synthase activity (Scanlon and Kashani-Sabet, 1988), reduction of DNA cross-linking as a consequence of decreased drug accessibility to DNA, and increased DNA repair (Hamilton *et al*, 1993; Eastman and Schulte, 1988; Scheibani *et al*, 1989; Lai *et al*, 1988).

The cancer cell response following the interaction of a cytotoxic agent with DNA can also play a significant role in cytotoxicity. Since cisplatin has been shown to induce programmed cell death or apoptosis (Barry *et al*, 1990; Sorenson *et al*, 1990; Ormerod *et al*, 1994), alterations in the pathway that leads from cisplatin-induced-DNA damage to apoptosis could also contribute to drug resistance. We have previously shown that, in a L1210 murine leukemia cell line, the selection for cisplatin-resistance resulted in the selection of a defective apoptotic process (Ségal-Bendirdjian and Jacquemin-Sablon, 1995).

The biochemical pathways that lead to cell death by apoptosis are complex and depend upon the cell type. To date, the principal feature that has been characterized is the non-random cleavage of DNA, a process thought to be associated with one of the most highly conserved morphological features of apoptosis, i.e., the condensation of chromatin. In most cases, DNA cleavage in cells undergoing apoptosis is mediated by the activity of (an) endonuclease(s), yielding internucleosomal integer DNA fragments of approximately 180–200 base pairs (Arends *et al*, 1990).

Apoptosis is regulated by a network of genes whose connection to cell cycle genes has not yet been fully elucidated (Hoffman and Liebermann, 1994; Oltvai and Korsmeyer, 1994). Among them, the p53 tumor suppressor gene is now widely recognized as a transducer of genome damage into growth arrest and/or apoptosis (Nelson and Kastan, 1994; Oren, 1994). Levels of p53 rapidly increase following DNA damage mainly because the normally short-lived p53 protein is stabilized (Kastan *et al*, 1991). Activation of the p53 protein can affect cell fate through the induction of either growth arrest at G₁/S or G₂/M cell cycle checkpoints or apoptotic cell death (Kuerbitz *et al*, 1992; Stewart *et al*, 1995; Agarwal *et al*, 1995). This G₁ arrest pathway involves p53-dependent transcriptional activation of the gene encoding the cyclin-dependent kinase inhibitor p21^{WAF1/Cip1} (p21) (El-Deiry *et al*, 1994). Tumor cells that have lost functional p53 exhibit resistance to induction of apoptosis by a range of genotoxic agents, including anticancer drugs and radiation (Livingstone *et al*, 1992; Clarke *et al*, 1993; Lowe *et al*, 1993a,b; Brown *et al*, 1993). In addition, these cells are frequently resistant to multiple chemotherapeutic agents (Fan *et al*, 1994), even though this has not been universally found (Biard *et al*,

1994; Hawkins *et al*, 1996). Although at this time the precise mechanism of apoptosis has not been fully elucidated, the regulation of the expression of the *p53* gene appears to be a major factor influencing the cell response to a number of chemotherapeutic agents.

A panel of sublines (L1210/DDP₂, L1210/DDP₅ and L1210/DDP₁₀), derived from a L1210/0 parental cell line by progressive adaptation to increasing doses of cisplatin and therefore representative of different stages in the development of cisplatin-resistance, has been previously characterized (Eastman and Schulte, 1988; Scheibani *et al*, 1989). These sublines presented mechanisms already known to be involved in the resistance to this drug including: a slightly reduced drug accumulation, a moderate increase of glutathione levels and an enhanced DNA repair. However, the extent of those mechanisms, observed as early as the second step of selection, did not correlate with the degree of resistance suggesting that additional defects have to be considered in the resistance development. Therefore, we analyzed the relationships between the development of cisplatin-resistance, the susceptibility to drug-induced apoptosis and the function of the *p53* transduction pathway in these cisplatin-resistance variants. We first assessed the ability of each cell line to undergo apoptosis after exposure to various cytotoxic agents, and to express modifications in the level of *p53* and *p21* upon drug exposure. Given the role of *p53* and *p21* in the control of the cell cycle, we finally investigated whether alterations in the cell-cycle checkpoints could be responsible for the phenotype of each cell line. We present evidence that although the selection of these cisplatin-resistant L1210 variants is associated with an altered *p53* expression, this defect does not determine their ability to undergo apoptosis.

Results

In a previous study (Ségal-Bendirdjian and Jacquemin-Sablon, 1995), we have shown that cisplatin-resistant L1210/DDP₁₀ cells, derived from the sensitive murine leukemia cell line L1210/0, were cross-resistant to 5-azacytidine, and failed to undergo typical apoptosis (defined by the oligonucleosomal fragmentation of the DNA and the morphological alterations detected by electron and confocal microscopy) after exposure to toxic doses of either drug. This failure was correlated with a loss of nuclear endonuclease activity which was present in the wild-type cell nuclei. However, the L1210/DDP₁₀ cells remained sensitive to staurosporine, a potent inhibitor of protein kinase C, which induced apoptosis in both cell lines. The induction of this pathway was associated with the presence of a cytoplasmic endonuclease whose specificity was different from that operating in the L1210/0 cells.

Cross-resistance pattern and apoptosis induction

In order to analyze the relationships between the development of cisplatin resistance and susceptibility to drug-induced apoptosis, we extend here our previous studies to a series of cisplatin resistant cell lines (including L1210/DDP₂ and

Table 1 Cytotoxicity of cisplatin, etoposide and staurosporine on the parental L1210/0 and the resistant sublines

Cell line	IC ₅₀ ^a (μ M)	cisplatin (μ M)	etoposide (μ M)	staurosporine (nM)
L1210/0	0.3 $\pm$ 0.02	0.08 $\pm$ 0.01	13.8 $\pm$ 1.0	
L1210/DDP ₂	7.3 $\pm$ 1.0	0.07 $\pm$ 0.01	12.0 $\pm$ 1.0	
L1210/DDP ₅	20.0 $\pm$ 2.0	0.1 $\pm$ 0.01	17.2 $\pm$ 2.0	
L1210/DDP ₁₀	50.0 $\pm$ 5.0	0.3 $\pm$ 0.03	18.0 $\pm$ 2.0	

^aIC₅₀ (Inhibitory Concentration 50%) values ($\pm$ S.D.) were estimated from MTT cytotoxicity assays after a 72 h exposure to the drug and represent mean values of at least three independent experiments

L1210/DDP₅), which were isolated at intermediate steps during the selection of the most resistant L1210/DDP₁₀ cells. In addition, we analyze the sensitivity of all the cells to another DNA damaging agent, etoposide, which is known as a topoisomerase II inhibitor.

Toxicity of cisplatin, etoposide and staurosporine on the parental L1210 cell line and cisplatin-resistant sublines was evaluated after a 72 h exposure to each drug by performing either growth inhibition assay or MMT assay (Table 1). This experiment shows that a high degree of cisplatin-resistance (24-fold) was gained during the first selection process (L1210/DDP₂) and each additional subclone represented a 2.5-fold increase in resistance as compared to the previous one. These increases led to a 66- and 166-fold increase in resistance to cisplatin, compared to the parental cell line, in the second and third step of selection, respectively. In the course of the last step of selection, L1210/DDP₁₀ cells acquired also a threefold increase in resistance to etoposide. However, all the cell lines were equally susceptible to staurosporine.

The ability of L1210/0 parental and cisplatin-resistant sublines to undergo apoptosis was evaluated by DNA fragmentation analysis in cells exposed for 72 h to doses of cisplatin that were known to induce more than 50% of growth inhibition in each cell line. As shown in Figure 1, DNA cleavage with a characteristic pattern of internucleosomal ladder (multiples of approximately 180-base pair fragments) was observed in L1210/0, L1210/DDP₂ and L1210/DDP₅ but not in the L1210/DDP₁₀. We have previously shown that this failure of DNA fragmentation in the L1210/DDP₁₀ cells was correlated with the absence of morphological alterations typical of apoptosis (Ségal-Bendirdjian and Jacquemin-Sablon, 1995). No fragmentation was observed after exposure of the L1210/DDP₁₀ cells to equitoxic doses of etoposide. However, staurosporine induced DNA fragmentation in the four cell lines. Thus, the acquisition of etoposide-resistance during the last step of selection for cisplatin-resistance parallels the inability of the L1210/DDP₁₀ cells to undergo apoptotic DNA fragmentation in the presence of these drugs.

AnnexinV staining

As analysis of DNA fragmentation on gel electrophoresis did not provide a suitable quantitative method of apoptosis, a quantitative analysis of apoptosis in the progressively resistant variants was performed using AnnexinV staining. This

technique offers the possibility of detecting early phases of apoptosis before the loss of cell membrane integrity (Vermes *et al*, 1995). Figure 2 shows a significant increase in AnnexinV staining in L1210/0, L1210/DDP₂ and L1210/DDP₅ cells treated with equitoxic doses of cisplatin (+35.8%, +26.7% and +37.2% respectively). In contrast, cisplatin-treated L1210/DDP₁₀ cells failed to show any similar increase in AnnexinV-FITC binding at either 50 μ M (+4%) or 66.7 μ M (9%, data not shown). However, staurosporine induced a significant AnnexinV-FITC binding in the four cell lines. This demonstrates a

close correlation between DNA fragmentation and AnnexinV binding. In addition, these results show the extent of cisplatin-induced apoptosis is not reduced in the resistant L1210/DDP₂ and L1210/DDP₅ cells relative to the parental cells.

Nuclear endonuclease activity

We have previously shown that a $\text{Ca}^{2+}/\text{Mg}^{2+}$ endonuclease present in the nuclei of the parental L1210/0 cells was lost in L1210/DDP₁₀ nuclei and we have suggested that this defect

Figure 1 Analysis of drug-induced internucleosomal DNA cleavage in wild-type L1210 and mutant cells. Cells were incubated for 72 h in the presence of equitoxic concentrations of cisplatin (p), etoposide (VP16) (e), or staurosporine (s). The DNAs were then extracted and separated on a 2% agarose gel. p1: 0.6 μ M cDDP; p2: 2.5 μ M cDDP; p3: 16.6 μ M cDDP; p4: 33.3 μ M cDDP; p5: 66.7 μ M cDDP; e1: 0.25 μ M etoposide; e2: 0.5 μ M etoposide; e3: 1.0 μ M etoposide; s1: 30 nM staurosporine; s2: 40 nM staurosporine; nt: non treated cells; m: 123-base pair ladder marker

Figure 2 AnnexinV staining of control and drug-treated cells. Cells were incubated for 72 h in the presence of cisplatin (cisPt) or staurosporine (STP). After treatment, cells were labeled with AnnexinV-FITC and analyzed by fluorescence-activated cell sorter. The percentages in each panel represent the number of cells exhibiting increased AnnexinV binding. 10 000 cells were analyzed under each condition

could be responsible for the failure of these cells to undergo cisplatin-induced apoptosis (Ségat-Bendirdjian and Jacquemin-Sablon, 1995). This hypothesis would predict that the endonuclease should be present in the nuclei of L1210/DDP₂ and L1210/DDP₅ mutant cells that retain the capacity to undergo cisplatin-induced apoptotic DNA fragmentation. This was directly tested by using a cell free assay as previously described (Ségat-Bendirdjian and Jacquemin-Sablon, 1995). Figure 3 (lane b) shows that incubation of L1210/0, L1210/DDP₂ and L1210/DDP₅ nuclei with 5 mM CaCl₂ and 10 mM MgCl₂ resulted in an extensive internucleosomal DNA fragmentation similar to that observed in cisplatin-treated cells. In contrast, incubation of isolated L1210/DDP₁₀ nuclei under the same conditions did not yield any DNA fragmentation. Therefore, the lack of DNA fragmentation in L1210/DDP₁₀ cells exposed to cisplatin or etoposide correlates with the loss of a nuclear endonuclease.

Analysis of p53 and p21

Alterations in p53 function have been shown to prevent DNA damage-induced apoptosis and to change the chemosensitivity in various cellular systems. Since these characteristics were observed in the L1210/DDP₁₀ cells, we analyzed the expression and the function of p53 in the resistant and sensitive cells. Protein extracts were prepared from each cell

line treated by either cisplatin, etoposide or staurosporine and analyzed for p53 and p21 expression.

Western blots analysis of p53 protein are shown in Figure 4. A low level of p53 was detected in the extracts from L1210/0 wild-type cells. This level began to increase after a 24 h exposure of these cells to staurosporine. This accumulation was more pronounced after a 48 h exposure. A similar accumulation was observed after etoposide or cisplatin treatment of the L1210/0 cells. As shown in Figure 5, an accumulation of p21 was also seen after exposure of this cell line to each drug.

A low level of p53 was also detected in L1210/DDP₂ mutant cells (Figure 4). As in wild-type cells, but to a lesser extent, p53 level was increased upon exposure to either staurosporine or etoposide. Interestingly, p53 level did not increase upon exposure to cisplatin doses equitoxic to those that increased p53 level in the parental cells. The accumulation of p21 paralleled that of p53 (Figure 5): it increased in the L1210/DDP₂ cells exposed to either staurosporine or etoposide but not in cells exposed to cisplatin. Thus, L1210/DDP₂ cells have retained, at least qualitatively, a wild-type response of p53 and p21 to staurosporine or etoposide, but not to cisplatin treatment.

In L1210/DDP₅ and L1210/DDP₁₀ cells, p53 and p21 were not detectable whatever the treatment of these cells (Figures 4 and 5).

Cell cycle analysis of cells exposed to cisplatin or staurosporine

p53 and p21 play a key role in the control of the G₁ to S-phase progression in response to DNA damage. Having observed that, in this cellular system, an alteration in p53 function was selected in parallel to the acquisition of a high level of resistance to cisplatin, we then analyzed the cell cycle distribution of the four cell lines after different times of exposure to cisplatin (Table 2).

After 16 h of exposure to equitoxic concentrations of cisplatin, a marked decrease of the G₁ population in advantage of G₂ population was observed in the sensitive L1210/0 and the three resistant L1210/DDP₂, L1210/DDP₅ and L1210/DDP₁₀ cells. This accumulation in G₂ phase of the cell cycle was inversely related to cisplatin sensitivity of the cell lines: the proportion of L1210/0, L1210/DDP₂, L1210/DDP₅ and L1210/DDP₁₀ cells present in G₂/M was 45%, 35%, 11% and 7% respectively. Moreover, the accumulation of cells in G₂/M was delayed in the resistant cell lines and this delay increased with the level of resistance. The accumulation of cells in G₂/M started after 16 h in the L1210/0 and in the L1210/DDP₂ cells compared to 24–31 h in the L1210/DDP₅ and in the L1210/DDP₁₀ cells. After longer treatment times, L1210/DDP₁₀ cells achieved a maximal arrest in G₂/M (64%) but apoptosis still did not occur (data not shown).

Exposure of L1210/0 cells to toxic doses of staurosporine resulted in a rapid cell arrest in G₂ phase (Table 3). Within 6 h of exposure of L1210/0 cells to 30 nM staurosporine, the fraction of G₁ phase cells dropped from 33.3% in control culture to 2%, while the proportion of cells with a DNA content equivalent to G₂/M increased from 9.7

Figure 3 Analysis of endonuclease activity in the nuclei of L1210/0, L1210/DDP₂, L1210/DDP₅ and L1210/DDP₁₀ cells. Isolated nuclei (4×10^6) from each cell line were incubated at pH 7.0 in the presence of 5 mM CaCl₂ and 10 mM MgCl₂ (lanes b and c) in the absence (lane b) or in the presence of 25 mM EDTA (lane c). DNAs were extracted after a 2 h incubation at 37°C, separated on a 2% agarose gel, and stained by ethidium bromide. Lane (a) contained control nuclei. A 123-base pair ladder marker (lane m) was included as molecular size markers

Figure 4 Detection of p53 protein in control, and drug-treated cells. Cells were treated with cisplatin, etoposide, or staurosporine for 24 or 48 h. After drug treatment, cell extracts were prepared and analyzed by Western blot using p53 specific antibodies. The identity of p53 was confirmed by simultaneous electrophoresis of molecular weight standards and an extract from 3T3 murine cells as a positive control

to 48.3%. At this point, the cell cycle distribution of staurosporine-treated cells was similar to that produced by agents (such as cisplatin) which cause cells to accumulate in G₂ phase. However, after a 16 h exposure to staurosporine, a fraction of the cells was observed to reside in S-phase but of a higher DNA ploidy (ST). These data suggest that, as already shown in Molt-4 cells (Traganos *et al*, 1994), in the continuous presence of staurosporine, some cells progressed through G₂, and instead of dividing, reentered the cycle at a tetraploid DNA level. The response of the L1210/DDP₂ and L1210 DDP₅ cells to staurosporine was very similar to that observed in the parental L1210/0 cells.

Exposure of L1210/DDP₁₀ cells to equitoxic doses of staurosporine (30 nM) for periods up to 8 h, resulted in cell accumulation in G₁ and G₂ phase without appearance of cells with more DNA than a G₂ content, even after a 72 h exposure. The possibility that these cells with a G₂/M DNA content were actually cells arrested in a G₁ phase of a

tetraploid cell cycle (G₁T) was considered and analyzed by flow cytometry using cyclin B₁ antibody. Cyclin B₁ is a marker of the G₂ phase and can be used in the discrimination of G₂ cells of lower DNA ploidy (high level of cyclin B₁) from G₁ cells with a 4C DNA content (low level of cyclin B₁) (Traganos *et al*, 1994).

L1210/0 and L1210/DDP₁₀ were treated with equitoxic doses of staurosporine or cisplatin and stained for DNA content by propidium iodide and for cyclin B₁ by FITC-antibody (Figure 6). Exposure of both cell lines to cisplatin resulted in an accumulation of cells with a G₂/M DNA content that strongly expressed cyclin B₁. These cells were accumulated in a true G₂/M phase. In cells exposed to staurosporine, this increase of cyclin B₁ was only transient, indicating that cells were accumulated transiently in G₂/M phase. Later a subpopulation of cells that did not express cyclin B₁ became prominent. This population of cells with a G₂/M DNA content that do not express cyclin B₁ represents tetraploid G₁/T cells.

Taken together, these cell cycle analyses indicate that in the presence of staurosporine, L1210/0, L1210/DDP₂ and L1210/DDP₅ cells can enter and progress into a tetraploid cell cycle while L1210/DDP₁₀ cells accumulate in a diploid and tetraploid G₁ phase.

Discussion

A variety of mechanisms of resistance to cisplatin have been identified in cell lines selected *in vitro* (Richon *et al*, 1987; Eastman and Schulte, 1988; Scheibani *et al*, 1989).

Figure 5 Detection of p21 protein in control, and drug-treated cells. Cells were treated as in Figure 3. The cell extracts prepared after 48 h of treatment were analyzed by Western blot using a p21 specific antibody. The identity of the p21 protein was confirmed by simultaneous electrophoresis of molecular weight standards

Table 2 Analysis of cell cycle distribution after cisplatin treatment of sensitive or resistant L1210 cells

Cells	Concentration (μM) ^a		Time of treatment							
			0 h	6 h	11 h	16 h	24 h	31 h	48 h	72 h
L/0	0.67	G1	32	31.5	27.7	5.7	6.1	2.7	— ^b	— ^b
		S	60.5	50.7	66.4	49.4	39.9	35	— ^b	— ^b
		G2/M	7.5	17.7	5.9	44.9	54.0	62.3	— ^b	— ^b
L/DDP ₂	20	G1	58.1	57.1	47	9.1	4.4	0.4	0.2	— ^b
		S	37.1	35.1	49.6	55.8	41.2	42.1	21.8	— ^b
		G2/M	4.8	7.8	3.4	35.1	54.4	57.6	78	— ^b
L/DDP ₅	50	G1	51.4	50.1	17.4	7.6	1	0.9	9.7	— ^b
		S	40.9	42.3	73.5	81.9	68.6	49.4	27.9	— ^b
		G2/M	7.7	7.6	9.1	10.5	30.4	49.7	62.4	— ^b
L/DDP ₁₀	66.7	G1	59.2	56.3	52.1	30.3	23.1	11.7	7.4	17.7
		S	34.3	35.7	39.5	69.8	58.4	59.3	34.8	36
		G2/M	6.6	8	8.4	6.9	18.5	29	57.7	46.3

^aCells were treated with equitoxic doses of cisplatin. ^bCell cycle analysis was not possible at these times because of the too high cytotoxicity

Most of them, directly or not, prevent the accumulation of DNA damage. Recent evidence suggests that most anti-cancer drugs, including cisplatin, exert their cytotoxic effects

through the induction of apoptosis. Since different DNA-damaging agents may use a common pathway to kill tumor cells, the inability to activate the apoptotic program has

Table 3 Analysis of cell cycle distribution after staurosporine treatment of sensitive or resistant L1210 cells

Cells	Concentration		Time of treatment							
	(nM) ^a		0 h	6 h	12 h	16 h	24 h	36 h	48 h	72 h
L/0	30	G1	33.3	2	18.2	15.5	28.7	27.9	28	26.6
		S	57.1	49.7	8.6	12.4	10.9	11.4	12.7	17.9
		G2M/G1T	9.7	48.3	73.2	54.3	39.7	38	40	35.7
		ST	0	0	0	11.3	10.6	6.6	6.3	7.4
		G2MT	0	0	0	6.7	9	15	12.4	12.3
L/DDP ₂	20	G1	51.5	26.2	32.7	24	29.8	37.5	37.3	29.8
		S	42.5	44	9.1	7.2	4.5	6.4	5.5	16.7
		G2M/G1T	6	29.8	58.2	68.7	61	51.9	54	47.8
		ST	0	0	0	0	2.2	1.9	1.2	1.7
		G2MT	0	0	0	0	2.2	2.1	1.7	2.4
L/DDP ₅	40	G1	48.3	23.6	9.6	7	6.2	7.6	— ^b	— ^b
		S	44.3	51	19	6.3	4.2	4.6	— ^b	— ^b
		G2M/G1T	7.5	25.4	71.4	86.7	63.3	58.3	— ^b	— ^b
		ST	0	0	0	0	18.3	10.8	— ^b	— ^b
		G2MT	0	0	0	0	7.9	18.6	— ^b	— ^b
L/DDP ₁₀	30	G1	56.8	38	25.7	48.8	33	34.4	32	25.1
		S	37.2	36.3	24.5	9.5	10.8	9.6	4.7	11.3
		G2M/G1T	6	25.7	49.8	41.6	56.2	56	63.3	63.5
		ST	0	0	0	0	0	0	0	0
		G2MT	0	0	0	0	0	0	0	0

^aCells were treated with equitoxic doses of staurosporine. ^bCell cycle analysis was not possible at those times because of the too high cytotoxicity

Figure 6 Expression of cyclin B₁ in cells exposed to cisplatin or staurosporine. Exponentially growing L1210/0 and L1210/DDP₁₀ cells were treated with 0.67 μ M and 66.7 μ M cisplatin (+cDDP), respectively, or 30 nM staurosporine (+STP), respectively, for 0 (untreated), 8, 24, 48 and 72 h and then stained with monoclonal antibodies to cyclin B₁ and PI. The results were plotted in the form of contour maps as a function of DNA content. The DNA distribution of the untreated cells is displayed above the contour map. The G₁, S and G₂ phases of the diploid cell cycle are indicated. The arrow shows the accumulation of G₁ cells with a 4C DNA content and a low level of cyclin B₁ (tetraploid G₁ phase; G₁T). Cell nb: cell number

been proposed as a mechanism of resistance to unrelated antineoplastic agents (Dive and Hickman, 1991; Eliopoulos *et al*, 1995; Perego *et al*, 1996). In agreement with this hypothesis, we have previously shown that a murine leukemia cell line selected for cisplatin-resistance (L1210/DDP₁₀) was defective in an apoptosis pathway, which involved a Ca²⁺/Mg²⁺ nuclear endonuclease activity required for the DNA fragmentation (Ségat-Bendirdjian and Jacquemin-Sablon, 1995). However, this cell line was isolated from the parental cell line (L1210/0) through multiple steps of selection and therefore was more likely to accumulate multiple genetic alterations.

In the present study, we also examined two other sublines (L1210/DDP₂ and L1210/DDP₅) which are intermediate variants in the progressive adaptation process used in the development of the most resistant L1210/DDP₁₀ line. In contrast with most studies investigating events associated with the occurrence of cisplatin resistance in only pairs of sensitive and resistant cell lines, the panel of sublines used in this study, with various degrees of cisplatin resistance, provide a unique cellular system allowing a reconstitution of the mechanisms that have occurred to lead to full cisplatin resistance.

The progressive accumulation of genetic defects which were selected during the adaptation process used in the development of the resistant L1210/DDP₁₀ cell line is summarized in Figure 7. In addition to mechanisms already known to be responsible for cisplatin resistance that were selected in the first step of selection (L1210/DDP₂), an alteration in p53 dependent functions were selected during the second step (L1210/DDP₅). Finally, a loss of a nuclear endonuclease activity was generated in the third step of the selection process (L1210/DDP₁₀). This last defect appears involved in the failure of apoptosis observed in the most resistant L1210/DDP₁₀ cells.

Figure 7 Accumulation of genetic defects during the multistep acquisition of cisplatin resistance. Mechanisms already known to be involved in the cisplatin-resistance were selected in the first step of selection for cisplatin resistance (L1210/DDP₂). Then, a defect in p53-dependent functions was selected during the second step (L1210/DDP₅). Finally, an alteration in an endonuclease activity is generated in the third step of the selection (L1210/DDP₁₀). This last defect may be involved in the failure of apoptosis observed in the L1210/DDP₁₀ cells

Role of apoptosis in drug-resistance

We first analyzed the link between the alteration of the apoptotic process and the occurrence of resistance to unrelated drugs. We observed that a threefold resistance for etoposide, as well as a similar increase in cisplatin resistance, was gained during the third step of the selection (L1210/DDP₁₀ cells). This property was associated with the loss of the capacity of these cells to undergo apoptosis after exposure to each of these drugs. In marked contrast, the L1210/DDP₁₀ cells were not resistant to staurosporine and underwent apoptosis when exposed to this drug. Thus, in this cell line, the resistance to DNA damaging agents, like cisplatin, etoposide and, as previously shown, 5-azacytidine (Ségat-Bendirdjian and Jacquemin-Sablon, 1995) correlates with a defect in apoptosis induction by these drugs. Analyzing the biochemical basis of this alteration, we found that a Ca²⁺/Mg²⁺ nuclear endonuclease, previously shown to be lost in L1210/DDP₁₀ cells, was present in nuclei isolated from both L1210/DDP₂ and L1210/DDP₅ cells. Therefore, this endonuclease activity was lost in the same selection step that resulted in full resistance of cells to apoptosis after exposure to DNA damaging agents. This suggests a strong association between the two events. The apoptotic process triggered by staurosporine was previously associated with the presence of a cytoplasmic endonuclease activity (Ségat-Bendirdjian and Jacquemin-Sablon, 1995).

Role of p53 in drug-resistance and in drug-induced apoptosis

p53 is recognized as an important component of the pathway leading from DNA damage to apoptosis and as a determinant in cellular chemosensitivity. The series of cisplatin-resistant variants allowed us to investigate the contribution of p53 in both the acquisition of drug resistance and the susceptibility to drug-induced apoptosis.

In the parental cells, etoposide or cisplatin treatment induced an accumulation of the p53 protein associated with an increased expression of p21, known as an inducible gene in cells expressing function p53. These results could suggest that drug-induced apoptosis is p53-mediated, in this cell line. Nevertheless, if there were a direct relationship between p53 expression and induction of apoptosis, one would expect to see a similar increase in p53 level in both L1210/DDP₂ and L1210/DDP₅ cells, which achieve apoptosis after treatment with these two drugs, and no increase in L1210/DDP₁₀ cells, which are deficient in apoptosis induced by the same compounds. Actually, L1210/DDP₁₀ resistant cells are deficient in p53 functions. However, this defect is also observed in the L1210/DDP₅ resistant cell line, which are proficient in apoptosis. Moreover, staurosporine, which activates p53 in the wild-type L1210/0 cells, induces apoptosis in the L1210/DDP₁₀ cells, although they are clearly deficient in p53-dependent functions. Interestingly, staurosporine and etoposide, but not cisplatin, activate p53-dependent function in L1210/DDP₂ cells. Thus, in this cell line, p53 pathway is still functional. The lack of p53 accumulation upon cisplatin exposure could likely be explained by the increase in DNA

repair already shown in this cell line (Eastman and Schulte, 1988; Scheibani *et al*, 1989) that would prevent the signal leading to the accumulation of the p53 protein. Nevertheless, in this cell line, either etoposide or cisplatin, which respectively activate or not p53-dependent functions, induce apoptosis. Although, there is a strong correlation between the accumulation of p53 and the accumulation of p21, these experiments do not exclude the possibility that during the selection procedure, L1210/DDP₅ or L1210/DDP₁₀ acquired multiple genetic lesions, one of them being the inactivation of p21 gene. We also cannot formally rule out the possibility that loss of p53 function reduced the extent of apoptosis in the cisplatin-treated L1210/DDP₅ cells relative to the less resistant variants. However, AnnexinV staining analysis shows that a same level of apoptosis can be achieved in cisplatin-treated L1210/0, L1210/DDP₂ and L1210/DDP₅ cells, demonstrating that loss of p53 functions fails to completely eliminate apoptosis. Thus, even if the alteration of p53 pathway may contribute to cisplatin-resistance, this defect is not sufficient by itself to prevent cisplatin-induced apoptosis. An additional mutation is in fact required to prevent apoptosis induced by cisplatin in the L1210/DDP₁₀ cells. Furthermore, these results demonstrate that p53 and p21 expression are neither necessary, nor sufficient, for apoptosis induction in these resistant cells.

This conclusion is in agreement with a previous work showing that other cell lines (e.g., SK-OV-3, a human ovarian cancer line) that are p53 null, still undergo death by apoptosis following exposure to cisplatin (Ormerod *et al*, 1996). Moreover, the loss of p53 function in L1210/DDP₅ cells did not affect the sensitivity of these cells to etoposide suggesting that p53 is not involved in etoposide resistance. The extent to which loss of p53-dependent function contributes to cisplatin-resistance in the various L1210 resistant cells is not clear. Either the deficiency in p53 pathway could directly contribute to cisplatin-resistance function by giving a selected property for growth, explaining why this alteration was actually selected, or it could be the result of random cisplatin-induced mutagenesis. The first hypothesis would be in agreement with recent studies (Griffiths *et al*, 1997) showing that lack of p53 function could promote the survival and proliferation of mutant cells after genotoxic treatments. One possible effect of the alteration in p53 functions in the L1210/DDP₅ cells is an increase in genomic instability leading to the appearance of further oncogenic lesions that could affect the apoptotic pathways by additional mechanisms.

Role of p53 in drug-induced cell cycle perturbations

Cell cycle analysis revealed that, in both the parental and resistant cell lines, cisplatin induced a G2/M arrest and staurosporine induced a strong G1 arrest in either a diploid or a tetraploid cell cycle. However, neither p53 nor p21 were involved in cell cycle perturbations since the different sublines exhibited the same cycle arrests, irrespective of the status of p53 or p21. In addition, L1210/DDP₁₀ cells failed to enter into a tetraploid cell cycle upon staurosporine treatment. These observations suggest that some specific cell cycle perturba-

tions could have some importance in either resistance or apoptosis.

Taken together, these studies have shown the successive accumulation of genetic defects that occurred during the selection process for resistance to cisplatin. The importance to use progressively resistant variants to analyze the molecular events involved in drug resistance and/or apoptosis was underlined. Indeed, we could demonstrate a strong association between a nuclear endonuclease activity and apoptosis susceptibility. In addition, it was possible to show that the resistance to DNA damaging agents, which is gained through a defect of apoptosis, and the deficiency in the p53 pathway, acquired during a selection in the presence of cisplatin, are independent events.

Materials and Methods

Chemicals

Cisplatin was a gift from R. Bellon Cie (France). Staurosporine and etoposide were purchased from Sigma Chemical Corp. (St Louis, MO) and Boehringer Mannheim (Meylan, France) respectively. Stock solutions were prepared as follows: 1.67 mM cisplatin in NaCl 0.15 M, 2.1 mM staurosporine in DMSO, 10 mM etoposide in DMSO. All agents were stored at -20°C after dissolution. MTT was obtained from Sigma Chemical Corp. Agarose electrophoresis grade was from Pharmacia (Biotech, Orsay, France). Proteinase K and DNase-free RNase were obtained from Boehringer Mannheim, and the DNA molecular weight marker (123 base-pair ladder) from Gibco BRL (Cergy-Pontoise, France).

Cell lines and culture conditions

L1210 murine leukemia cells (L1210/0 sensitive and L1210/DDP₂, L1210/DDP₅ and L1210/DDP₁₀ cisplatin-resistant cells) kindly provided by Dr A Eastman (Department of Pharmacology, Dartmouth Medical School, Hanover, NH, USA) have been previously described (Eastman and Schulte, 1988; Scheibani *et al*, 1989; Lai *et al*, 1988). The resistant cell lines were selected by continuous exposure of the L1210/0 cell line to increasing concentrations of cisplatin (6.6, 16.6 and 33.3 µM) for L1210/DDP₂, L1210/DDP₅ and L1210/DDP₁₀, respectively. They were cultured in drug-free medium. All cell lines were grown in suspension in Dulbecco minimal essential medium (DMEM, Eurobio, France) supplemented with 15% foetal calf serum (Eurobio), 2 mM glutamine and antibiotics (streptomycin, 200 µg/ml; penicillin, 200 U/ml). The cells were grown in a humidified 5% CO₂ atmosphere.

Drug exposure and cytotoxicity evaluation

Exponentially growing cells were treated with various drug concentrations for 3 days. Cell numbers were determined with a Coulter counter and the fraction of viable cells was assayed by Trypan blue dye exclusion assay. The IC₅₀ (Inhibitory Concentration 50%) values correspond to the drug concentrations that inhibit cell growth by 50%.

Drug-induced cytotoxicity was also estimated using the MTT colorimetric assay. In brief, 1 ml of exponentially growing cells were incubated in a 24 well plate in the presence of various concentrations of drug. Seventy-two hours later, 100 µl of 5 mg/ml MTT in PBS were added in each well, incubated for 4 h at 37°C and the formazan

crystals were dissolved in 10% SDS, 10 mM HCl. The optical density was recorded at 550 nm. The IC₅₀ values were the drug concentration inducing 50% reduction in the optical density.

Analysis of DNA fragmentation

DNA was isolated from 2×10^6 cells by the salting out procedure described by Miller *et al* (1988) modified as previously described (Ségat-Bendirdjian and Jacquemin-Sablon, 1995).

AnnexinV binding analysis

Staining for AnnexinV-FITC binding was performed using AnnexinV-FITC kit (Boehringer Ingelheim, Bender MedSystem). After washing once in PBS, the cultured (7×10^5 cells) were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). AnnexinV-FITC was added to a final concentration of 1 µg/ml and the cells were incubated at room temperature in the dark for 15 min. The cells were then washed again in binding buffer, centrifuged and resuspended in the same buffer. The cells were analyzed with a Coulter Epics-Profile II flow cytometer. Histograms of the change of the mean fluorescence intensity of the Annexin-FITC in control and treated cells were generated.

Cell cycle analysis

Cell cycle distribution was assessed by flow cytometry. Cells were fixed for at least 14 h, in 80% ice-cold methanol at -20°C . Fixed cells were washed once in PBS and resuspended in PBS containing 10 µg/ml propidium iodide and 250 µg/ml RNase A for 30 min. Nuclear fluorescence was measured by quantitative flow cytometry and profiles were generated on a Coulter Epics-Profile II analyser.

Western analysis

Cell protein extracts were prepared by lysing cells in 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, and 50 mM Tris-HCl (pH 7.4) for 30 min at 4°C . Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent. For p53 detection, samples (80 µg/lane) were fractionated on a 10% SDS-PAGE in a Tris-Glycine running buffer and blotted on nitrocellulose sheets (Schleicher and Schuell). The loading homogeneity and transfer efficiency were checked by staining the membrane with red Ponceau and the gel with Coomassie blue. Blots were preblocked for 4 h at room temperature in PBS containing 0.1% Tween 20 (v/v) and 5% dried nonfat milk (w/v). Filters were incubated overnight at 4°C with a mixture of Ab-1 and Ab-3 antibodies to human p53 (Oncogene Science, Genzyme SA, Cergy St Christophe, France). For p21 detection, samples (40 µg/lane) were fractionated on 15% SDS-PAGE, and blotted on polyvinylidene fluoride membranes (Boehringer Mannheim). Blots were treated as described above and reached with a polyclonal antibody to human p21 (Santa Cruz Biotechnology Inc, Tebu, Le Perray en Yvelines, France). Subsequently, membranes were probed with horseradish peroxidase-labeled secondary antibodies (Amersham, Les Ulis, France). Detection was performed using chemiluminescence procedure (ECL), according to the manufacturer's recommendations.

Expression of cyclin B₁

Aliquots of control and treated cells were fixed with 80% ethanol for at least 4 h at -20°C centrifuged and washed with PBS. Triton X-

100, at a concentration of 0.25%, was added to the cell suspensions for 5 min at 4°C . The cell suspensions were then centrifuged and washed with PBS. The mouse monoclonal antibody to human cyclin B₁ (Santa Cruz Biotechnology, Inc) was diluted 1:50 in PBS containing 1% of bovine serum albumin (BSA; Sigma); 200 µl of the diluted antibody were added to each sample and incubated overnight at 4°C . The cells were then washed with PBS containing 1% BSA, centrifuged, and stained with FITC-labelled goat anti-mouse IgG antibody (1:60) for 30 min in the dark at room temperature. Following an additional centrifugation, the cells were resuspended in 10 µg/ml of propidium iodide (PI) and 0.1% RNase A in PBS and incubated at room temperature for 30 min. Cellular fluorescence was measured with a Coulter Epics-Profile II analyser. The red (PI) and green (FITC) fluorescence emission from each cell were separated and recorded using standard optical setup.

Nuclei isolation and endogenous endonuclease activity

Nuclei were isolated and assayed for endogenous endonuclease activity according to the method of Nieto and Lopez-Rivas (Nieto and Lopez-Rivas, 1989) as previously described (Ségat-Bendirdjian and Jacquemin-Sablon, 1995).

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