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Preclinical report

Resistance to paclitaxel induces time-delayed multinucleation and DNA fragmentation into large fragments in MCF-7 human breast adenocarcinoma cells

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DNA fragmentation was investigated in MCF7 and the MCF7^{TAX19} paclitaxel-resistant subline exposed to paclitaxel for 24 h. No nucleosome-sized DNA fragmentation was observed by DNA agarose gel electrophoresis in both cell lines. However, DNA fragmentation was detected by flow cytometry sub-G_1 peak analysis in both cell lines immediately after paclitaxel exposure. Nuclear abnormalities were observed in both cell lines in the range of 35–40% of the total cell population. This value was reached immediately in MCF7 cells but was time-delayed in MCF7^{TAX19} cells. Significant morphologic differences were observed between sensitive and resistant cell lines, 24 h after exposure to 50 nmol/l paclitaxel. Although no difference in the sub-G_1 cell population was observed between sensitive and resistant cells, a significantly higher rate of multinucleated cell features was observed in resistant cells. [© 2000 Lippincott Williams & Wilkins.]

Key words: Apoptosis, DNA fragmentation, micronucleation, paclitaxel.

Introduction

Paclitaxel (Taxol®) has demonstrated high chemotherapeutic activity in the treatment of ovarian and breast tumors. Paclitaxel is an antimitotic agent which is active in the G_2/M phase of the cell cycle where it tightly binds microtubules, inhibiting their depolymerization and forming abnormal cytoplamic bundles and nuclear asters, thus blocking cell division.[1] These microtubular changes affect cells mostly in G_2 and M, preventing completion of cell division, which results in accumulation of cells in G_2 and M.[2] More recently, the complexity of cell cycle arrest in G_1 and/or G_2 phase was investigated, and results showed that the death rate induced by paclitaxel could be higher in G_1 than in G_2 blocked cells.[3] Defective G_1/S checkpoint function was reported to sensitize colon cancer cells to paclitaxel-induced apoptosis.[4] Paclitaxel-induced apoptosis was reported to be related to drug concentration in human tumor cells treated with a low drug concentration, i.e. unable to alter the microtubule mass but blocking mitosis by stabilizing microtubule dynamics.[5] Induction of apoptosis was found to be not only related to cell cycle arrest but to the concomitant phosphorylation of p34^{cdc2} protein controlling mitosis.[6] Inadequate activation of this protein could induce apoptosis in two different manners, being directly related to p53 or not. Independently of p53, apoptosis could be initiated through the cell rheostat. Activation of c-Raf-1 leading to the phosphorylation of Bcl-2[7,8] could consequently inhibit its anti-apoptotic activity. The second pathway for induction of apoptosis by paclitaxel is p53 dependent. After being blocked in G_2/M phases and consequently to the activation of p34^{cdc2}, the cells would undergo aberrant mitosis without cytokinesis leading to multinucleated cell features. Cell death would then occur within 3–6 days after G_1/S cell cycle arrest and p53 induction.[9] More recently, paclitaxel sensitivity was reported to correlate with p53 status and DNA fragmentation, but not G_2/M accumulation.[10]

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In MCF7 breast cancer cells,[11] paclitaxel-induced apoptosis was documented and found to account for part of the paclitaxel cytotoxicity and suggested that in the MCF7 cell line, DNA degradation appears to be characterized by infrequent DNA fragmentation yielding large (50–300 kbp) DNA fragments. The *in vivo* relevance of apoptosis induction in paclitaxel antitumor activity was confirmed in murine mammary and ovarian tumors.[12,13]

Resistance to paclitaxel can be mediated by several mechanisms. The multidrug resistance phenotype has been largely described as a potent resistance mechanism to paclitaxel.[14] However, in cells selected after exposure to paclitaxel, resistance to paclitaxel is usually not related to P-glycoprotein (P-gp) or multidrug resistance-related protein (MRP) overexpression, thus indicating other mechanisms of resistance. Some reports demonstrated that modifications of tubulin subunits could be implicated in paclitaxel resistance. Recently, in H69 human small cell lung cancer cell line, alteration of the α-tubulin isoform with an increase in acetylation has been reported as a possible resistance pathway.[15] Some other papers indicate that in K562 cells selected by concomitant exposure to paclitaxel and to the non-immunosuppressive cyclosporin analog, P-glycoprotein modulator SDZ-PSC833, an increase in 5-β-tubulin isoform was observed and induced a decrease in the cellular sensitivity to paclitaxel.[16]

In addition to these mechanisms, the potential implication of alterations of the apoptotic pathway in paclitaxel-resistant cell models has been suggested as a potent mechanism of resistance to paclitaxel.[17] Previous studies revealed that in leukemic cell models[18] paclitaxel resistance was not related to its inability to target microtubules or to block cell cycle progression in the G_2 phase but to the inability of resistant cells to enter the apoptotic pathway. More recently, paclitaxel resistance was reported to be mediated by caspase inhibition, leading to multimininucleation in the PC3 prostate cancer cell line as compared to paclitaxel relatively sensitive HeLa cells.[19]

In the present paper, DNA fragmentation induction after exposure to paclitaxel was studied in a paclitaxel-resistant subline established from the MCF7 human breast adenocarcinoma cell line and exhibiting a low resistance index.

Materials and methods

Drugs

Paclitaxel was purchased from Sigma (St Quentin-Fallavier, France), and was first solubilized in ethanol and then diluted in sterile water. Therefore, control experiments always included samples treated with the equivalent ethanol concentration.

Cell lines

MCF7 human breast adenocarcinoma cell line and a subline $MCF7^{TAX19}$ displaying resistance to paclitaxel were used. The resistant subline, $MCF7^{TAX19}$, was established in our laboratory[17] after culturing parental MCF7 cells in increasing paclitaxel concentrations. The $MCF7^{TAX19}$ subline was then maintained in paclitaxel-free medium. Neither P-gp nor MDR1 mRNA expression was detected in both cell lines.[20]

Both cell lines were cultured at 37°C in phenol red-free RPMI 1640 medium supplemented with 10% fetal calf serum and in 5% CO_2 atmosphere.

Cytotoxicity assays

The sulforhodamine-B (SRB) assay was performed as previously reported.[21] The cells were harvested from exponential phase culture by trypsinization, counted and plated in 96-well microtiter plates. The optimal seeding density of each cell line was determined to ensure adequate absorbance readings in control wells during a 5-day assay. An initial concentration of 10^4 cells/ml was found suitable for all cell lines. All experiments were performed in exponentially growing cells, 3 days after seeding, treated with paclitaxel for 24 h and then either assayed immediately (T_0) or incubated for 24 h (T_{24}) in paclitaxel-free culture medium before performing the SRB cytotoxicity assay. Briefly, the cells were washed with PBS and fixed by means of protein precipitation with trichloroacetic acid at 4°C for 1 h. After washing with water, the cells were stained with SRB (Aldrich-Chimie, St Quentin Fallavier, France). Protein-bound stain was solubilized with unbuffered Tris base (Merck, Darmstadt, Germany). The absorbance of each well was then measured at 540 nm using a MultiskanMCC/340 microplate reader (Flow, Les Ulis, France).

Each concentration was assayed in sextuplicate and each experiment was repeated 3 times. Results were expressed as relative absorbance as compared with untreated controls. The paclitaxel concentration inhibiting the cellular growth by 50% (IC_{50}) was calculated using the median-effect principle.[22]

Cell cycle analysis

Cell suspensions were prepared according to Liebmann.[23] Briefly, about 10^6 cells were stained in 2 ml of 0.1% sodium citrate/0.1% Triton X-100 solution containing 50 μg/ml propidium iodide. After at least 24 h in the

staining solution at 4°C, RNase A (50 μg/ml) was added and all samples (5000 cells minimum) were analyzed by flow cytometry (Orthocyte; Ortho Diagnostic System, Roissy, France). Cell cycle phase distribution was calculated using Multicycle analysis software (Phoenix, CA) and Dean and Jett's modelization.

Determination of apoptosis

Flow cytometry sub-G_1 peak analysis using propidium iodide DNA labeling. DNA content of apoptotic cells was analyzed according to Ormerod *et al.*[24] with slight modifications. After collection by trypsinization and centrifugation, $2–4 \times 10^6$ cells were fixed on ice in 70% cold ethanol and stored at −20°C until experiments were performed. Before staining, 10^6 cells were washed with PBS and then resuspended in 4 ml PBS for 15 min at room temperature to elute cleaved DNA small fragments. After centrifugation, cells were stained with a solution of PBS containing propidium iodide (50 μg/ml), EDTA (0.1 mmol/l), Triton X-100 (0.1%) and RNase A (25 μg/ml). Cell cycle phases analysis was then performed as described above.

Fluorescence microscopy using Hoechst 33342 DNA labeling. After completion of paclitaxel exposure in slideflasks (Nunc, Polylabo, Strasbourg, France), cells were rinsed twice in PBS and then fixed with 1% paraformaldehyde solution for 20 min at room temperature. The slide was then washed with PBS and then stained with Hoechst 33342 (Molecular Probes, Interchim, Asnières, France) 5 μg/ml solution in PBS according to Lizard *et al.*[25] Fluorescence microscopy analysis was then performed with excitation at 330–380 nm and emission at 420–460 nm using an AX70 Provis epifluorescence microscope (Olympus, Rungis, France) fitted with a LH1600 cooled CCD camera (Lhesa, Cergy-Pontoise, France). Cells were classified as 'intact cells' with uncondensed chromatin, 'multinucleated cells' exhibiting more than three nuclei with uncondensed chromatin and 'apoptotic cells' exhibiting apoptotic bodies.

DNA agarose gel electrophoresis. Cells were collected after completion of paclitaxel exposure. For DNA extraction the cells were lyzed in Tris buffer (pH 8) containing 0.5% sodium dodecylsulfate, and supernatants were treated with RNAse A (10 μg/ml) and proteinase K (25 μg/ml). DNA was phenol–chloroform extracted and precipitated in 10 volumes of ethanol and 10 mmol/l $MgCl_2$ washed in 1 ml ethanol, dried and resuspended in 100 μl diethylpyrocarbonate solution. DNA (1 μg/sample, determined by absorbance at 260 nm) was analyzed by agarose gel electrophoresis (1.8% agarose, 25 V, 4 h) using 0.89 mol/l Tris, 0.89 sodium borate, 0.2 mol/l EDTA, pH 8 running buffer. A 100 bp size standard was electrophoretized in parallel. DNA was stained with ethidium bromide (1 μg/ml) in water, analyzed using UV transillumination and image analysis (Gel-Doc 1000 fitted with Molecular Analyst software; BioRad, Ivry Sur Seine, France). The positive control consisted of HT29 human colon carcinoma cells exposed for 24 h to paclitaxel.

Statistical analysis

Student's *t*-test was employed to determine the statistical significance with a limit set to $p < 0.05$ using Statview 4.02 software (Abacus Concepts, Berkeley, CA).

Results

Cytotoxicity assays

Paclitaxel IC_{50} values were calculated from cytotoxicity plots (Figure 1) as 291 (32) nmol/l (SD) in the parental MCF7 cells while being significantly increased in $MCF7^{TAX19}$ cells to 1177 (120) ($p < 0.02$).

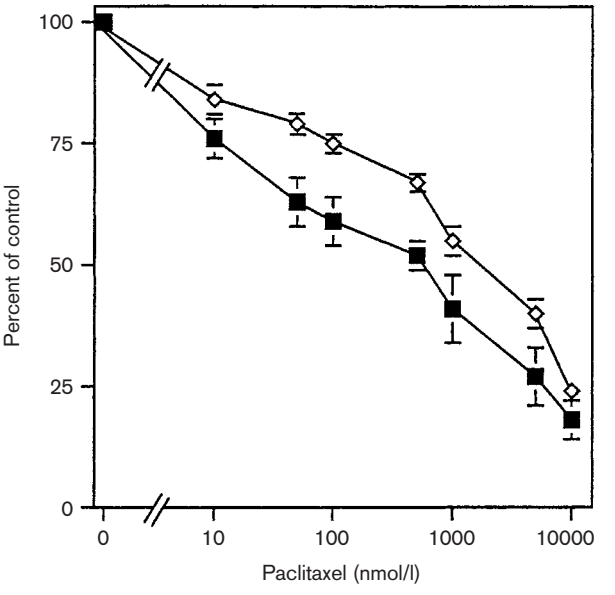

Figure 1. Dose–response plots of MCF7 (■) and $MCF7^{TAX19}$ (◇) cells exposed to paclitaxel for 24 h. Cytotoxicity was assessed using SRB assays, performed immediately after completion of paclitaxel exposure.

The resistance index to paclitaxel was calculated by comparing the IC_{50} achieved in $MCF7^{TAX19}$ with that of the parental taxol-sensitive MCF7 cell line and found to be 4.0.

Paclitaxel-induced G_2/M cell cycle arrest

G_2/M phase cell accumulation (Table 1) appeared immediately after paclitaxel exposure (T_0) in both MCF7 and $MCF7^{TAX19}$ cells, and to a significantly ($p < 0.05$) lower extent in $MCF7^{TAX19}$ cells at the lowest concentration (20 nmol/l). The G_2/M cell population increased significantly ($p < 0.01$) as the concentration was set to 50 nmol/l in both cell lines. No significant difference remained at 50 nmol/l between MCF7 and $MCF7^{TAX19}$ cells (Figure 2).

Table 1. G_2/M phase cell fraction (expressed as percent of the total cell population) detected immediately (T_0) or 24 h (T_{24}) after exposure of MCF7 and $MCF7^{TAX19}$ cells to paclitaxel (20 or 50 nmol/l for 24 h)

		MCF7	$MCF7^{TAX19}$
T_0	20 nmol/l	34 (5)	22 (3)
	50 nmol/l	61 (8)	56 (8)
T_{24}	20 nmol/l	10 (3)	7 (2)
	50 nmol/l	23 (4)	29 (5)

Results are mean values (SD) of a minimum of three independent experiments. Cell cycle analysis was performed using flow cytometry after propidium iodide DNA labeling (for details, see Materials and methods).

Twenty-four hours after paclitaxel exposure (T_{24}), G_2/M cell fractions significantly ($p < 0.01$) decreased as compared to T_0 values, in both cell lines, and for 20 and 50 nmol/l concentrations, probably in relation to apoptosis induction in cells arrested in G_2/M. Significantly higher G_2/M cell fractions were detected at 50 than at 20 nmol/l ($p < 0.02$). No significant difference was detected between MCF7 and $MCF7^{TAX19}$ cells.

DNA fragmentation

Apoptosis induction was first screened using sub-G_1 peak analysis for paclitaxel concentrations of 10, 20, 50 and 100 nmol/l, immediately or 24 h after completion of paclitaxel exposure in MCF7 cells. The results show that significant apoptosis induction was

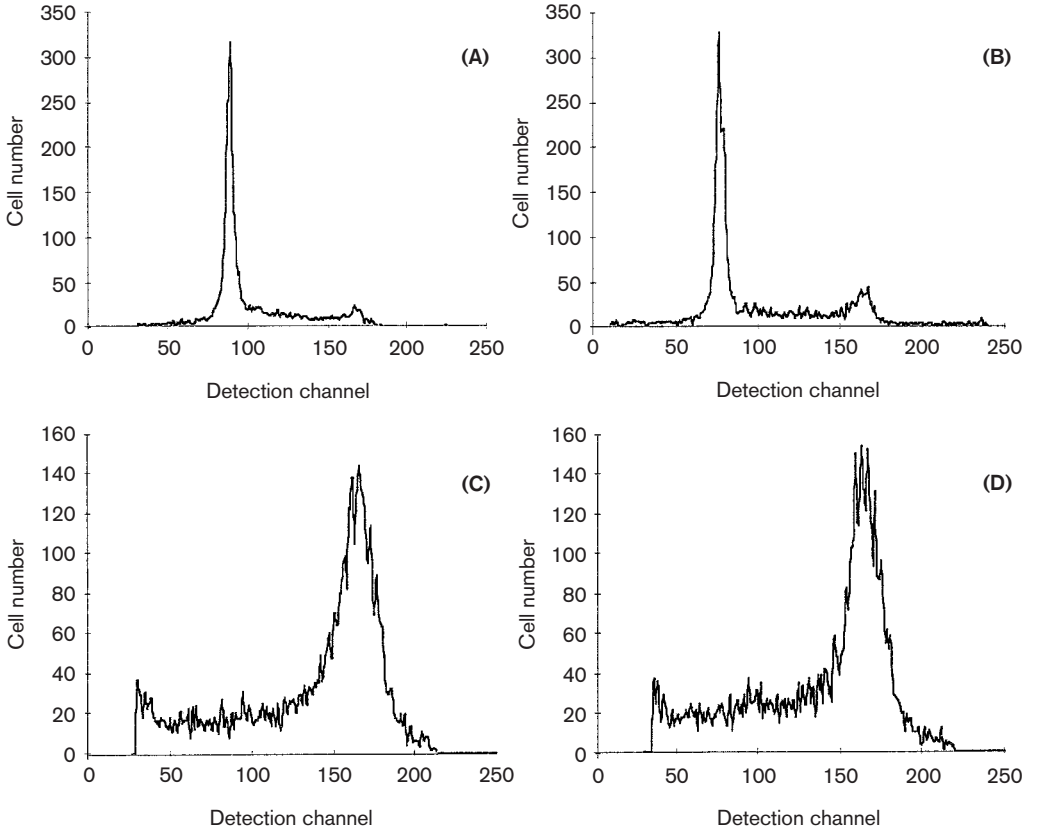

Figure 2. Representative histograms illustrating G_2/M cell cycle arrest in MCF7 (A and C) and $MCF7^{TAX19}$ (B and D) cells. Untreated controls (A and B) and cells exposed to paclitaxel (50 nmol/l) for 24 h (C and D). Analyses were performed using flow cytometry, immediately after completion of paclitaxel exposure.

achieved within a 20–50 nmol/l concentration range. Below (i.e. 10 nmol/l), no significant variation was observed as compared to untreated controls (data not shown). Above (i.e. 100 nmol/l), necrosis appeared through a large proportion of broken cells. Therefore, 20 and 50 nmol/l concentrations were further used for apoptosis analysis using flow cytometry and fluorescence microscopy.

Flow cytometry sub-G_1 peak analysis. Using flow cytometry sub-G_1 peak analysis (Table 2), a low proportion of cells (approximately 10%) was detected immediately after 20 nmol/l paclitaxel exposure (T_0) in both MCF7 and MCF7^{TAX19} cells. When the concentration was set to 50 nmol/l, the number of DNA-fragmented cells increased significantly in both cases ($p < 0.01$), although remaining significantly lower in MCF7^{TAX19} cells, as illustrated Figure 3 ($p < 0.01$).

Table 2. Sub-G_1 cell fraction (expressed as percent of the total cell population) detected immediately (T_0) or 24 h (T_{24}) after exposure of MCF7 and MCF7^{TAX19} cells to paclitaxel (20 or 50 nmol/l for 24 h)

		MCF7	MCF7^{TAX19}
T_0	20 nmol/l	10 (1)	9 (1)
	50 nmol/l	26 (4)	13 (1)
T_{24}	20 nmol/l	38 (5)	43 (3)
	50 nmol/l	34 (4)	40 (5)

Results are mean values (SD) of a minimum of three independent experiments. Cell populations were analyzed using flow cytometry after propidium iodide DNA labeling (for details, see Materials and methods).

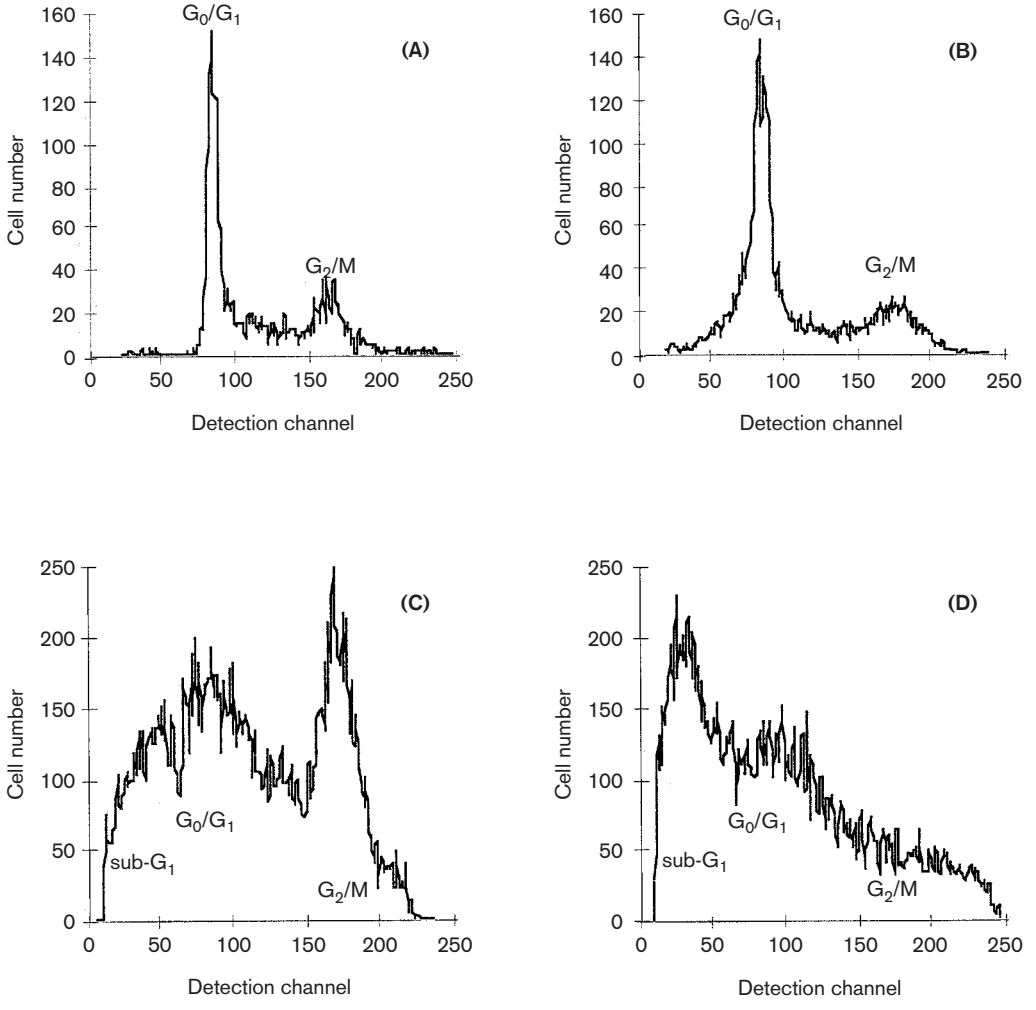

Figure 3. Representative histograms illustrating DNA fragmentation induced in MCF7^{TAX19}. Untreated controls (A and B) and cells exposed to paclitaxel (50 nmol/l) for 24 h (C and D). Sub-G_1 peak analyses were performed using flow cytometry immediately (A and C) or 24 h (B and D) after completion of paclitaxel exposure.

Twenty-four hours after paclitaxel exposure (T_{24}), the number of DNA-fragmented cells significantly increased in MCF7 cells incubated in 20 nmol/l paclitaxel ($p < 0.005$) while no significant effect was observed at 50 nmol/l. In MCF7^{TAX19} cells, significant increases were observed for both concentrations ($p < 0.005$). In all cases, no significant difference remained between cell populations incubated in either 20 or 50 nmol/l paclitaxel and between MCF7 and MCF7^{TAX19} cells, thus indicating that maximal DNA fragmentation was achieved in the range of 35–40% of the total cell population. This value was reached at T_0 in MCF7 cells but was time-delayed to T_{24} in MCF7^{TAX19} cells in which DNA fragmentation appeared to be associated with stabilization of G_2/M-arrested cells, unable to undergo DNA fragmentation at T_0 (Figure 3).

Fluorescence microscopy cell morphology analysis. Using fluorescence microscopy with Hoescht 33342 DNA labeling enabled us to distinguish multinucleated cells from late-apoptotic cells exhibiting apoptotic bodies (Figure 4). One hour after paclitaxel exposure (T_0), multinucleated cells were mostly observed (Table 3) in both cell lines, whatever the concentration. As

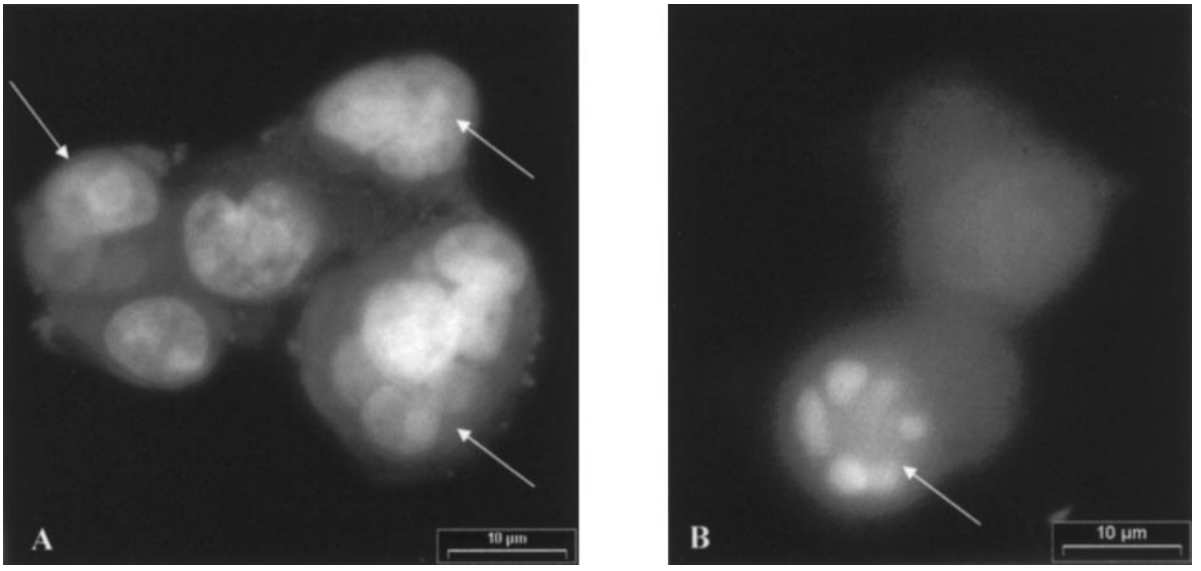

Figure 4. Representative fluorescence microscopy images of multinucleation (A) and apoptosis (B) in MCF7^{TAX19} cells exposed to paclitaxel (50 nmol/l) for 24 h. Analyses were performed 24 h (T_{24}) after completion of paclitaxel exposure after Hoescht 33342 DNA labeling.

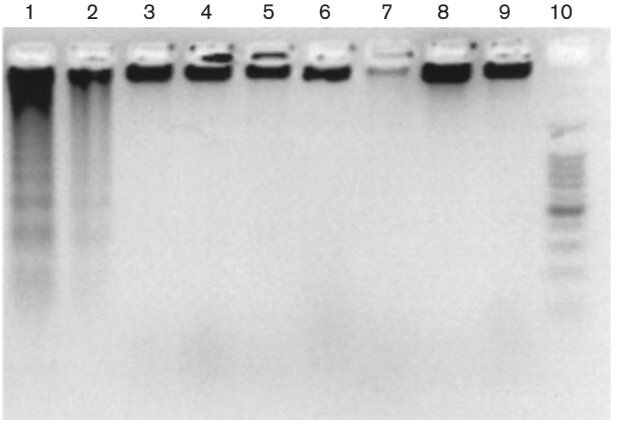

Figure 5. Agarose gel electrophoresis pattern of DNA extracted from MCF7 (lanes 3–6) and MCF7^{TAX19} cells (lanes 7–9), immediately (lanes 3, 5 and 7) or 24 h (lanes 4, 6, 8 and 9) after completion of exposure to 20 (lanes 3, 4, 7 and 8) and 50 (lanes 5, 6 and 9) nmol/l paclitaxel. Lanes 1 and 2, positive controls (HT-29 cells exposed to 50 nmol/l paclitaxel for 24 h). Lane 10, molecular weight standard.

observed previously, no significant difference was found between MCF7 and MCF7^{TAX19} cells when exposed to 20 nmol/l paclitaxel. In cell populations exposed to 50 nmol/l paclitaxel, the proportion of apoptotic figures observed in MCF7^{TAX19} was significantly lower ($p < 0.02$) than in MCF7 cells. Twenty-four hours after paclitaxel exposure (T_{24}), the proportions of either multinucleated cells or cells with apoptotic bodies increased significantly ($p < 0.01$) in both cell lines excepted in MCF7 cells exposed to 50 nmol/l paclitaxel in which the proportion of multinucleated cells variation was not significant. Although no significant difference remained between MCF7 and MCF7^{TAX19} cells when the proportion of multinucleated cells was considered, a significantly ($p < 0.02$) lower proportion of cells exhibiting apoptotic bodies was detected in MCF7^{TAX19} cells.

DNA agarose gel electrophoresis. Figure 5 illustrates the agarose gel electrophoresis pattern of DNA extracted from MCF7 and MCF7^{TAX19} cells. No DNA ladder was observed either in the parental sensitive or in the paclitaxel-resistant cell line, being analyzed immediately and 24 h after exposure to paclitaxel (20 and 50 nmol/l).

Discussion

Paclitaxel has been reported to induce apoptosis in several experimental models including leukemic cells, fibroblasts, gastric, cervix and breast carcinoma cell lines[5,6,11,26] grown *in vitro*, head and neck tumor specimens from patients exposed *ex vivo* to paclitaxel[27] as well as *in vivo* murine mammary tumor models.[12,13]

In the present paper, apoptosis induction by paclitaxel in the MCF7 cell line and its variant expressing 4-fold resistance to paclitaxel (MCF7^{TAX19}) was demonstrated using morphologic criteria by fluorescence microscopy after DNA labeling by Hoechst 33342 and confirmed by assessing DNA fragmentation using sub-G_1 flow cytometry peak analysis after DNA labeling by propidium iodide. The results achieved in sensitive MCF7 cells confirmed those recently reported by Saunders *et al.*,[11] qualitatively and quantitatively, with apoptosis induction representing 30–40% of the whole cell population, achieved with approximately 20 nmol/l paclitaxel 24 h after incubation with the drug. Maximal paclitaxel-induced apoptosis was achieved at 50 nmol/l and represented 50% of the cell population. In the MCF7^{TAX19} paclitaxel-resistant subline, no difference in the maximal rate of apoptosis was observed but a significant time delay appeared in the resistant subline, tending to show that low paclitaxel resistance could be associated with a time delay to apoptosis induction.

The results achieved using fluorescence microscopy were consistent with those achieved using flow cytometry. No significant difference was found when the sub-G_1 peak population was compared with the proportion of multinucleated cells and cells bearing apoptotic bodies. Only in cell populations exposed to 50 nmol/l paclitaxel and 24 h after paclitaxel exposure did a significant difference appear between the results achieved with the two methods. This difference was mostly related to the increase in the proportion of cells bearing apoptotic bodies that are not being considered in the flow cytometry analyses based on cell size population selection.

Twenty-four hours after paclitaxel exposure, DNA fragmentation was illustrated by a high proportion of multinucleated cells and a very low amount of apoptotic cells. The relative proportion of multinucleated cell features was consistent with the sub-G_1 DNA cell population and coincided with the lack of 200 bp DNA fragmentation. This confirmed the results achieved by Panvichian *et al.*[19] using two different cell models and showing that multimininucleation was related to paclitaxel resistance.

As already noted by Saunders *et al.*[11] in the same cell model, apoptosis-related DNA fragmentation seems to be complex. Among the large variety of cell models that were used to study paclitaxel-induced apoptosis, two modes of DNA degradation were reported to occur. The most characterized mode of DNA breakage during apoptosis, i.e. nucleosome-sized fragmentation illustrated by a DNA ladder structure with agarose gel electrophoresis,[28] was reported in several models.[5,18] The present results showed that in the MCF7 cell line this event was not evidenced, as opposed to the HT29 colon carcinoma model used as positive control, and therefore is probably not necessary to trigger apoptosis. This confirms that paclitaxel should induce an alternative DNA fragmentation mechanism, yielding larger DNA fragments,[29] that, in the present case, could not be evidenced with agarose electrophoresis but could be detected with sub-G_1 DNA content analysis. This was consistent with the results achieved previously in MCF7 cells[11,18] as well as in K562 leukemic cells.[18]

Investigations of paclitaxel-induced apoptosis molecular pathways have been reported. Bcl-2 apoptosis regulation[30] appears to be an important event in paclitaxel-induced apoptosis. Results achieved in MCF7 cells[8] suggest that the mitogen-stimulated serine/threonine kinase Raf-1 could form a molecular

complex with Bcl-2 after paclitaxel exposure and represents a distinct Raf-1-mediated signaling pathway whose activation leads to apoptosis. Recent results[31] envision a pathway of Raf-1 activation and Bcl-2 activation following disruption of microtubular architecture that could play a role similar to that of p53 after DNA damage. Similar investigations could be reproduced in the $MCF7^{TAX19}$ paclitaxel-resistant cell model to investigate whether molecular alterations of this pathway might be involved in apoptosis induction.

References

1. Parness J, Horwitz SB. Taxol binds to polymerized tubulin *in vitro*. *J Cell Biol* 1981; **91**: 479–87.
2. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci USA* 1980; **77**: 1561–5.
3. Sena G, Onado C, Cappella P, Montalenti F, Ubezio P. Measuring the complexity of cell cycle arrest and killing of drugs: kinetics of phase-specific effects induced by taxol. *Cytometry* 1999; **37**: 113–24.
4. Stewart ZA, Mays D, Pietenpol JA. Defective G_1–S cell cycle checkpoint function sensitizes cells to microtubule inhibitor-induced apoptosis. *Cancer Res* 1999; **59**: 3831–7.
5. Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L. Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results inabnormal mitotic exit and apoptotic cell death. *Cancer Res* 1996; **56**: 816–25.
6. Donaldson KL, Goolsby GL, Wahl AF. Cytotoxicity of the anticancer agents cisplatin and taxol during cell proliferation and the cell cycle. *Int J Cancer* 1994; **57**: 847–55.
7. Blagosklonny MV, Schulte TW, Nguyen P. Taxol induction of p21WAF1 and p53 requires c-*raf*-1. *Cancer Res* 1995; **55**: 4623–6.
8. Blagosklonny MV, Schulte TW, Nguyen P, Trepel J, Neckers LM. Taxol-induced apoptosis and phosphorylation of *bcl-2* protein involves c-*raf*-1 and represents a novel c-*raf*-1 signal transduction pathway. *Cancer Res* 1996; **56**: 1851–4.
9. Woods CM, Zhu J, McQueney PA, Bollag D, Lazarides E. Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway. *Mol Med* 1995; **1**: 506–26.
10. Rakovitch E, Mellado W, Hall EJ, Pandita TK, Sawant S, Geard CR. Paclitaxel sensitivity correlates with p53 status and DNA fragmentation, but not G_2/M accumulation. *Int J Radiat Oncol Biol Phys* 1999; **44**: 1119–24.
11. Saunders DE, Lawrence WD, Christensen C, Wappler NL, Ruan HM, Deppe G. Paclitaxel-induced apoptosis in MCF-7 breast-cancer cells. *Int J Cancer* 1997 **70**: 214–20.
12. Meyn RE, Stephens LC, Hunter NR, Milas L. Kinetics of cisplatin-induced apoptosis in murine mammary and ovarian adenocarcinomas. *Int J Cancer* 1995; **60**: 725–9.
13. Milas L, Hunter NR, Kurdoglu B, *et al*. Kinetics of mitotic arrest and apoptosis in murine mammary and ovarian tumors treated with taxol. *Cancer Chemother Pharmacol* 1995 **35**: 297–303.
14. Horwitz SB, Cohen D, Rao S, Ringel I, Shen HJ, Yang CP. Taxol: mechanisms of action and resistance. *J Natl Cancer Inst* 1993 **15**: 55–61.
15. Ohta S, Nishio K, Kubota N, *et al*. Characterization of a taxol-resistant human small-cell lung cancer cell line. *Jpn J Cancer Res* 1994; **85**: 290–7.
16. Jaffrezou JP, Dumontet C, Derry WB, *et al*. Novel mechanism of resistance to paclitaxel (Taxol) in human K562 leukemia cells by combined selection with PSC 833. *Oncol Res* 1995; **75**: 17–27.
17. Di Betta D, Colosetti P, Merlin JL. Altered induction of apoptosis in paclitaxel resistant human carcinoma cell lines. *Proc Am Ass Cancer Res* 1997; **38**: 486.
18. Gangemi RM, Tiso M, Marchetti C, Severi AB, Fabbi M. Taxol cytotoxicity on human leukemia cell lines is a function of their susceptibility to programmed cell death. *Cancer Chemother Pharmacol* 1995; **36**: 385–92.
19. Panvichian R, Orth K, Day ML, Day KC, Pilat MJ, Pienta KJ. Paclitaxel-associated multimininucleation is permitted by the inhibition of caspase activation: a potential early step in drug resistance. *Cancer Res* 1998 **58**: 4667–72.
20. Merlin JL, Aubé O, Bastien L, Bour C, Gramain MP, Marchal S. Paclitaxel-mediated induction of apoptosis in human tumor cell lines. *Ann Oncol* 1998; **9** (suppl 2): 93.
21. Griffon-Etienne G, Merlin JL, Marchal C. Association of docetaxel/paclitaxel with irradiation in ovarian carcinoma cell lines in bidimensional (sulforhodamine B assay) and tridimensional (spheroids) cultures. *Anti-Cancer Drugs* 1996; **7**: 660–70.
22. Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enz Reg* 1984; **22**: 27–55.
23. Liebmann J, Cook JA, Lipschultz C, Teague D, Fisher J, Mitchell JB. The influence of Cremophor EL on the cell cycle effects of paclitaxel (Taxol) in human tumor cell lines. *Cancer Chemother Pharmacol* 1994; **33**: 331–9.
24. Ormerod MG, Collins MK, Rodriguez-Tarduchy G, Robertson D. Apoptosis in interleukin-3-dependent haemopoietic cells. Quantification by two flow cytometric methods. *J Immunol Methods* 1992; **153**: 57–65.
25. Lizard G, Fournel S, Genestier L. Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. *Cytometry* 1995; **21**: 275–83.
26. Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME, Huang Y. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. *Leukemia* 1993; **7**: 563–8.
27. Gan Y, Wientjes MG, Lu J, Au JLS. Cytostatic and apoptotic effects of paclitaxel in human breast tumors. *Cancer Chemother Pharmacol* 1996; **42**: 177–82.
28. Hickman JA. Apoptotis induced by anticancer drugs. *Cancer Metastasis Rev* 1992; **11**: 121–39.
29. Walker PR, Weaver VM, Lach B, Leblanc J, Sikorska M. Endonuclease activities associated with high molecular weight and internucleosomal DNA fragmentation in apoptosis. *Exp Cell Res* 1994; **213**: 100–6.
30. Haldar S, Jena N, Croce CM. Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci USA* 1995; **92**: 4507–11.
31. Blagosklonny MV, Giannakakou P, El-Deiry WS, *et al*. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res* 1997; **57**: 130–5.

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