G_2/M blockade by paclitaxel induces caveolin-1 expression in A549 lung cancer cells: caveolin-1 as a marker of cytotoxicity

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Caveolins are highly expressed in terminally differentiated cells, but this expression is down-regulated in various cancer cell lines. Exposure to low doses of paclitaxel (taxol) is sufficient to up-regulate caveolin-1, suggesting that a mild cytotoxic stress induces a response implying caveolin and caveolae. Here we show that this up-regulation is sustained even after the cessation of paclitaxel treatment. After exposure to a cytostatic dose of paclitaxel (50 nM), A549 lung cancer cells are blocked in the G_2/M cell cycle phase. After removal of paclitaxel, cell death occurs, accompanied with an increase in caveolin expression, suggesting an effect of caveolin in this process. Three days post-paclitaxel treatment, surviving A549 cells were passaged and only a half of them adhered to the culture dish. Adhering cells (still mainly in the G_2/M cell cycle phase) were still unable to grow and progressively entered in an apoptotic state. This study suggests that effects of a low dose of paclitaxel were still present even 1 week after drug removal and that caveolin-1 is a good marker of cytotoxicity. ***Anti-Cancer Drugs*** **15:961–967 © 2004 Lippincott Williams & Wilkins.**

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Introduction

Caveolae are plasma membrane organelles which are present in almost all cell types, and are abundant in terminally differentiated cells like endothelial cells, fibroblasts, adipocytes and muscle cells [1]. Caveolae act as signaling protein concentrators and thus allow cross-talk between different signaling pathways [2,3]. Caveolins, 21- to 24-kDa membrane proteins, are the principal component and structural proteins of caveolae [1]. Caveolin-1 has been shown to bind with signaling molecules (G-proteins, Src, Ras, epidermal growth factor receptor, protein kinase C, adenylyl cyclase and others) that are retrieved inside the caveolae and controls their activation [1,4–7]. Caveolin maintains these proteins in an inactivated state to limit cell proliferation and in this way contributes to avoiding oncogenic cell transformation. In fact, a targeted down-regulation of caveolin expression is proven to cause hyperactivation of the p42/44 MAP kinase cascade [8]. Conversely, recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth [8–12]. Caveolin-1 expression was shown to be down-regulated in several lung carcinoma cell lines [13,14].

Paclitaxel is a chemotherapeutic agent that is mainly used in the treatment of breast, ovary, esophageal and lung cancer. Paclitaxel acts by its fixation to microtubules. This fixation blocks the cell cycle in G_2/M phase because paclitaxel inhibits microtubule depolymerization during chromatid separation. Depending on the concentration, different effects could be obtained. At low doses (below 5 nM) for 3–4 days, paclitaxel only slows down proliferation. Under therapeutic conditions (5–200 nM), the cell cycle is blocked. Higher doses of paclitaxel induce non-cell-cycle-dependent death [15,16].

A previous study by our research group showed that exposure of A549 cells to chemotherapeutic drugs like VP-16 and doxorubicin induced up-regulation of caveolin-1 mRNA and protein expression. This up-regulation was dose related [14]. Up-regulation of caveolin-1 expression was also reported in A549 cells treated with paclitaxel for a period of 48 h [17].

In this study, we wanted to study the kinetics of caveolin up-regulation in A549 lung cancer cells treated with paclitaxel.

Material and methods

Materials

Paclitaxel, stock concentration 7 mM, was from Bristol-Myers Squibb (Montreal, Québec, Canada). Anti-caveolin-1 mouse IgG (clone 2297), rabbit polyclonal

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anti-caveolin-1 and anti-caveolin-2 mouse IgG were from BD Biosciences (Mississauga, Ontario, Canada).

Cell culture

A549 cell line used in this report were obtained from the ATCC (Manassas, VA) and cultured as suggested by the supplier.

Cell proliferation studies

Cells were cultured at a predetermined density of 125 000 cells/well in six-well plates for 18 h in DMEM. After this pre-treatment period, control cells were harvested using trypsin/EDTA, counted and pelleted by centrifugation after which they were lysed in Laemmli buffer (100 mM Tris, 1% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.02% phenol red) at 1×10^6 cells/ml. Paclitaxel at the indicated concentration was added to the culture medium in DMEM and renewed every 2 days.

Immunoblotting

Cultured cells were collected using trypsin/EDTA, counted and pelleted by centrifugation. The pellet was then resuspended in Laemmli sample buffer, boiled for 5 min and analyzed by SDS–PAGE using caveolin-1 or -2 antibodies for Western blotting. Volumes of samples loaded on gels were corrected for the number of cells. Quantification of bands by densitometry was performed using Scion Image software (Fredericks, MD).

Immunofluorescence staining

One hundred thousand cells were cultured in LabTech slides (Nalge Nunc, Naperville, IL) for 4 days in the presence or not of paclitaxel 25 nM. After the incubation period, cells were washed with phosphate-buffered saline (PBS) and fixed in 100% methanol at −20°C for 15 min. Non-specific sites were blocked with 0.5% bovine serum albumin (BSA) for 15 min. Anti-caveolin-1 mouse IgG (dilution 1/200) was incubated for 1 h at room temperature in 0.5% BSA. Anti-mouse Alexa 488-coupled secondary antibody (Molecular Probes, Leiden, The Netherlands) was used at a 1/1000 dilution in PBS and incubated for 30 min at room temperature in the dark. Following each incubation, cells were washed with PBS. Finally, cells were covered with mounting media (90% glycerol, 10% PBS and 0.1% *p*-phenylenediamine) and a microscope slide. Cells were then observed with an epifluorescence microscope (Eclipse E600; Nikon) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

Cell cycle analysis

Cells were collected, counted and pelleted by centrifugation. Cells were then washed twice with PBS without Ca^{2+} and Mg^{2+}, and fixed in 70% ethanol at –20°C for 24 h. Cells were stained in a propidium iodide (PI) solution (200 U of DNase-free RNase and 50 mg/ml PI in water) for 30 min at room temperature and then stored at 4°C for at least 48 h before flow cytometry analysis. Analysis was performed using a Coulter Elite flow cytometer (Becton Dickinson, Fullerton, CA). Ten thousand events were recorded for each sample.

Proliferation study by flow cytometry

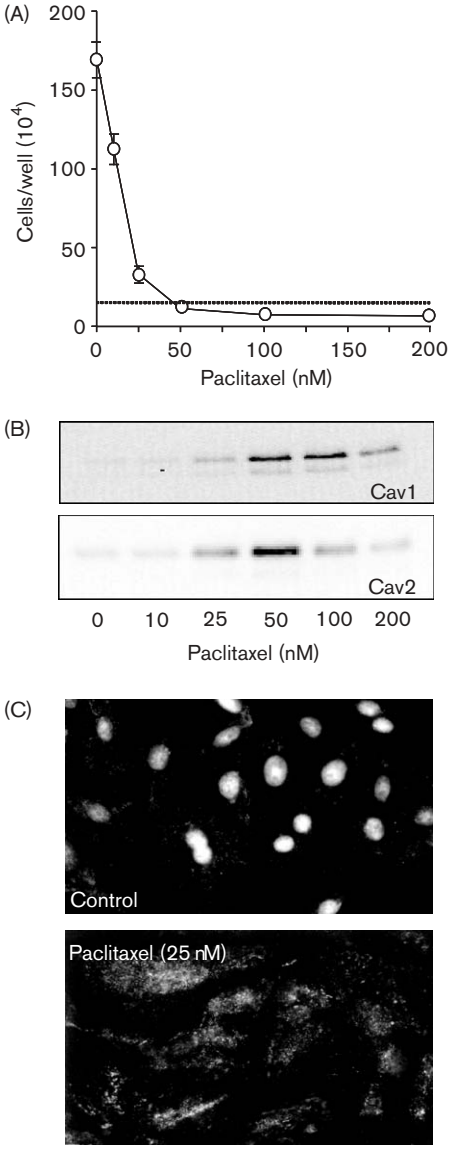
Cells were cultured has described previously and in the presence or not of 5-bromo-2-deoxyuridine (BrdU) 60 μM (Sigma) for 3 h. Cells are then harvested, fixed in 70% ethanol and placed at 4°C for at least 24 h. Cells were treated with RNase A 20 U/ml for 30 min at 37°C and incubated in HCl 2 N for 20 min at room temperature. Cells are then immunostained or not with an anti-BrdU mouse antibody conjugated with Alexa 488 dye (Molecular Probes) at 5 μg/ml in Hank's balanced salt solution with 0.1% Triton X-100 and 0.5% BSA in the dark at room temperature for 30 min. Cells were then incubated with PI 50 μg/ml. For cell cycle analysis, the same parameters were studied and 10 000 events were captured. For proliferation analysis, autofluorescence was discriminated from specific fluorescence. Each individual positive staining (green staining for BrdU and red staining for PI) was determined and gated. After these adjustments, double-stained samples were analyzed to evaluate the proliferation level on a logarithmic scale as a function of cell DNA content (indicator of cell cycle phase).

Results and discussion

To evaluate the effect of a paclitaxel treatment on cell proliferation and caveolin-1 and -2 expression in A549 cells, 125 000 cells were treated for 4 days treatment with increasing concentrations of paclitaxel from 0 to 200 nM. As illustrated in Figure 1(A), cell proliferation was possible but reduced at concentrations lower than 50 nM, whereas significant cell death was observed at higher concentrations. At 50 nM paclitaxel, the total cell count at the end of the experiment was unchanged and this dose was therefore designated 'cytostatic'. This cytotoxic concentration of paclitaxel was used for the rest of the study. As described by others [14,17], both caveolin-1 and -2 expression was up-regulated in the presence of a cytotoxic drug such as paclitaxel (Fig. 1B) as demonstrated by the increased protein content. The induction of caveolin-1 expression (grainy staining) can be visualized by immunofluorescence (Fig. 1C).

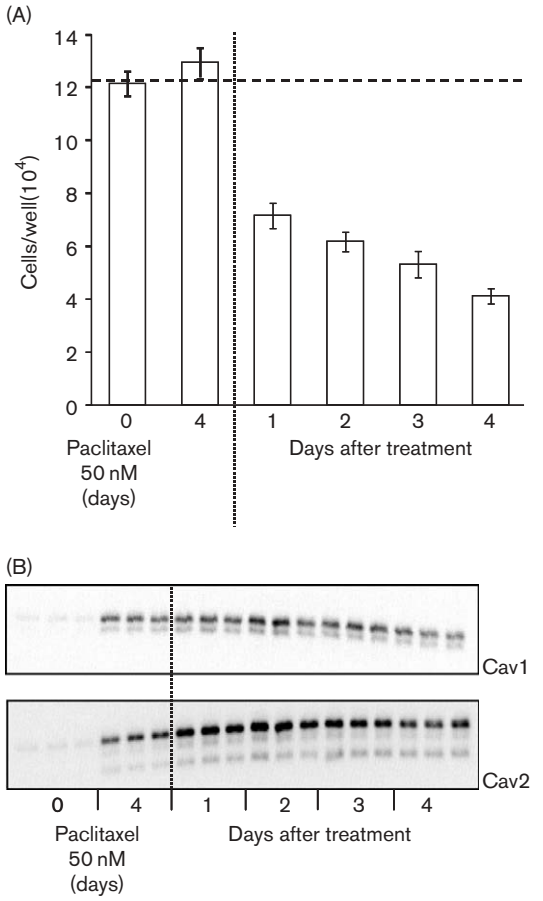
We wanted to investigate more thoroughly the induction of caveolin expression in the presence of a cytotoxic stress and if this up-regulation is sustained after drug removal. A549 cells were treated for 4 days with a cytostatic dose of paclitaxel (50 nM) and the cells were left in culture for different recovery periods in the absence of drug. Interestingly, 1 day after the end of treatment, significant cell death was observed as illustrated in Figure 2(A) and cell counts slowly declined afterwards. A similar

Fig. 1



Paclitaxel induces caveolin expression in A549 cells. (A) Cells were treated with increasing doses of paclitaxel for 4 days. Two hundred thousand cells (dotted line) were plated per well on day 0 in triplicate. Cells were trypsinized and counted on a hemocytometer. Cell counts are expressed as cells/well ± SEM (n = 3). (B) Volumes of crude cell homogenates corresponding to 30 000 cells were separated by SDS–PAGE and Western blot analysis was performed using caveolin-1 or -2 monoclonal antibodies. (C) Cells were cultured in chamber slides with the indicated concentration of paclitaxel for 48 h. Cells were then fixed and immunofluorescence for caveolin-1 was performed as described in Material and methods. Note the more abundant grainy labeling characteristic of caveolin in the treated cells.

Fig. 2

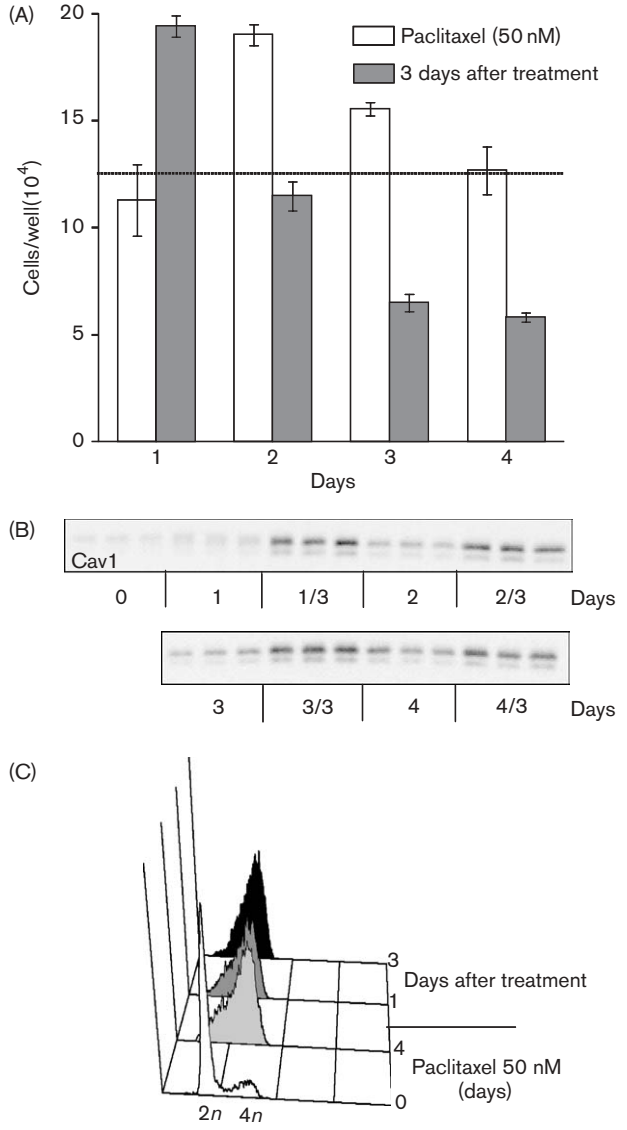


Cessation of a cytostatic paclitaxel treatment induces increased cell death and a sustained expression of caveolins. (A) Cells were treated as indicated then cultured for 1–3 days without drugs. Adhering cells at the end were counted as indicated above and results expressed as cells/well ± SEM (n = 3). The horizontal dotted line indicates the number of cells plated at day 0. The vertical line shows when the treatment has been stopped. (B) Caveolin-1 and -2 protein level increase caused by paclitaxel treatment is sustained afterwards.

observation had been made previously by others [18]. Caveolin-1 expression remained high for all the recovery period (Fig. 2B). We then studied the effect of length of treatment on the recovery period and caveolin expression. Cell counts as illustrated in Figure 3(A) remained unchanged after a 24-h paclitaxel treatment, and were increased after 2 and 3 days of treatment. Cells were then left to recover for 3 days without drug. Only cells treated for 24 h with paclitaxel showed a moderate increase in number, whereas longer durations of treatment were associated with accelerated cell death. Caveolin-1 protein content increased gradually from 1 to 4 days of treatment. Interestingly, drug removal was associated with a sharp increase in caveolin-1 protein content (Fig. 3B). Cell cycle analysis of A549 cells treated with paclitaxel shows a typical G_2/M blockade. Interestingly, this blockade persists 3 days after drug removal, and a slight shift of the 4n cell population is indicative of increased DNA degradation and apoptosis (Fig. 3C).

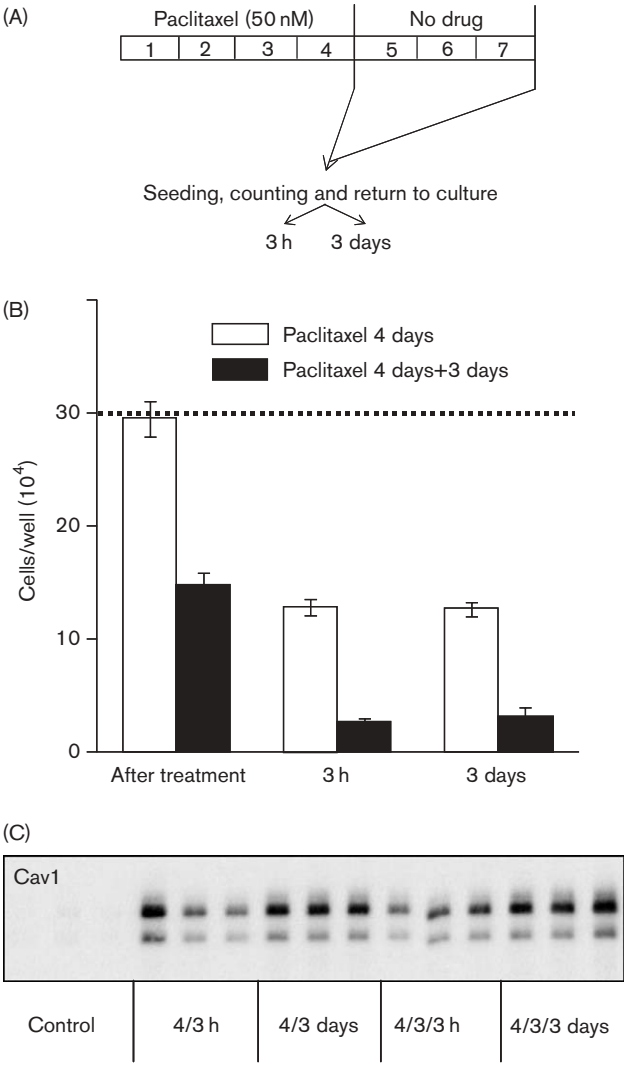
We then evaluated cell viability after paclitaxel treatment and recovery period. We seeded 300 000 cells and treated

Fig. 3



Cessation of paclitaxel increased caveolin expression. (A) Cells were treated with 50 nM of paclitaxel for up to 4 days, then cultured or not for 3 days without drug. Open bars indicate cell counts after paclitaxel, whereas hatched bars indicate cell counts after the additional 3 days of culture under normal conditions. Adhering cells were counted as indicated above and results are expressed as cells/well ± SEM ($n=3$). The horizontal dotted line indicates the number of cells plated at day 0. (B) Caveolin-1 protein levels are increased after return to normal culture conditions in A549 cells treated with paclitaxel for up to 4 days. (C) Paclitaxel causes cell cycle blockade in the G_2/M phase and this blockade is sustained after return to normal culture conditions. Cell cycle analysis was realized as described in Material and methods.

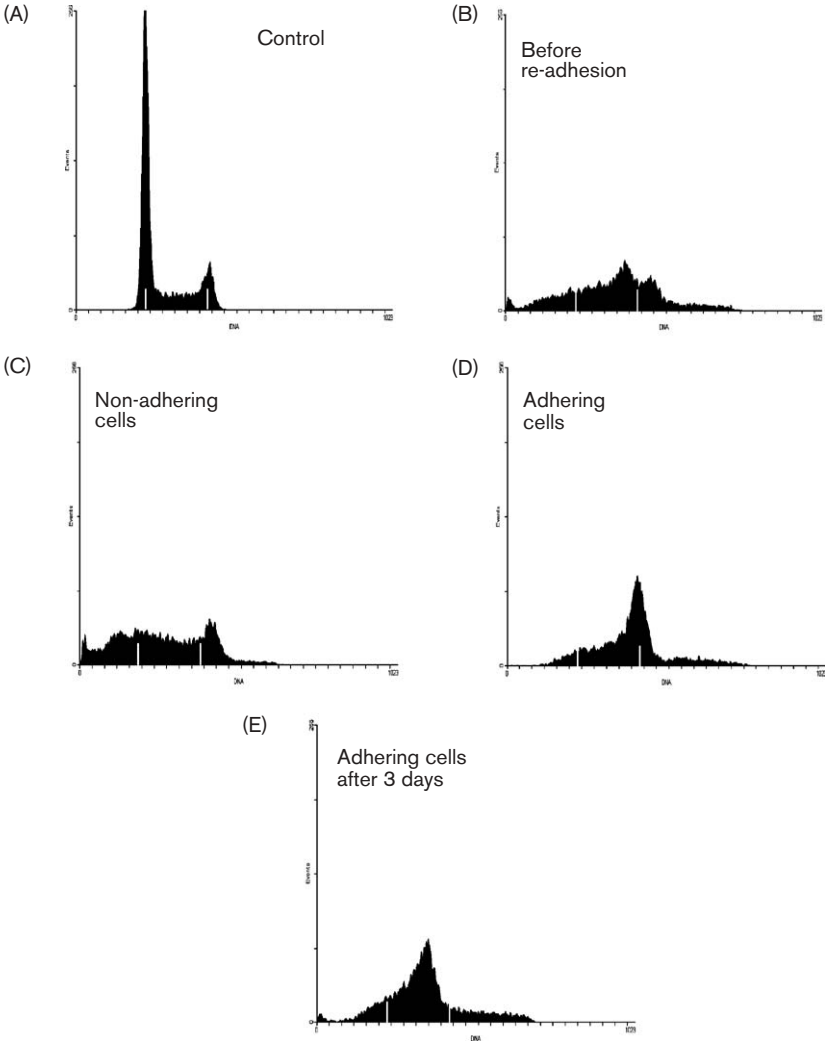
Fig. 4



A slow loss of viability is observed after a cytostatic treatment with paclitaxel in A549 cells. (A) A549 cells were cultured for 4 days (open bars) with paclitaxel (50 nM) or for 4 days plus an additional 3 days of normal culture conditions (hatched bars) (left). Cells were then trypsinized and put back in culture for 3 h or 3 days. Adhering cells were counted as indicated above and results are expressed as cells/well ± SEM ($n=3$). (B) Caveolin-1 levels are increased in viable cells after paclitaxel treatment.

them with paclitaxel 50 nM for 4 days (Fig. 4A). Two experiments were done in parallel. For one experiment, cells were counted after 4 days and put back in culture for 3 h or 3 days. For the other experiment, paclitaxel was removed and cells were kept in DMEM without paclitaxel for 3 days. After this post-treatment period, cells were counted and put back in culture for 3 h or 3 days. As illustrated in Figure 4(B), only half of the paclitaxel-treated cells were able to adhere to the dish after 3 h and these cells did not proliferate after 3 days in culture. Cells incubated without paclitaxel for 3 days after treatment died in a large proportion as we expected. After re-seeding, only 25% of the cells could adhere to the plastic after 3 h and they did not proliferate after 3 days. By immunoblotting analysis (Fig. 4C), we can see for both experiments that treated and post-treated cells express high levels of caveolin-1 after a re-seeding period of 3 h in

Fig. 5



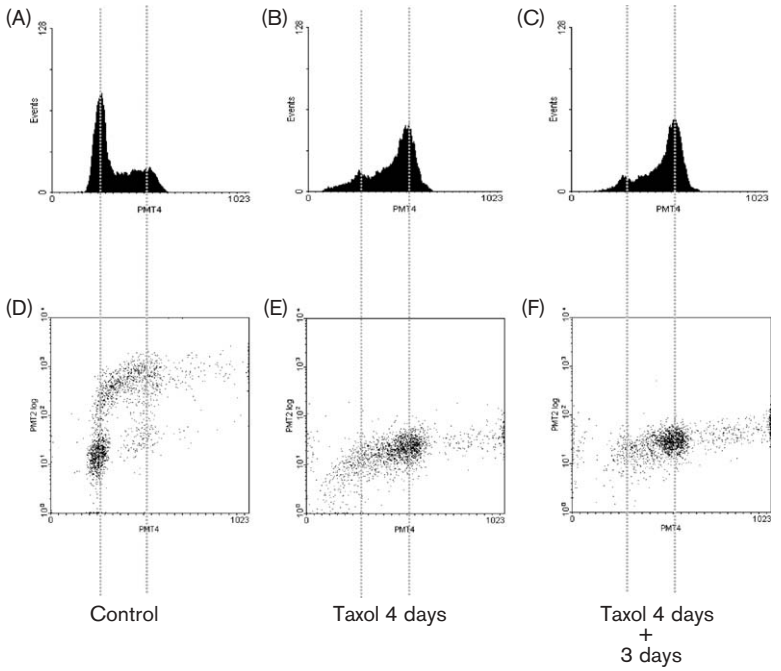
Cell cycle analysis of non-adhering and adhering cells from the experiment described in Fig. 4 (paclitaxel 4 days + no drug for 3 days). (A) Proliferating A549 cells. Note the small white lines indicating the G_0/G_1 (left) and the G_2/M (right) phases of the cycle determined from (A). (B) A549 cells after 7 days (4 + 3) of treatment. Cells were let to adhere under normal culture conditions for 3 h. The cell cycles of non-adhering (C) and adhering cells (D) were then analyzed. (E) Adhering cells after an additional 3 days of culture. Note the left shift of the G_2/M peak present in (D).

comparison with control cells. Levels of caveolin-1 expression after 3 days of incubation were even higher compared to those at 3 h.

The effect of paclitaxel treatment on cell viability was also studied by cell cycle analysis. Cells were harvested at different times: 0, 4 days of paclitaxel 50 nM, 3 h after re-seeding and 3 days after re-seeding. The non-adhering cells were also harvested and analyzed. All these cells were fixed and stained with PI. Figure 5(A) illustrates the cell cycle distribution of normally proliferating A549 cells with a majority of cells in G_0/G_1 phase. After 4 days of treatment (Fig. 5B) paclitaxel treatment caused the expected G_2/M blockade, with an important DNA degradation. The non-adhering cells (Fig. 5C) exhibit an important DNA degradation with some cells in the G_2/M phase. On the hand, adhering cells after 3 h (Fig. 5D) are mainly blocked in the G_2/M phase. After 3 days of incubation, the G_2/M peak is shifted toward the G_1 phase, probably due to DNA degradation.

In order to confirm that no proliferation was taking place after paclitaxel treatment and the recovery period, cells were incubated in the presence of BrdU. Cell cycle analysis is illustrated for A549 cells treated (Fig. 6B) or not (Fig. 6A) with paclitaxel and after a 3-day recovery period (Fig. 6C). The bottom row shows the results of BrdU staining. Cell cycle phases are superimposed on those of the top row. Control cells show the normal distribution of cell cycle with a peak in the G_1 phase

Fig. 6



DNA synthesis is blocked by paclitaxel treatment and remains blocked after drug removal in A549 cells. Cells were cultured for 4 days with (B and E) or without (A and D) paclitaxel 50 nM. Treatment was followed by a 3-day recovery period (C and F). Cells were incubated with BrdU for 3 h before they were collected, counted and fixed. Cells were then double-stained with an anti-BrdU Alexa 488-conjugated antibody and with PI. Flow cytometry analyses were performed to study the cell cycle (A–C) and to evaluate cell proliferation through the cell cycle (D–F). Top histograms show number of events as a function of PI concentration per cell. Bottom histograms show the concentration of BrdU (proliferation) as a function of PI concentration (cell cycle phase).

(Fig. 6A) and a positive staining of BrdU for cells mostly in the S phase (Fig. 6D). Cells treated with paclitaxel for 4 days show the typical cell cycle blockade in the G_2/M phase (Fig. 6B) and cells present in all phases remained negative for BrdU incorporation (Fig. 6E). In cells treated for 4 days and kept in paclitaxel-free medium for 3 additional days, the same pattern is repeated for cell cycle distribution (Fig. 6C) and for evaluation of proliferation with BrdU (Fig. 6E). Cells treated with paclitaxel and found in S phase are probably apoptotic cells rather than proliferating cells when considering the negative staining with BrdU.

Caveolin-1 expression is known to be abolished in transformed fibroblasts as well as in some cancer cell lines [8,12,19]. As mentioned above, we and others have observed that treatment with paclitaxel and other chemotherapeutic agents induces an up-regulation of caveolin-1 expression [14,17]. These results lead us to the hypothesis that caveolin-1 could be a cytotoxicity marker [14]. In the present study, we confirmed that caveolin-1 expression increase was directly related to cell death itself. A link between caveolin-1 and apoptosis has been proposed in the past [20–23]. On the other hand, caveolin has also been associated with cell survival, and its expression is known to be induced in multidrug resistance in cancer cell lines and in metastases of some type of cancers [24–29]. Our results suggest that caveolin-1 was expressed in cells that were viable but unable to undergo division. This situation is reminiscent of high levels of caveolin expression usually found in terminally differentiated cells [1].

We observed that the effects of paclitaxel treatment were still present 6 days after the end of A549 lung cancer cells. We did not observed large amounts of cells undergoing another cycle of DNA replication without division, like others [30,31]. Viable paclitaxel-treated A549 cells were thus unable to initiate DNA replication and normal division, suggesting that paclitaxel binding to microtubules is a long process to reverse.

In conclusion, we observed that caveolin-1 expression is increased in paclitaxel-treated A549 lung cancer cells, suggesting that caveolin has an implication with cell death associated with microtubule-interacting drugs.

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