Apoptosis 2006; 11: 589–605
© 2006 *Springer Science + Business Media, LLC. Manufactured in The United States.*
DOI: 10.1007/s10495-006-4603-7

Regulation of survivin by retinoic acid and its role in paclitaxel-mediated cytotoxicity in MCF-7 breast cancer cells

M. A. Christine Pratt, Min-Ying Niu and L. Isabel Renart

Department Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON, Canada K1H 8M5

Published online: 9 March 2006

The chemotherapeutic drug paclitaxel induces microtubular stabilization and mitotic arrest associated with increased survivin expression. Survivin is a member of the inhibitor of apoptosis (iap) family which is highly expressed in during G2/M phase where it regulates spindle formation during mitosis. It is also constitutively overexpressed in most cancer cells where it may play a role in chemotherapeutic resistance. MCF-7 breast cancer cells stably overexpressing the sense strand of survivin (MCF-7(survivin-S) cells) were more resistant to paclitaxel than cells depleted of survivin (MCF-7(survivin-AS) despite G2/M arrest in both cell lines. However, survivin overexpression did not protect cells relative to control MCF-7(pcDNA3) cells. Paclitaxel-induced cytotoxicity can be enhanced by retinoic acid and here we show that RA strongly reduces survivin expression in MCF-7 cells and prevents paclitaxel-mediated induction of survivin expression. Mitochondrial release of cytochrome c after paclitaxel alone or in combination with RA was weak, however robust Smac release was observed. While RA/paclitaxel-treated MCF-7 (pcDNA3) cultures contained condensed apoptotic nuclei, MCF-7(survivin-S) nuclei were morphologically distinct with hypercondensed disorganized chromatin and released mitochondrial AIF-1. RA also reduced paclitaxel-associated levels of cyclin B1 expression consistent with mitotic exit. MCF-7(survivin-S) cells displayed a 30% increase in >2N/<4N ploidy while there was no change in this compartment in vector control cells following RA/paclitaxel. We propose that RA sensitizes MCF-7 cells to paclitaxel at least in part through survivin downregulation and the promotion of aberrant mitotic progression resulting in apoptosis. In addition we provide biochemical and morphological data which suggest that RA-treated MCF-7(survivin-S) cells can also undergo catastrophic mitosis when exposed to paclitaxel.

Keywords: apoptosis; mitotic catastrophe; paclitaxel; retinoids; survivin.

Correspondence to: M. A. Christine Pratt, Department Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON, Canada K1H 8M5. Tel.: 613-562-5800 x8366; Fax: 613-562-5636; e-mail: cpratt@uottawa.ca

Introduction

The regulation of the intrinsic apoptotic pathway involves control over mitochondrial membrane permeability and release of cytochrome c into the cytoplasm where it is free to interact with Apaf-1 and caspase-9 to form an active "apoptosome" complex inducing a cascade of caspase activity and ensuing cell death.[1] Regulation of caspase activity is afforded by a family of inhibitor of apoptosis (IAP) gene products,[2,3] which contain from one to three domains homologous to the initially described baculovirus IAP repeat (BIR) domain. The proteins cIAP1, cIAP2 and XIAP contain three BIR domains[4] and all inhibit the activity of several caspases.[5] Survivin is the smallest member of the IAP family and encodes only one BIR domain. Interestingly, survivin is overexpressed in most cancer cells but not in most post-mitotic differentiated cells.[6] Although it has been reported that survivin inhibits caspase-9,[7,8] there is no direct binding data and others have disputed this finding.[9] Survivin has however been shown to bind the proapoptotic protein Smac/DIABLO.[10] Following an apoptotic signal the Smac protein is released from the mitochondria and functions by binding to and inhibiting the activity of cytosolic IAPs thus allowing the apoptosome complex to initiate caspase-9 activation and subsequent apoptosis.[11,12] Thus, binding of survivin to Smac could serve to functionally remove the blocking effect of Smac on IAPs thus preventing caspase activation. Survivin is regulated in a cell cycle dependent manner through the regulatory regions of its promoter[13] as well as through stablization in G2/M as a result of cdc2 phosphorylation of Thr 34.[14] The best studied function of survivin has been its role at the mitotic checkpoint. Early in mitosis survivin is located with the microtubules of the mitotic spindle associated with proteins of the centromere. Survivin has been shown to be necessary to maintain mitotic arrest in response to signals from kinetochore proteins which sense spindle tension.[15,16]

The chemotherapeutic drug, paclitaxel (Taxol™) binds to β-tubulin and, in sufficient quantities, can induce microtubule polymerization (stabilization) activating the mitotic spindle checkpoint resulting in a transient mitotic arrest.

With time cells may undergo an abnormal metaphase and proceed into G1 where they can contain either multiple micronuclei or multilobed nuclei. During this tetraploid G1 state, although the cells appear to initiate DNA repair, many proceed to classical apoptosis.[17] Some cells may also be released from the spindle checkpoint as a result of defective cell cycle checkpoints and undergo cell death while progressing through an abnormal mitosis. This form of cell death is known as mitotic catastrophe (reviewed in Castedo *et al.*).[18] These cells become extremely large and, similar to the G1 cells that have exited mitosis without proper chromosome separation and cytokinesis, cells undergoing mitotic catastrophe may also contain multiple micronuclei.[19–21] Consistent with the ability of paclitaxel to induce mitotic arrest, survivin levels are increased following paclitaxel treatment,[22] although this induction may be independent of the G2/M arrest.[23] Based on this observation it has been suggested that paclitaxel may itself induce drug resistance through increasing survivin levels.[22,23] Recent evidence suggests that paclitaxel-mediated mitotic arrest is dependent on survivin based on the observation that survivin expression in survivin defective ovarian carcinoma cells restores paclitaxel-induced mitotic arrest. However, survivin expression did not alter the overall levels of cell death induced by paclitaxel in these cells.[24]

Retinoids such as all-trans retinoic acid (RA) act through α, β and γ isoforms of their receptors which are ligand-dependent transcription factors to promote or inhibit the transcription of genes whose regulatory sequences contain retinoid receptor binding sequences or whose transcription requires a common coactivator.[25] In addition to growth inhibition, RA can also induce apoptosis during normal development and in several cancer cell types (reviewed in Nagy *et al.*)[26] Exposure to retinoids can induce apoptosis in breast cancer cell lines both *in vitro*[27,28] and in mice.[29] Importantly, retinoids have been shown to sensitize MCF-7 breast cancer cells[30,31] as well as prostate cancer cells[32] to paclitaxel.

Based on the ability of paclitaxel to regulate survivin and the observed potentiation of paclitaxel-induced cell death by RA, we wished to determine the role of survivin in paclitaxel-induced cytotoxicity as well as the mechanism of RA-mediated taxol sensitization. In the present study we show that breast cancer cells overexpressing survivin are relatively protected from cell death induced by paclitaxel compared to cells expressing antisense survivin but undergo similar levels of paclitaxel-induced death as cells expressing endogenous survivin levels. Enforced survivin expression did not protect cells from RA-mediated augmentation of paclitaxel-induced cytotoxity relative to cells with endogenous survivin. Collectively, our results suggest that RA promotes mitotic exit of paclitaxel-treated cells resulting in death by differential mechanisms depending on the expression level of survivin.

Materials and methods

Cell Culture: MCF-7 cells and ZR-75 cells were grown in DMEM (high glucose) at 37C/5% CO_2 with 5% fetal bovine serum, 1% non-essential amino acids, 110 μg/ml sodium pyruvate and 10 μg/ml gentamicin. RA was used from a 1mM stock solution in 100% ethanol and paclitaxel added to medium from a 100 uM stock in Me_2SO_4. For treatment with RA, 100 mm plates were seeded with 5×10^5 cells and made 1 μM with RA after attachment for 72 h or for the indicated times. Paclitaxel was added alone to medium for 24 h at 100 nM or for the last 24 h of the incubation in RA (RA/paclitaxel). Controls were treated with the equivalent concentrations of vehicle(s).

Clonogenic assays: Following the RA and paclitaxel treatments 2×10^3 cells from each of the MCF-7 stable cell lines or the untransfected ZR-75 cells were plated on 60 mm dishes and colonies allowed to form over 7 days. For these experiments paclitaxel was added for only 3 h since 24 h exposure results in almost complete cell death in longer term culture. Colonies were fixed with methanol acetone (3:1) and stained with 2% crystal violet then enumerated on a grid. Only colonies with greater than fifty cells were counted.

Isolation of stable transfectants: MCF-7 cells were transfected with empty pcDNA3 or that into which the full length cDNA for survivin was cloned into either in the sense or antisense orientation using Fugene transfection reagent (Roche Diagnostics, Laval, Quebec) according to the manufacturer's directions. Stable colonies were selected in G418 until there was no further cell death then isolated colonies were picked and expanded in the presence of G418 prior to immunoblot analysis for survivin protein.

Plasmids and antibodies: We thank Dr. Alex MacKenzie (CHEO Research Institute, Ottawa) for human survivin cDNA. Anti-cyclin B1 monoclonal and anti-AIF-1 were purchased from Santa Cruz Biotech (Santa Cruz, CA); anti-Smac/DIABLO (CT) was from Pro Sci (Poway, CA); anti-cytochrome c monoclonal antibody was purchased from Pharmingen (Mississauga, ON); anti-mHSP-70 and anti-cytochrome c oxidase was purchased from Molecular Probes (Eugene, OR); anti-survivin was obtained from R&D Systems (Minneapolis, MN); anti-phospho histone H3 was purchased from Sigma-Aldrich, Canada (Oakville, ON); secondary antibodies conjugated to horseradish peroxidase (HRP) or CY3 were obtained from Jackson Laboratories (West Grove, PA).

Mitochondrial fractionation: Subconfluent cultures were collected after trypsinization, centrifuged in medium containing 5% serum and washed in phosphate-buffered saline (PBS)(137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO4, KH_2PO_4). The insoluble microsomal fraction containing mitochondria was isolated as we previously described[33] except that final supernatants were transferred to ultra centrifuge tubes and further clarified following a 30 min centrifugation at $100{,}000 \times g$.

Immunoblot: Cultures were harvested into pre-chilled microfuge tubes in 500 μL RIPA buffer containing a protease inhibitor cocktail. Lysates were processed, subjected to SDS-PAGE and transferred to PVDF membranes for immunodetection and visualization by chemiluminescence. Denistometry was performed using a Kodak Image-Station.

Immunocytochemistry: For Hoechst staining, cells were fixed on coverslips with cold methanol at −20C for 5 min then incubated in a 1:1500 dilution of 1 mg/ml Hoechst 33258 at room temperature, washed with phosphate-buffered saline (PBS) then mounted on coverslips with anti-fade. For immunohistochemistry, cells on coverslips were fixed in 3.7% formaldehyde in methanol prior to reaction with anti-phospho-histone H3. Positive cells were detected by reaction with CY3 conjugated goat anti-rat IgG. Image capture and slide evaluations were performed using a Zeiss Axiophot fluorescence microscope equipped with Northern Eclipse image analysis software (EMPIX Imaging Inc., Mississauga, ON). All histogram bars represent results from evaluation of at least 1200 cells counted in multiple microscopic fields from each coverslip and are representative of at least two separate experiments.

Viable cell staining and enumeration: Culture medium was removed and floating cells were collected by centrifugation. The cell monolayer was rinsed with PBS, trypsinized and pooled with the floating cells. After washing with PBS cells were resuspended in medium and 0.2% trypan blue prior to enumeration using a haemocytometer.

Analysis of caspase activity: Cells were trypsinized and washed twice in PBS then resuspended in PBS at a concentration of 10^6 ml. Detection of caspase activity was performed using the CaspaTag fluorescein caspase VAD activity kit (Intergen, Purchase, NY) containing the inhibitor FAM-VAD-FMK according to the manufacturer's instructions. Caspase activity in camptothecin-treated cells was used as a positive control for caspase activation while cells exposed only to FAM-VAD-FMK and propidium iodide (PI) were used as a negative control to which results were normalized. A total of 3×10^5 cells were analysed in each sample. Flow analysis of labelled cells was performed on a Coulter Epics Altra cytometer equipped with an Argon laser and EXPO II software (Applied Cytometry Systems).

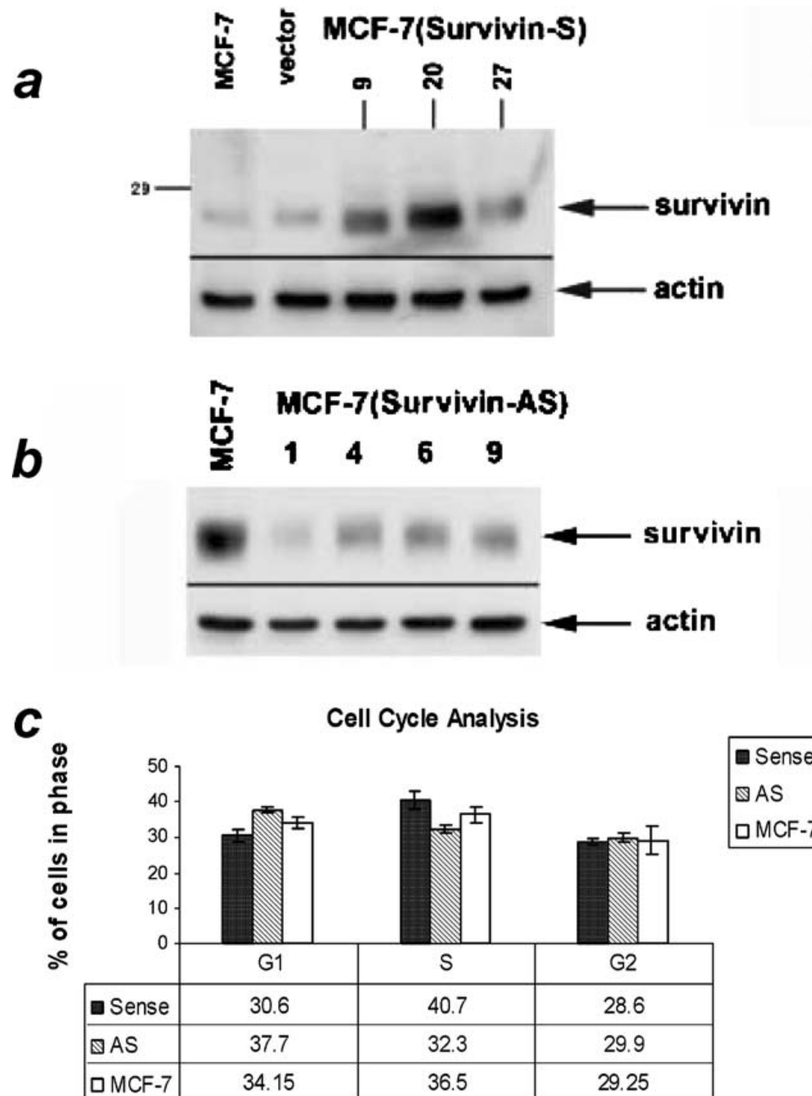
Flow cytometry: Cell monolayers were rinsed with PBS, trypsinized and pelleted then washed twice with PBS. Cell pellets were resuspended in 1ml PBS containing 1mM EDTA and 3 ml of 70% ice cold ethanol then frozen at −20C overnight. Just prior to analysis, cells were pelleted, washed once with cold PBS then resuspended in 1ml PBS/EDTA containing RNAse A at a concentration of 100 ug/ml for 20 min at room temperature. Cells were then transferred to ice and PI added to final concentration of 50 ug/ml. Analysis of DNA contents was performed on the Coulter Epics Altra flow cytometer and data was analyzed using the MultiCycle AV program for Windows™ (Phoenix Flow Systems).

Results

Generation and cell cycle parameters of MCF-7 cells expressing elevated and reduced survivin protein levels

In order to examine the role of survivin in paclitaxel-induced death and RA-mediated sensitization we first generated MCF-7 cell lines constitutively expressing elevated or reduced levels of survivin. Previous studies have shown that cancer cells express higher levels of survivin than non-transformed cells and paclitaxel induces a G2/M arrest and further enhances survivin expression.[22,23] To generate cells with altered cellular survivin levels we generated stable clones of MCF-7 cells expressing survivin cDNA in either the sense or anti-sense orientation. Figure 1(a) shows immunoblot analysis of three MCF-7(survivin-S) stable clones compared with three pooled clones containing empty vector or MCF-7 cells. Clones 9 and 20 were routinely pooled for the experiments described below and contained 3 and 7-fold higher survivin protein levels respectively compared with control cells as determined densitometrically (not shown). The western blot in Figure 1(b) depicts the results of isolation of four clones of MCF-7 cells expressing full-length antisense orientation of survivin from the CMV promoter. Survivin levels in the clones were at least 50% lower than control cells. Note that this blot was exposed longer than that shown in Figure 1a in order to visualize residual survivin. Clones 1, 4 and 9 were routinely pooled for the experiments described below. We next performed cell cycle analysis on the clones in order to both determine what effects differential survivin might have on the cell cycle of MCF-7 cells and also to determine if higher or reduced survivin levels were associated with increases or decreases in G2/M cells. The graph in Figure 1(c) shows that MCF-7(survivin-S) clones display a small decrease in G1 concurrent with a proportional increase in S phase cells when compared with MCF-7(pcDNA3) cells, results which concur with the effects of survivin expression in hepatoma cells.[34] Conversely, MCF-7(survivin-AS) cells display a small increase in G1 phase cells and a similar decrease in S phase cell percentages compared with MCF-7(pcDNA3) clones. Importantly neither sets of clones displayed drastic changes in cell cycle nor any alterations in G2/M phase proportions suggesting that survivin expression alone cannot significantly alter the cell cycle in this model. These results also show that levels of survivin protein expression were primarily the result of ectopic expression of the sense or antisense survivin cDNA and G2/M arrest or prolongation did not contribute to survivin expression.

Figure 1. Generation of MCF-7 clones expressing sense and anti-sense survivin. Individual clones were transfected then selected for G418 resistance, expanded and monitored by immunoblot for survivin expression as described in Methods. (a) Immunoblot analysis of 20 ug of whole cell lysates from three MCF-7 cell stable clones constitutively overexpressing survivin (MCF-7(survivin-S). Samples from three pooled G418-resistant clones transfected with empty pcDNA3 and untransfected MCF-7 cells are labelled vector and MCF-7. (b) Immunoblot of four MCF-7 stable cell clones transfected with the antisense survivin cDNA. Untransfected MCF-7 cells are shown as a positive control. MCF-7(survivin-S) clones 9 and 20 were pooled together and MCF-7(survivin-AS) clones 1, 4 and 9 were pooled together for all subsequent experiments. The same three pooled MCF-7(pcDNA3) clones were used in all experiments. (c) Effects of survivin overexpression and depletion on MCF-7 cell cycle. Subconfluent cultures of MCF-7(survivin-S) and MCF-7(survivin-AS) were analyzed by flow cytometry as described in Methods and compared with MCF-7 untransfected control cells. Bars represent standard error of the mean.



	G1	S	G2
Sense	30.6	40.7	28.6
AS	37.7	32.3	29.9
MCF-7	34.15	36.5	29.25

Depletion of survivin protein enhances paclitaxel-induced apoptotic events but elevated survivin levels do not protect cells relative to endogenous levels

As an IAP family member survivin potentially has direct anti-apoptotic activity and increased expression of survivin may afford protection from cytotoxicity. Although the original experiments described by Wang *et al.*[30] demonstrating RA-mediated sensitization to paclitaxel were performed over a period of 6 days including a 3 day pre-treatment with RA followed by a one hr exposure to paclitaxel then another 3 day culture period prior to determination of cytotoxicity, we have included paclitaxel in the last 24 h of the 3 day RA treatment prior to harvesting of samples for biochemical/morphological analysis or viable cell determinations. This was done in order that we would not miss early/intermediate processes involved in the death of cells induced by these conditions. It has been shown that survivin overexpression can prevent apoptosis in NIH3T3 cells induced by paclitaxel,[34] however these studies did not consider direct effects of survivin expression on

paclitaxel-induced apoptotic events. We therefore isolated mitochondrial and cytoplasmic fractions from MCF-7(pcDNA3), MCF-7(survivin-S) and MCF-7(survivin-AS) clones treated with paclitaxel. The immunoblot in Figure 2(a) depicts release of both cytochrome c and Smac in untreated MCF-7(pcDNA3) and MCF-7(survivin-S) cells and following paclitaxel or RA treatment alone or RA and paclitaxel combination treatment. The panel in (b) shows the same experiment with MCF-7(survivin-AS) cells. Under control untreated conditions the cell lines had little or no cytoplasmic cytochrome c. Release of cytochrome c after paclitaxel or RA alone or in combination with RA was relatively weak in both MCF-7(pcDNA3) and MCF-7(survivin-S) cells. The ratio of mitochondrial cytochrome c to

Figure 2. Effects of RA, paclitaxel and cotreatment with paclitaxel and RA on mitochondrial events. *a and b* Mitochondrial release of cytochrome c, Smac/DIABLO and mHSP-70 in cells with differential survivin expression. MCF-7(pcDNA3), (survivin-S) (a) and (survivin-AS) (b) cells were treated with vehicle, 1 μM RA for 72 h or 100 nM paclitaxel for 24 h either alone or for the last 24 h of RA treatment as described in Methods. Soluble (S) and membrane (M) fractions were isolated as described in methods and subjected to immunoblot for cytochrome c, Smac/DIABLO and mHSP-70. Immunoblotting with cytochrome c oxidase and PCNA was used as a control for loading and integrity of the mitochondrial and soluble fractions respectively. Positions of molecular weight markers are shown on the left and the calculated relative molecular weight of each protein is given (c) Effects of differential survivin expression on caspase activation by paclitaxel and RA treatments. MCF-7(pcDNA3), (survivin-S) and (survivin-AS) cells were treated with 1 μM RA for 72 h, 100 nM paclitaxel or treated with RA for 72 hrs with the addition of paclitaxel for the final 24 h. Caspase activity was detected using the pan-caspase CaspaTag detection kit as described in Methods. The caspase-positive population includes cells that were positive for caspase activity only (FAM+) as well as those that had dual positivity (propidium iodide (PI+) and FAM+) and those stained with PI+ only which represent late stage apoptotic cells.

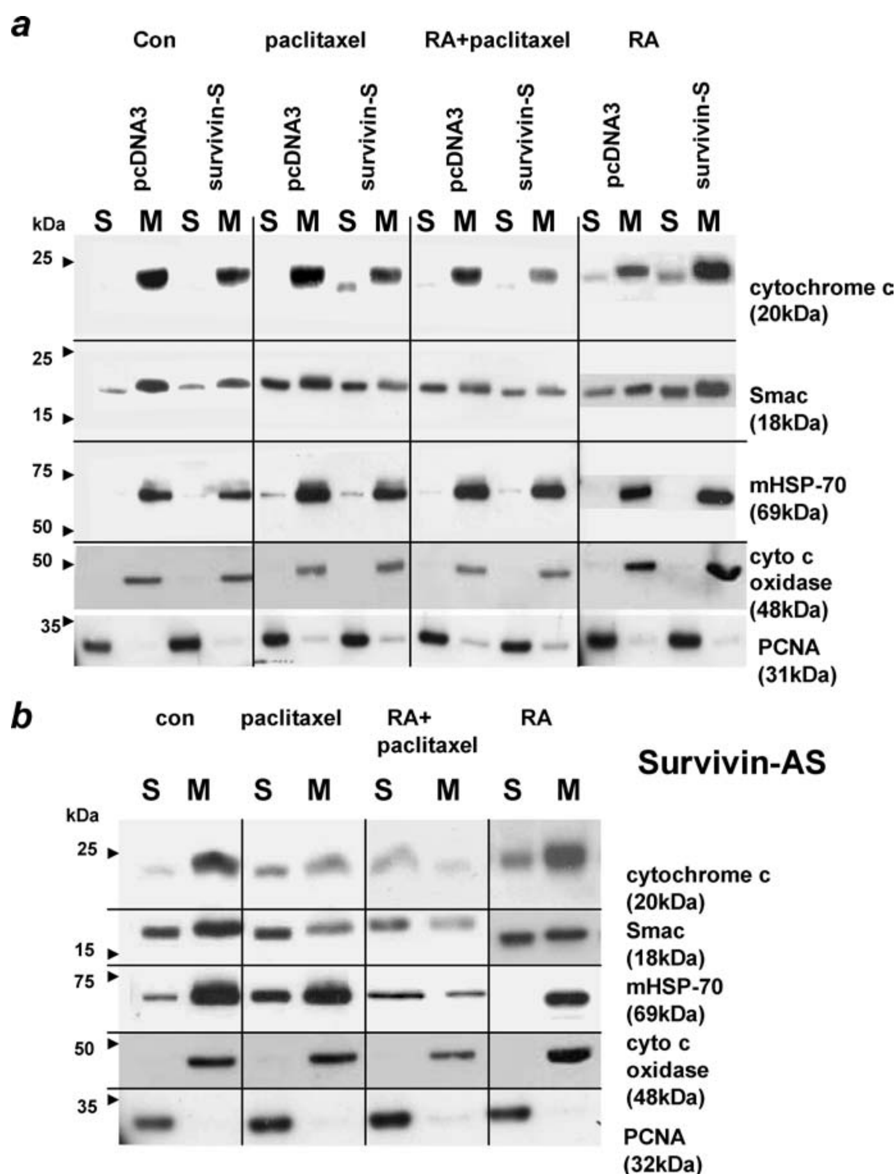
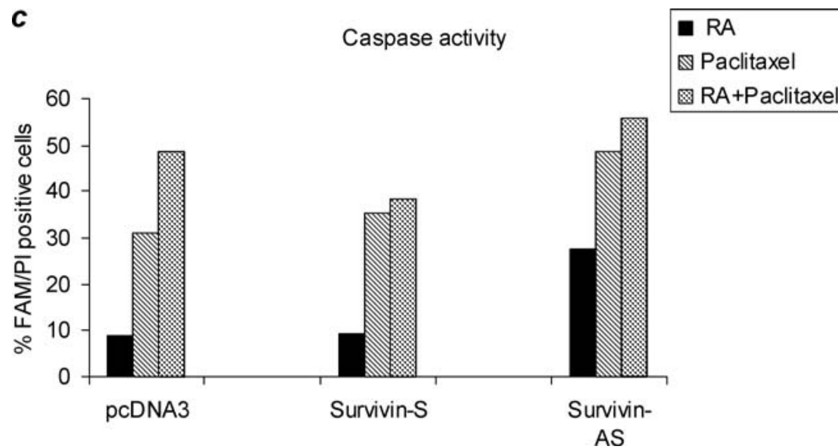


Figure 2. Continued.



cytoplasmic cytochrome c was slightly reduced following RA treatment alone relative to control conditions. Significantly more release was observed in MCF-7(survivin-AS) cells following paclitaxel or the combination treatment. Although very little cytochrome c was visible in the cytoplasmic fraction of MCF-7(survivin-AS) cells there was a significant decrease in mitochondrial cytochrome c levels. This may be indicative of degradation of released cytochrome c since, protein loading of each sample fraction appeared equivalent as determined by cytochrome c oxidase and PCNA reactivity. Although it is not clear why this is, we note that MCF-7(survivin-AS) cells were more fragile than the other two cell lines and amounts of derived protein lysates/volume of cells were lower overall from treated antisense cultures.

Although there were small amounts of Smac detected in the cytoplasmic fraction of both MCF-7(pcDNA3) and (survivin-S) cells, in MCF-7(survivin-AS) cells significant cytoplasmic Smac was tonically present in untreated cells (a mitochondrial:cytoplasmic ratio of approximately 2, determined densitometrically). The ratio of mitochondrial to soluble Smac was lower in MCF-7(survivin-S) cells compared with MCF-7(pcDNA3) following paclitaxel treatment. As with release of cytochrome c, paclitaxel treatment resulted in the strongest Smac release in MCF-7(survivin-AS) cells such that the mitochondrial:cytoplasmic ratio at culture termination was approximately 0.5. Overall cellular levels of Smac appeared decreased in these cells under these conditions. A reduction in released cytoplasmic Smac levels is most likely the result of proteasomal Smac degradation induced by IAP-mediated ubiquitination.[35] The combination of RA and paclitaxel further reduced mitochondrial Smac as well as overall Smac levels in all cell lines. While RA alone increased cytoplasmic Smac levels relative to mitochondrial Smac when compared with untreated cells, paclitaxel alone had a more profound effect on Smac release than did RA alone in all cell lines.

Mitochondrial Hsp70 is localized in the mitochondrial matrix where it functions as a molecular chaperone for protein translocation[36] and serves as a marker for intact mitochondria. Loss of integrity of the outer mitochondrial membrane in late stage apoptosis results in cytoplasmic accumulation of mHsp70. Immunoblot with anti-mHsp70 demonstrated no significant paclitaxel-induced cytoplasmic localization of mHsp70 in MCF-7(pcDNA3) or (survivin-S) cells. As was the case for Smac, some mHsp70 was also present in the cytoplasm of untreated MCF-7(survivin-AS) cells and cytoplasmic mHsp70 increased progressively following paclitaxel and RA/ paclitaxel treatment respectively.

Thus these results indicate that cells depleted of survivin are more sensitive to paclitaxel-induced mitochondrial events than cells expressing endogenous levels of survivin or cells constitutively overexpressing survivin. They also demonstrate that RA treatment further exacerbates release of Smac as well as reduces both cytochrome c and Smac protein levels. Remarkably the data also show that constitutive overexpression of survivin does not reduce mitochondrial release of either cytochrome c or Smac in response to paclitaxel or RA/ paclitaxel.

Differential caspase activation by paclitaxel in cells with altered survivin levels

Since one of the proposed ways in which survivin acts is through caspase inhibition the results above suggested that this protein may prevent caspase-dependent feedback amplification of mitochondrial apoptotic events. To determine if this was the case we measured caspase activity using a fluorescence-tagged FAM-VAD-FMK inhibitor which binds irreversibly to all active caspases except caspase-4 and -10 (Figure 2c). Less than 10% of MCF-7(pcDNA3) and MCF-7(survivin-S) cells were positive for active caspase after a 72 h exposure to RA while this number was significantly higher at 28% in MCF-7(survivin-AS) cells. Taxol alone produced a much higher percentage of caspase-positive cells in both MCF-7(pcDNA3) and (survivin-S) cells with

approximately 30% and 35% positive respectively. This number was increased to nearly 50% in the MCF-7(survivin-AS) cells. Following concomitant treatment with RA and paclitaxel there was a marked increase in caspase activation in MCF-7(pcDNA3) cells up to 50% of the total cell population. In contrast there was only a small increase in caspase activation in the MCF-7(survivin-S) cultures. In MCF-7(survivin-AS) clones in which paclitaxel had already produced a high level of caspase activity, the RA-mediated increase was considerably less than that observed for MCF-7(pcDNA3) cells although the numbers approached 55% of cells. Thus overexpression of survivin does not alter the activation of caspases in response to paclitaxel relative to control cells however these two differ in that the combination of RA and paclitaxel mediates an increase in caspase activity in vector control cells but not in MCF-7(survivin-S) cells suggesting potentially different types of cell death.

Retinoic acid inhibits basal and paclitaxel-induced survivin expression

As discussed above two reports have indicated that RA can sensitize cancer cells including MCF-7 cells such that lower concentrations of paclitaxel can produce similar levels of cell death as those achieved in the presence of higher concentrations of the drug alone.[30,31] Since paclitaxel is known to increase survivin expression, RA might promote paclitaxel cytotoxicity by reducing survivin levels. To assess this we collected protein extracts from MCF-7 cells at various times following RA treatment. The immunoblot in Figure 3(a) shows that levels of survivin began to diminish by 72 h and by 96 h survivin was almost absent in RA-treated cultures. This time frame is similar to the kinetics of RA-induced G1 accumulation,[37] therefore survivin downregulation may have been the result of a reduction in G2/M phase cells. To investigate this, we immunoblotted cellular extracts from MCF-7 and ZR-75 cells as well as stable clones expressing cyclin D1 which, although they contain apoptotic cells after RA-treatment, are partially resistant to the G1 block.[38] If G1 accumulation was the sole factor determining survivin levels, we expected to see a diminution in the RA-mediated decreases in survivin. Figure 3(b) shows that survivin expression was profoundly reduced by RA in both cell lines and was similarly decreased in MCF-7(cycD1) and ZR-75(cycD1) stable cell lines.

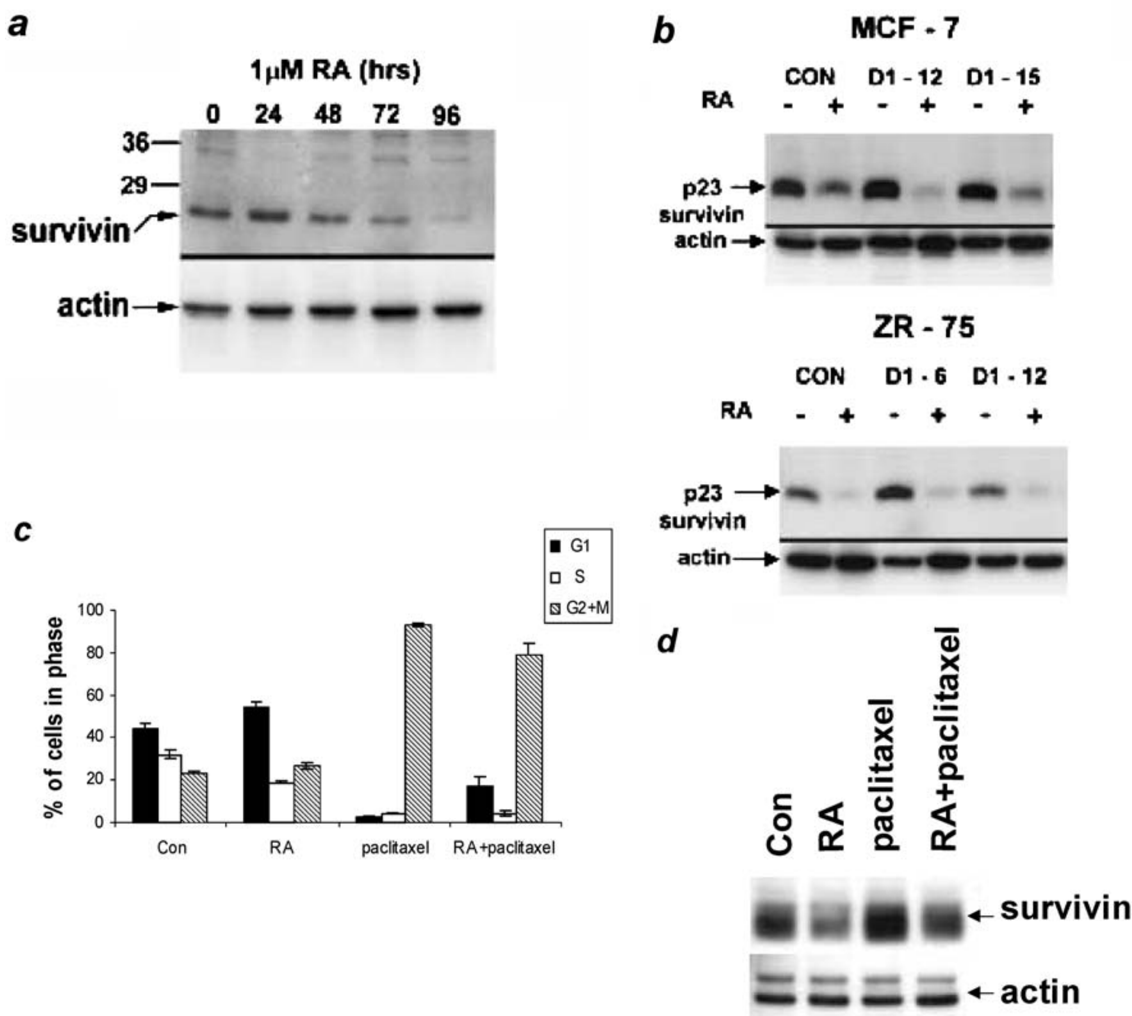
In order to determine the effects of RA on the cell cycle of paclitaxel treated cells we performed flow cytometry. Figure 3(c) indicates that over this time period RA by itself increased the percentage of G1 cells by about 10%. A similar increase was seen after concurrent treatment with paclitaxel and RA essentially at the expense of G2/M cells. However, the percentage of cells in G2/M was still substantial (80%). In agreement with other reports,[22] Figure 3(d) shows that survivin levels were almost doubled following paclitaxel treatment while dual treatment with both RA and paclitaxel reduced survivin levels to those of untreated control cells. Importantly, just over 20% of control cells were in G2/M while in RA and paclitaxel cotreated cells 80% remained in G2/M. (Figure 3c). Thus this result clearly shows that RA reduces paclitaxel-mediated increases in survivin independent of the small reduction in G2/M cells and also supports the possibility that RA may sensitize cells to paclitaxel at least in part by reduction of survivin protein expression.

Exposure to RA prior to paclitaxel treatment results in survivin-dependent differential nuclear morphological changes and increased cell death

Survivin protein levels are normally rapidly reduced during anaphase. Given that survivin is required to stabilize the spindle checkpoint we predicted that an RA-mediated reduction in survivin levels in MCF-7(pcDNA3) cells could result in aberrant progression into G1 rendering them susceptible to apoptotic cell death. On the other hand constitutive expression of survivin in MCF-7(survivin-S) cells might stabilize the paclitaxel-induced mitotic arrest and RA may enforce cell death from mitosis. To get an assessment of the combined effects of RA and paclitaxel on cell death, we calculated the percentage of dead cells using Trypan blue exclusion following treatment with either RA alone, paclitaxel or RA/paclitaxel relative to live cells. These experiments included floating cells and are necessarily performed after only 24 h in paclitaxel with or without RA since longer periods result in significant amounts of cell debris such that the enumeration of detached dead cells would be inaccurate. The results in the graph in Figure 4(a) show that approximately between 15 and 20% of cells were dead after a 24 h exposure to paclitaxel in both the pcDNA3 and survivin-S-transfected cells. This number was nearly 40% in MCF-7(survivin-AS) cell cultures. In MCF-7(pcDNA3) and MCF-7(survivin-S) cells the combination of RA and paclitaxel induced a 35% increase in the population of dead cells above that in the paclitaxel-treated cells. Thus, even over this 24 h period the RA-mediated increases in paclitaxel-induced dead cells were almost additive in the MCF-7(pcDNA3) and MCF-7(survivin-S) cells and were more than additive in the MCF-7(survivin-AS) cells.

As discussed previously, the studies reported by Vivat-Hannah *et al.*[31] and Wang *et al.*[30] demonstrated more significant synergy between RA and paclitaxel than what we have obtained with shorter paclitaxel exposure. To determine the effects of survivin overexpression or depletion on the longer term RA/paclitaxel toxicities, we performed clonogenic assays over a period of 6 days post-paclitaxel treatment. The results in Figure 4(b) show that over the extended period of culture the effects of RA/paclitaxel treatment on colony formation are much more pronounced than those observed with either agent alone. However, consistent with

Figure 3. RA reduces survivin expression in a cell cycle-independent manner and alters paclitaxel-mediated survivin induction and cell cycle effects. (a) Immunoblot analysis of survivin in whole cell extracts of MCF-7 cells treated for the indicated times with 1 μM RA. (b) Extracts from MCF-7 and ZR-75 stable cell lines constitutively expressing cyclin D1 described in Niu *et al.*,[38] treated with RA or vehicle for 96 h were immunoblotted with anti-survivin. (c) Effects of RA and paclitaxel on the cell cycle of MCF-7 cells. MCF-7 cells were treated with ethanol (Con), 1 uM RA for a total of 72 hrs (RA); 10 nM paclitaxel for 48 h, (paclitaxel) or RA for 72 h with the addition of 10 nM paclitaxel for the final 48 h of culture, (RA+paclitaxel). Flow cytometry to determine cell cycle phase was performed as described in Methods. Results shown are means of triplicate determinations and bars represent standard error. *d* Effects of paclitaxel and RA cotreatment on survivin expression. Immunoblot analysis of survivin expression in MCF-7 cells treated with 1 uM RA for 72 h, 100 nM paclitaxel for 24 h or RA for 72 h with the addition of paclitaxel for the final 24 h. Immunoblot with an α-actin antibody was used as an internal control for protein loading.

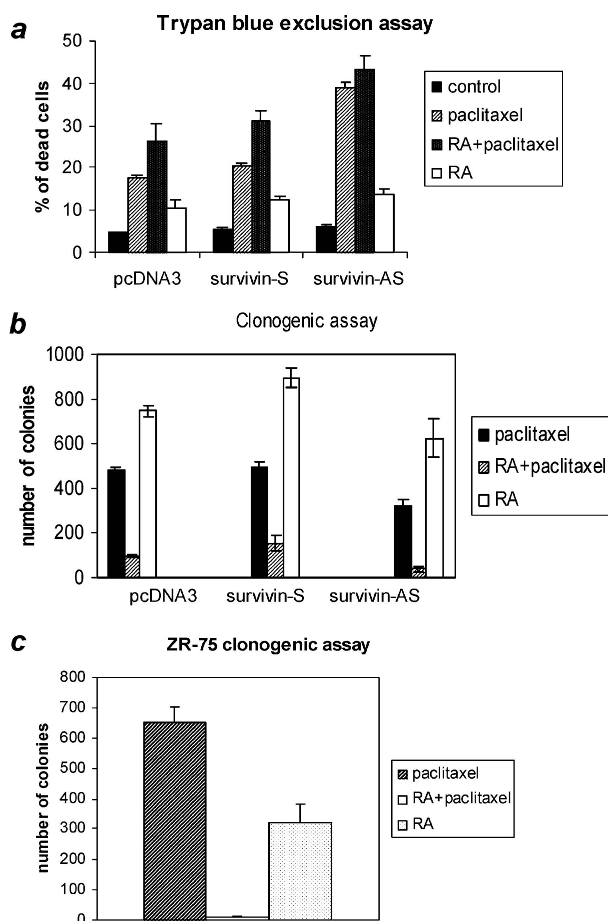


what we observed at the earlier post-treatment time point, survivin overexpression did not confer significant resistance to either paclitaxel alone or RA/paclitaxel relative to MCF-7(pcDNA3) cells in terms of clonogenicity.

In order to confirm the sensitization by RA to paclitaxel cytotoxicity we performed the same experiment on another estrogen receptor positive cell line, ZR-75. The clonogenic assays in Figure 4(c) show that unlike MCF-7 cells which were more sensitive to paclitaxel alone than RA alone, in ZR-75 cells this rank order of sensitivity was reversed. However, as was the case with MCF-7 cells, the combination of RA and paclitaxel reduced colonies significantly over either agent alone. Thus both ER+ cell lines are subject to paclitaxel sensitization by RA despite differences in caspase-3 expression.

In order determine if there were any gross nuclear morphological differences between paclitaxel and combination RA/paclitaxel- treated in the three cell lines we enumerated Hoechst-stained nuclei both for nuclear morphologies as well as cells with highly condensed nuclear DNA as a method to determine the level of apoptosis in each cell line (Figure 5a and b). Following the 24 h paclitaxel treatment many cells with clear mitotic figures were present in all cultures with little evidence of adherent apoptotic cells. Many cells in the MCF-7(pcDNA3) and MCF-7(survivin-S) cultures contained multiple micronuclei which is typical of cells

Figure 4. Effects of paclitaxel, RA and combination RA/paclitaxel on viable cell numbers and clonogenicity in cells with differential survivin expression. (a) The percentage of dead cells were enumerated by trypan blue exclusion following exposure to vehicle, 1 μM RA, 100 nM paclitaxel or the combination RA/paclitaxel treatment as described in Methods. Results are derived from triplicate determinations and are representative of 3 separate experiments. Bars indicate standard error. (b) Clonogenic assay of MCF-7 cell lines: The indicated MCF-7 cell lines were treated as described in Methods, trypsinized following treatment and plated at equivalent densities to allow colony formation for 7 days as described in Methods. Colonies were then stained and enumerated. Bars represent standard errors derived from triplicate determinations. (c) The same experiment as in (b) was performed on ZR-75 cells and the results of the clonogenic assays are shown. Bars represent standard errors based on triplicate determinations.



that have undergone an aberrant cytokinesis. Fewer MCF-7(survivin-AS) cells were multinucleated but notably these cells were capable of morphologically undergoing mitotic arrest in response to the 24 h paclitaxel treatment. Dual RA/paclitaxel treatment resulted in either a reduction or alteration in the appearance of mitotic figures as well as the appearance of larger numbers of apoptotic and floating cells. In RA/paclitaxel-treated MCF-7(pcDNA3) cell cultures mitotic cells were reduced by about 50% although there still remained a large number of cells that retained a mitotic chromatin morphology. This treatment also induced numbers of cells with typical apoptotic morphologies consisting primarily of highly condensed round apoptotic nuclei. In contrast, RA markedly reduced numbers of morphologically mitotic MCF-7(survivin-S) cells relative to paclitaxel alone but gave rise instead to cells containing hypercondensed chromatin aggregates that stained intensely with Hoechst as well as some typical apoptotic nuclei. Hyper-condensation of chromatin is characteristic of cells undergoing mitotic catastrophe.[18] As expected, numbers of mitotic cells were markedly lower in RA-only-treated cells and numbers of apoptotic nuclei were similar to those produced by paclitaxel alone. MCF-7(survivin-AS) cultures contained clearly apoptotic nuclei usually consisting of two adjacent nuclei consistent with telophasic or early G1 nuclei. To confirm the effects of RA and paclitaxel on mitotic cell numbers we stained cells with anti-phospho-histone H3 in a separate experiment to detect cells in M phase (Figure 5c). In general the percentages of mitotic cells positive for phospho-histone H3 under the various culture conditions were similar to what was determined morphologically following Hoechst staining, although we obtained a somewhat higher percentage of anti-phospho-histone H3 positive MCF-7(survivin-S) cells relative to the morphological determinations. Despite this, the effect of RA/paclitaxel treatment relative to paclitaxel alone remained the same in that mitotic cells were significantly reduced by 40 to 50%.

Thus, our results show that in MCF-7(survivin-S) and MCF-7(pcDNA3) cells paclitaxel-induced cytotoxicity is similar as is the level of caspase activation. However, while RA/paclitaxel increased caspase activity in MCF-7(pcDNA3) cells above that obtained with paclitaxel alone, further increases in caspase positive cells were not observed in MCF-7(survivin-S) cultures demonstrating that survivin overexpression is associated with differential caspase activation and nuclear morphologies following paclitaxel/RA treatment relative to control cells.

Taken together these data suggest that RA may promote mitotic progression of paclitaxel-treated cells resulting in morphologically distinct forms of cell death contingent on levels of survivin expression. In particular, RA/paclitaxel treatment of survivin-S expressing cells might promote death through elimination of mitotic cells.

RA differentially alters the percentages of paclitaxel-treated MCF-7(survivin-S) cells with higher order and intermediate DNA ploidy

We reasoned that cells that die out of mitosis with a 4N DNA content will produce DNA degradation products that would be found in a sub-4N population. In order to characterize the effects of paclitaxel and the addition of RA to paclitaxel-treated cells on cellular DNA content, we performed flow cytometry on each of the different cell lines. The histograms in Figure 6a depict the 2N, 4N and 8N DNA peaks obtained and regions of cellular debris/apoptosis are indicated. Note that it is not possible to designate 4N cells

exclusively as G2/M phase since some of these cells may represent tetraploid G1 cells. Cells overexpressing survivin show evidence of a large number of polyploid cells and cells depleted of survivin also have a tendency to become polyploid. In contrast only a very small percentage of control pcDNA-3 transfected cells appeared polyploid.

The addition of paclitaxel strongly increased the percentage of tetraploid cells and those with a >4N DNA content in all cell lines the latter of which can be the result of endoreplication, the process in which cells progress into another S phase in the absence of cytokinesis.[39] Interestingly, MCF-7(survivin-S) cells demonstrated two defined >4N peaks as did (pcDNA3) cells (although to a lesser extent) while MCF-7(survivin-AS) and show a continuous range of >4N DNA content. Assuming that these are tetraploid S phase cells, it is possible that higher cellular levels of survivin may act to stall replication at discreet points. Indeed S phase fractions were also slightly increased in untreated MCF-7(survivin-S) cells. A second interpretation is that these peaks represent aneuploid cells of greater than 4N and less than 8N DNA content induced by paclitaxel treatment in combination with relatively high levels of survivin. This would further stabilize microtubule function and prevent normal sister chromatid exchange. These results contrast with depletion of survivin which would be likely to result in random chromosomal non-disjunction thus generating a more continuous inter-4N to 8N region.

During cell death cells display a reduction in cellular DNA content as a result of DNA degradation. Cells dying from a diploid state will thus generate sub 2N DNA while cells dying from a tetraploid or higher state will, in the same time period, result in higher order DNA degradation products. Thus dying 4N and greater cells will manifest as an increase in the sub-tetraploid level of DNA also occupied by diploid S phase cells. On first inspection, the DNA content histograms are not grossly different between all the cell lines for each treatment group. This is perhaps not unexpected given that changes in DNA content in polyploid cells might be spread over a wide range of values. We therefore quantified changes in the cellular DNA content between and including 2N to 4N, 4N to 8N and 4N as defined in the histograms. Figure 6b shows that, in keeping with the notion that MCF-7(survivin-AS) and (pcDNA3) cells may undergo cell division prior to cell death while those overexpressing

Figure 5. Survivin-dependent nuclear morphological changes following paclitaxel and RA/paclitaxel combination treatment. MCF-7(pcDNA3), (survivin-AS) and (survivin-S) were cultured in RA or paclitaxel alone or in combination as described in Methods. (a) Micrographs of representative Hoechst-stained MCF-7 cells. Paclitaxel-treated cells were predominantly mitotic in all cell lines while RA treated cells were predominantly interphase. Combined treatment of MCF-7(pcDNA3) cells with RA and paclitaxel resulted in increased numbers of apoptotic nuclei (white arrowheads) while many cells remained mitotic. Apoptotic cells were also visible in cultures of MCF-7(survivin-S) cells along with many cells containing hypercondensed chromatin aggregates (black arrows). MCF-7(survivin-AS) cultures contain interphase cells along with many intensely stained apoptotic nuclei. (b) Enumeration of mitotic, apoptotic and hypercondensed chromatin aggregates (HCCA). For each set of cell line data at least 1200 cells were enumerated per slide. (c) Reactivity with anti-phospho-histone H3 was used to confirm the effects of paclitaxel and RA on the percentages of mitotic cells. Values represent percentages of positive cells derived from counting a minimum of 1200 cells per slide.

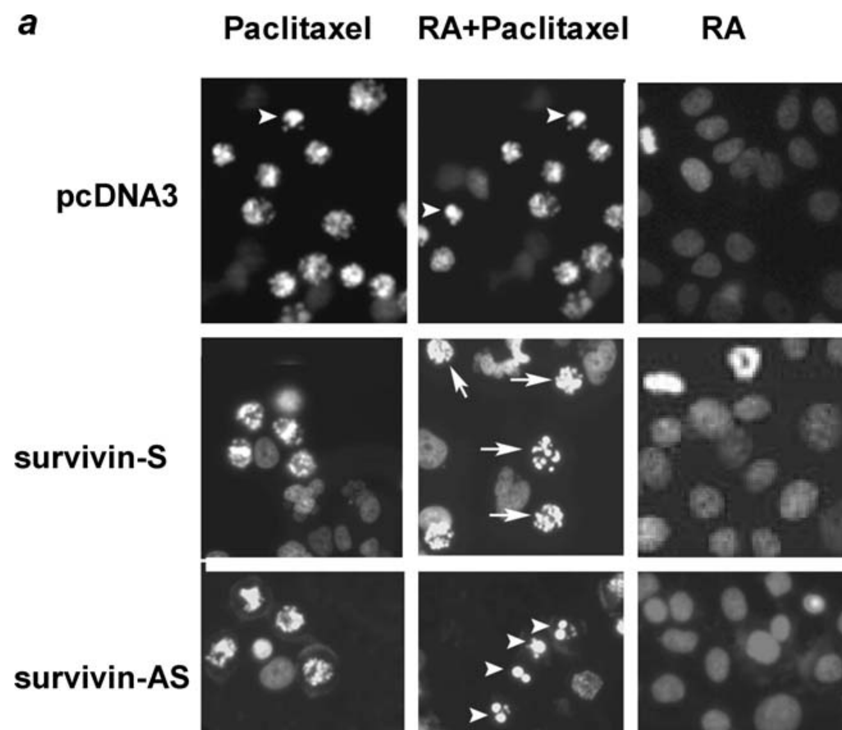
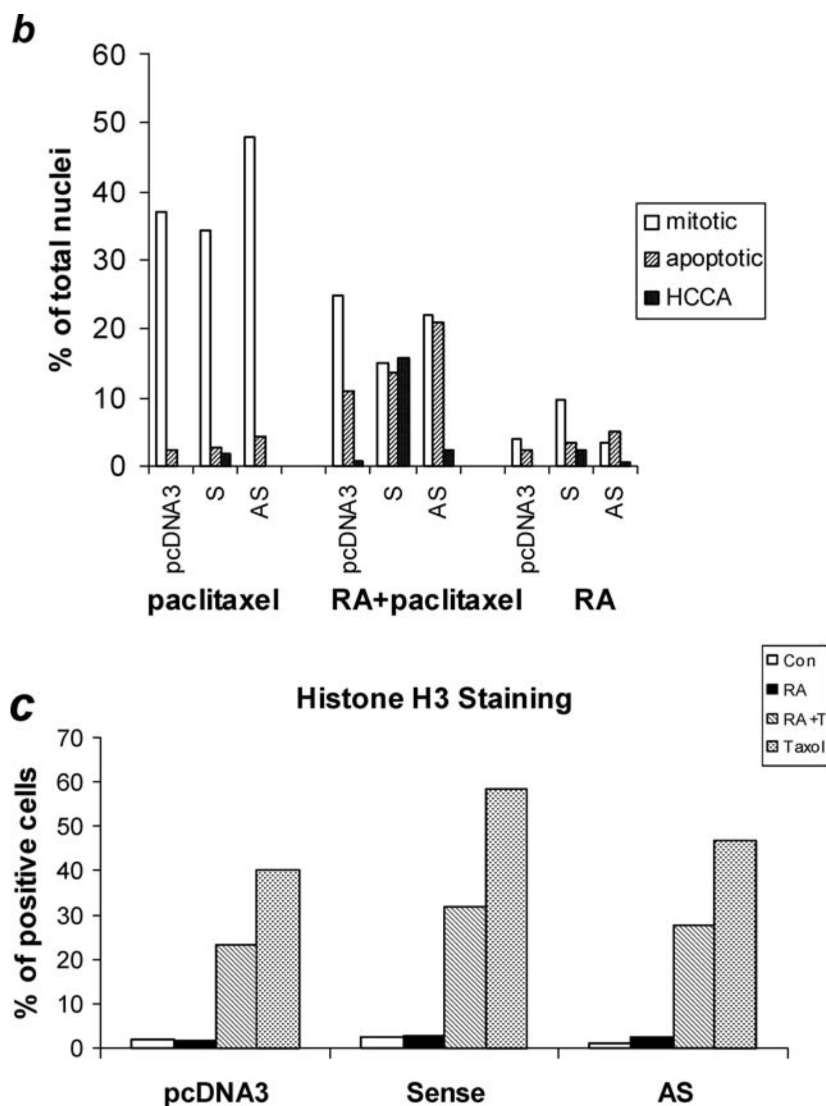


Figure 5. Continued.



survivin are more likely to die in a polyploid state without prior mitosis, RA and paclitaxel strongly reduced the ≥4N population of MCF-7(survivin-S) cells while the ≥2N and <4N levels increased. In contrast RA and paclitaxel had no effect on the same populations of MCF-7(pcDNA3) and (survivin-AS). The large tetraploid population of MCF-7(survivin-AS) cells was also significantly reduced which, based on the nuclear morphology data, appears to be due to post-mitotic apoptosis.

Overall, the significant decrease in the proportion of MCF-7(survivin-S) cells with tetraploid and higher DNA content combined with the increase in cells ranging from 2N up to 4N DNA content accompanied by the nuclear morphological data above are consistent with the survivin-associated induction of mitotic cell death following combination RA and paclitaxel treatment. The same treatment in cells in which RA downregulates survivin (MCF-7(pcDNA3) or in cells depleted of survivin (survivin-AS) cells results in cell division prior to apoptosis.

RA reduces cyclin B1 content and differentially increases cytoplasmic AIF-1

Based on our finding that RA reduces survivin expression, we surmised that RA might facilitate a M→ G1 transition in paclitaxel-treated cells MCF-7(pcDNA3) and MCF-7(survivin-AS) cells. In MCF-7(survivin-S) cells there may be sufficient stabilization of the mitotic spindle to prevent sister chromatid exchange and interfere with cytokinesis, then RA treatment, by promoting mitotic exit might result in mitotic catastrophe. Progression into G1 is expected to be accompanied by the a decrease in the mitotic cyclin B1. Figure 7(a) is an immunoblot of cyclin B1 and survivin from treated cultures. Previous studies determined

that survivin is required for the mitotic spindle checkpoint and accordingly paclitaxel-treated MCF-7(survivin-AS) cells contain the lowest levels of cyclin B1 while MCF-7(pcDNA3) and MCF-7(survivin-S) cells both have similarly higher levels. Consistent with the hypothesis, cyclin B1 levels were strongly reduced in both the latter cells, especially in the MCF-7(pcDNA3) cultures following RA/paclitaxel cotreatment. As expected, RA reduced survivin expression in all cells compared with those cultured in the absence of RA. The lower reduction of survivin expression in MCF-7(survivin-S) cells is due to downregulation of endogenous protein.

Apoptosis inducing factor (AIF-1) is a mitochondrial flavoprotein that can induce cytochrome c release and caspase activation but can also translocate to the nucleus via the cytoplasm causing nuclear fragmentation in a caspase-independent manner.[40,41] Mitotic catastrophe has been reported to occur in the absence of caspase activation and can involve the mitochondrial release of AIF-1.[18] Mitochondrial release of AIF-1 was determined by immunoblot of soluble cytoplasmic fractions from cell lines subjected to these treatments with AIF-1 antibody. Figure 7(b) shows that vehicle-treated control and paclitaxel-treated cells displayed little evidence of AIF-1 release. Similarly RA and

Figure 6. Combined RA and paclitaxel increases sub 4N DNA content. (a) Flow cytometric analysis of DNA content. MCF-7(pcDNA3), (survivin-AS) and (survivin-S) cells were treated with 100 nM paclitaxel for 48 h with or without RA as described in Methods and fixed with ethanol prior to staining with PI. Cells were then analyzed for DNA content using the Multi-cycle program for Windows as described in Methods. DNA content is represented on a linear-log scale and the position of 2N, 4N and 8N cells were determined by calculating the peak mean for each population. Sub G1 DNA is depicted as apoptotic cells and debris (apop/debris). Histograms typical of triplicate determinations are shown. (b) Graphs depicting the percentages of cells with 2N to <4N and >4N to ≤8N intermediate DNA content. Percentages are the means of triplicate samples ± standard error. The percentage change in each of the cell populations following RA and paclitaxel treatment relative to paclitaxel alone is shown.

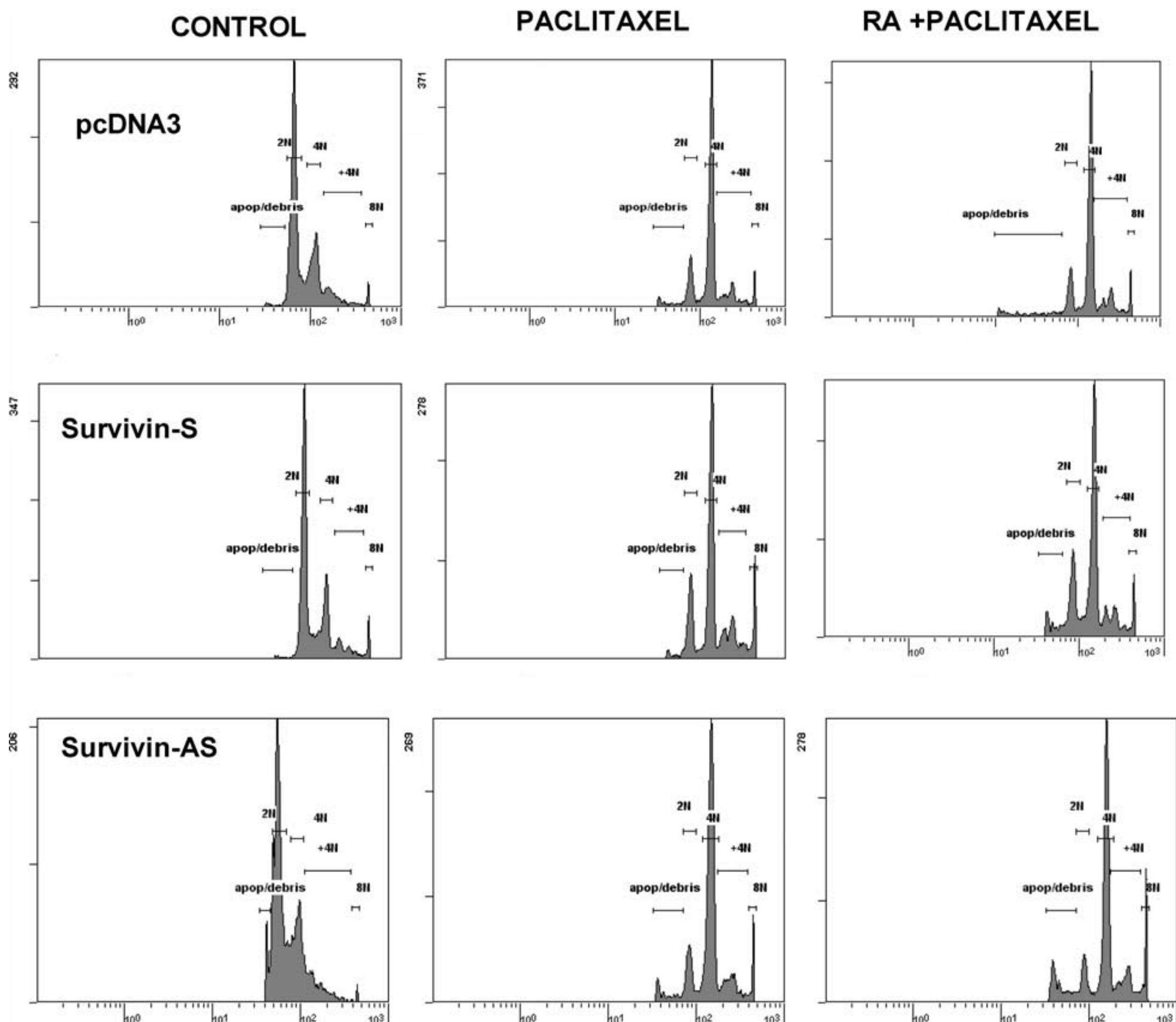
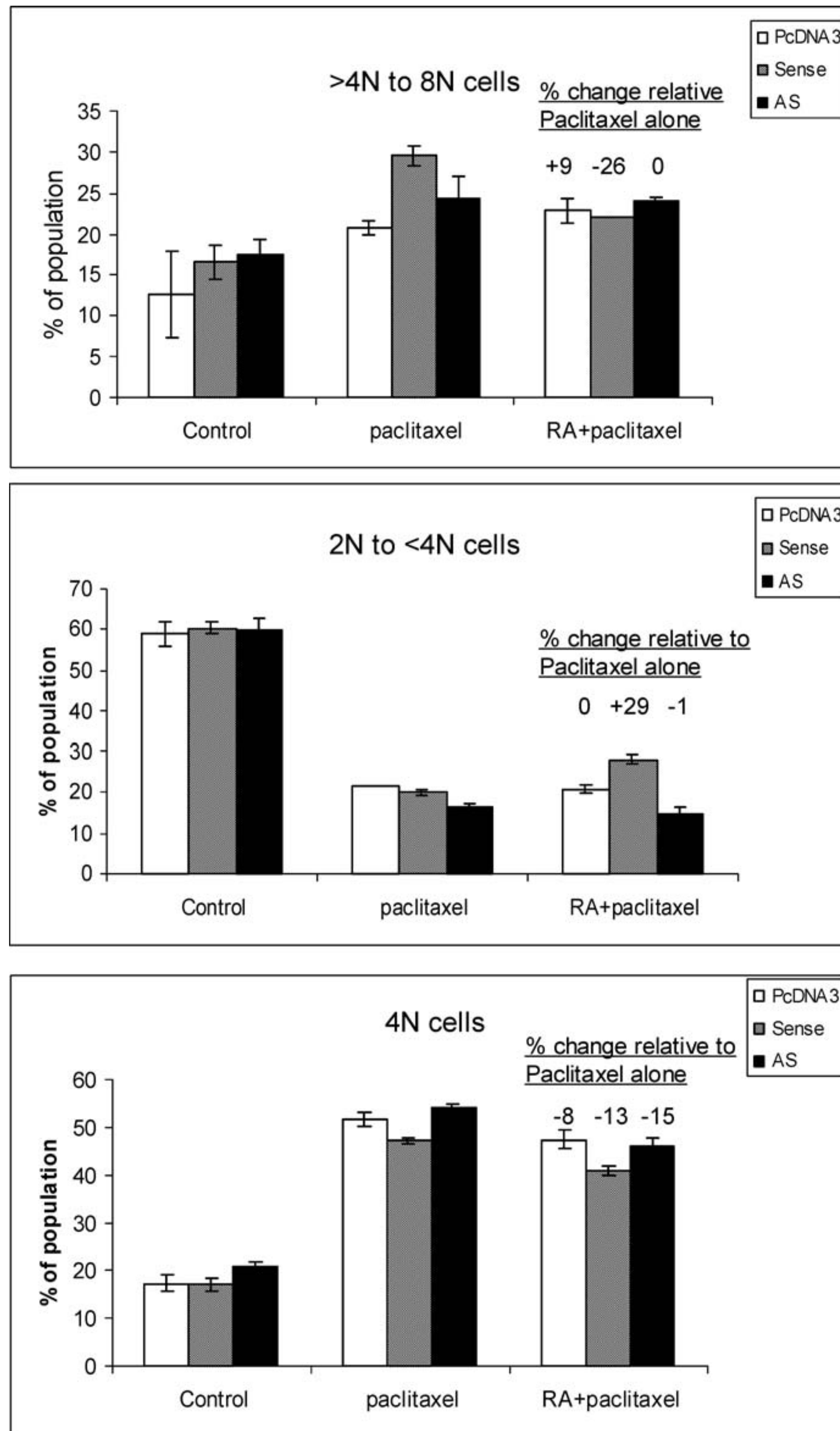
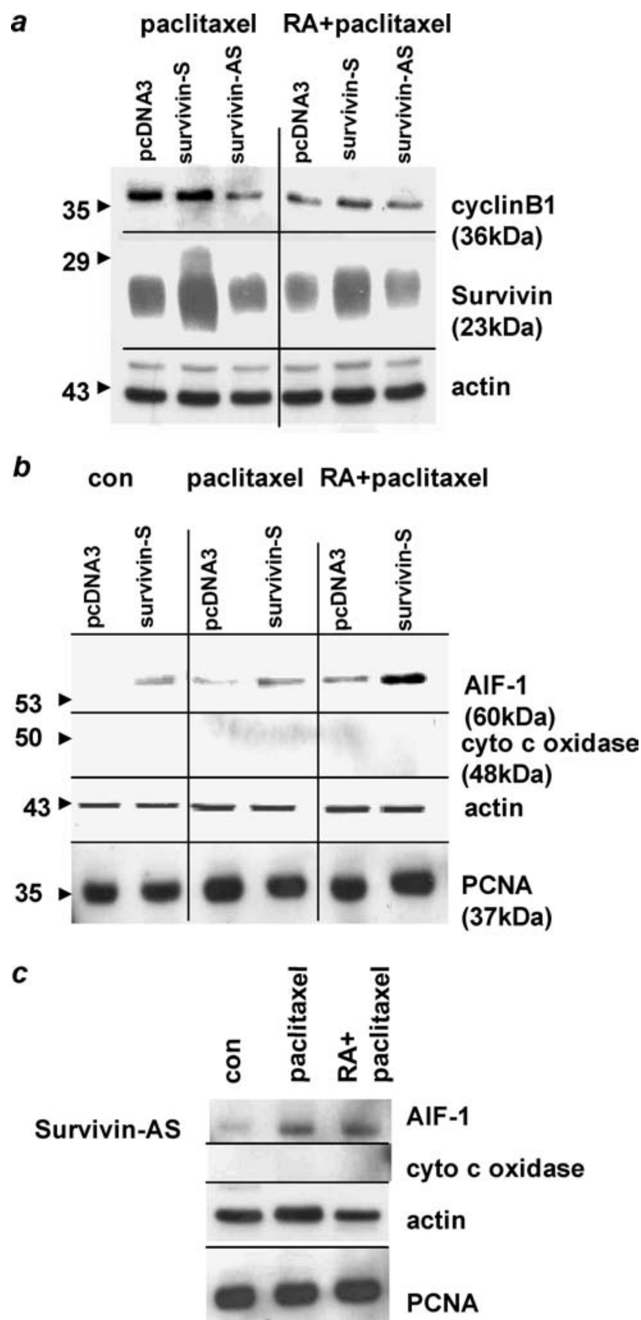


Figure 6. Continued.



paclitaxel combination treatment of MCF-7(pcDNA3) cells induced release of only low levels of AIF-1. In contrast AIF-1 was reproducibly released into the cytoplasm in MCF-7(survivin-S) cells treated with both agents. Thus, under conditions of differential survivin expression, the addition of RA to paclitaxel can induce AIF-1 release which may contribute to a caspase-independent increase in cell death. In MCF-7(survivin-AS) cells AIF-1 was found in the cytoplasm of both paclitaxel-treated and combination RA/paclitaxel-treated cells (Figure 7c) suggesting a role for AIF-1 in

Figure 7. RA reduces cyclin B1 expression in paclitaxel-arrested cells and differentially induces AIF-1 mitochondrial release in MCF-7(survivin-S) cells. (a) Whole cell protein extracts from MCF-7(pcDNA3), (survivin-S) and (survivin-AS) cells treated with 100 nM paclitaxel or cotreated with RA and paclitaxel as described in Methods were subjected to immunoblot analysis with antibodies to cyclin B1 and survivin. Anti-α-actin was used as a control for protein loading. (b) The soluble fractions shown in Figure 2 from MCF-7(pcDNA3), MCF-7(survivin-S) and from MCF-7(survivin-AS) cell lines (c), treated with 100 nM paclitaxel and RA/paclitaxel were subjected to immunoblot analysis with anti-AIF-1. The internal control for mitochondrial contamination reactivity with anti-cytochrome c oxidase and protein loading was standardized with anti-actin and anti-PCNA. The positions of molecular weight markers from each gel is shown on the left and the calculated relative molecular weights of each protein are indicated.



mediating the higher levels of paclitaxel-induced cell death not seen in the other two cell lines. Despite the presence of soluble AIF-1 it is important to note that there were virtually no cells with morphologies consistent with mitotic catastrophe following RA/paclitaxel treatment in this population. It is also noteable that mitochondrial release of both cytochrome c and Smac was considerably greater in MCF-7(surivivin-AS) cells than in the other cell lines after either paclitaxel or RA/paclitaxel. Moreover there was a decrease in the levels of these proteins suggesting that in some cells there may be complete mitochondrial membrane potential collapse. Thus differential AIF-1 release distinguishes survivin-overexpressing cells from those with endogenous survivin but not those depleted of survivin protein.

Together with the reductions in mitotic cells, nuclear morphological data and flow cytometric data these results support the notion that RA promotes mitotic progression facilitating death out of mitosis in cells constitutively overexpressing survivin. On the other hand the vector control clones and cells depleted of survivin appear to preferentially undergo apoptosis.

Discussion

Elevated survivin protein expression is a feature of many cancers.[42,43] Moreover, paclitaxel has been shown to increase survivin expression through a mechanism that may be independent of the G2/M block[23] and this has been proposed to be a mechanism by which paclitaxel itself restricts cytotoxicity. Our finding that survivin overexpression did not protect cells from paclitaxel-induced cytotoxicity shows that increased survivin does not promote paclitaxel resistance. The importance of survivin in the paclitaxel response derives from the mitotic function of survivin wherein it forms a complex with INCENP (inner centromere binding protein) and the aurora B kinase.[22] The mitotic checkpoint is regulated by proteins of the MAD family (mitotic arrest deficient) which bind kinetochores of unattached chromosomes and sense spindle tension[44,45] Survivin is required for normal mitosis since in its absence microtubule formation is disrupted and MAD proteins are prematurely released from kinetochores. It is also been demonstrated to be required for maintaining mitotic arrest in response to microtubule disrupting agents such as paclitaxel[15,16] which reduce spindle tension. Paclitaxel-treated HeLa cells depleted of survivin can progress out of M phase without undergoing cytokinesis resulting in essentially tetraploid G1 phase cells with multiple micronuclei and undergo apoptosis.[16] On the other hand it has been proposed that cells overexpressing survivin may not undergo cell death in G1, but instead be highly susceptible to death by mitotic catastrophe- a function of the ability of overexpressed survivin to stabilize the paclitaxel-induced spindle checkpoint.[18] It is interesting that at least after the 24 h treatment period in 100 nM paclitaxel, MCF-7(survivin-AS) cells depleted of

survivin were still able to undergo mitotic arrest and this was associated with substantial induction of cell death. It is possible that paclitaxel induces sufficient survivin even in these cells to promote M phase arrest. The MCF-7(survivin-S) cells, wherein the paclitaxel-induced mitotic arrest would be predicted to be highly stabilized, underwent significantly less cell death than MCF-7(survivin-AS) cells after paclitaxel treatment. Together these results suggest that the mitotic arrest in paclitaxel-treated cells can be dissociated from its cytotoxicity. This conclusion parallels that of Zhou *et al.*[24] who found that while survivin expression could restore mitotic arrest to paclitaxel-resistant ovarian cancer cells it did not improve cytotoxicity.

Our data suggest that the combination of paclitaxel and RA would result in cells which have stabilized microtubules yet proceed through mitosis into G1 as a result of enforced mitotic slippage, a hypothesis supported by the decreased levels of cyclin B1 as well as the observed reduction in mitotic cells. This would amount to an increase in the rate of mitotic exit in the absence of proper segregation of sister chromatids and cytokinesis. The G1 checkpoint is subsequently activated in these cells and most will then undergo classical apoptosis.[17] However, in cells constitutively overexpressing survivin, RA may alter gene expression to promote mitotic progression while the high survivin levels stabilize the mitotic arrest preventing successful entry into G1, resulting instead in enforced catastrophic mitosis accompanied by the production of higher order DNA degradation products.

Release of AIF-1 has been associated, albeit not exclusively, with mitotic catastrophe and paclitaxel-induced apoptosis[46,47] and its preferential release from MCF-7(survivin-S) cells versus MCF-7(pcDNA3) cells following RA/paclitaxel combination treatment, further supports the contention that significant proportions of these cells undergo the latter form of cell death. Unlike MCF-7(survivin-S) and (pcDNA3) cells, AIF-1 was present in the soluble fraction of MCF-7(survivin-AS) cells after either paclitaxel or RA+paclitaxel treatment. It is possible that the depletion of survivin may result in the priming of a broader spectrum of apoptotic events after cytotoxic challenge, especially given the fact that most cancer cells have elevated levels of survivin wherein it plays a role beyond mitosis.

In both MCF-7(pcDNA3) cells and MCF-7(survivin-S) cells the level of paclitaxel-induced cytochrome c release was relatively low compared with untreated cells. Unlike its minimal effects on cytochrome c release, however, paclitaxel induced a substantial release of Smac from MCF-7(survivin-S) and MCF-7(pcDNA3) cells relative to untreated controls. This suggests that, at least following paclitaxel, differential mitochondrial release of these proteins can occur. Similar to our findings, paclitaxel-arrested G2/M leukemic U937 cells also release little cytochrome c.[48] However, paclitaxel-treated B lymphoid BJAB cells display caspase-3 and -8-dependent release of cytochrome c but little Smac release.[49] Moreover, cell death in these cells was prevented by caspase-3 inhibition. One explanation for the very different pattern of Smac and cytochrome c release we observed in MCF-7 cells may be related to the lack of caspase-3 in MCF-7 cells which could impact on amplification of mitochondrial cytochrome c release. However, the lack of endogenous caspase-3 in MCF-7 cells, does not effect the eventual cytotoxic response of these cells to paclitaxel and RA after longer term culture post-treatment. In fact Wang *et al.*[30] showed that MCF-7 cells treated with RA for 72 h followed by a 1 h exposure to 100 uM paclitaxel resulted in nearly 60% of cells staining positive for Trypan blue after an additional 3 days in culture compared with 40% following RA alone and 20% for paclitaxel only. Thus the combined effect was essentially additive at these concentrations. The RA sensitization was more fully realized at higher concentrations of paclitaxel and after longer culture periods which is consistent with our results following clonogenic assays and those of others[30,31] wherein clear sensitization was observed. In order to observe early apoptotic changes prior to large scale cell death, we performed assays after only 24 h of exposure to paclitaxel (approximately one cell cycle). Thus, as expected, the percentages of dead cells as determined by trypan blue exclusion and enumeration of morphologically apoptotic nuclei in our experiments do not reflect the major sensitization effects of RA on paclitaxel-induced cytotoxicity.

Although caspases are clearly induced by paclitaxel in MCF-7 cells without significant cytochrome c release, it is interesting to note that high levels of cytoplasmic Smac have been shown in ovarian cancer cells to sequester sufficient hIAP-1/-2 activity to result in autoprocessing of caspase-9.[50] The role of caspases in the eventual cell death induced by paclitaxel in MCF-7 cells has been shown to be minimal in at least one study.[51] In fact, collectively, studies have shown that the mechanism of taxane-induced cancer cell death is cell-type dependent and can be either caspase-independent (NSCLC (lung), SKOV (ovarian) and MCF-7), or caspase-dependent (lymphoma).[51,52]

The possibility that retinoids may find utility in combination with standard chemotherapeutics has recently been explored.[53,54] From the standpoint of tumour-specific therapy, RA-mediated downregulation of survivin may be especially important given that survivin is expressed in most common tumors but not normal tissue.[6] While survivin may actually predispose cells to paclitaxel- induced cytotoxicity, it has been shown to inhibit apoptosis induced in many ways including a spectrum of anticancer drugs.[8] In this way survivin downregulation may be a novel mechanism by which retinoids act in conjunction with chemotherapy to effectively lower the survival threshold in cancer cells. Retinoids have also long been recognized as chemopreventive agents.[55] Although it is not yet clear at what point in transformation deregulation of survivin expression begins, based on its ability to impinge on mitotic function, it is possible that survivin may contribute to cancer progression through mitotic dysfunction resulting in polyploidy which has been suggested to result in genomic instability and

eventual aneuploidy[39] Thus retinoid-mediated inhibition of survivin expression might attenuate this phenomenon.

Two groups have shown that survivin overexpression can prevent apoptosis in NIH3T3 cells induced by paclitaxel[16,34] However, as suggested by our data, sufficiently high levels of survivin in cancer cells may also make them susceptible to death through mitotic catastrophe following paclitaxel treatment and therefore confer a degree of cancer cell specificity to paclitaxel-induced cytotoxicity. We propose that RA may, through downregulation of survivin and cyclin B1, succeed in accelerating cell death by enforcing G1 progression resulting in mitotic death in cells with high survivin levels or in apoptosis in cells with lower survivin levels.

Acknowledgments

This work supported by grants 98-B062 and 04-B093 to M.A.C.P. from The American Institute for Cancer Research. We thank Rosanna Lau for expert technical assistance.

References

1. Li P, Nijhawan D, Budihardjo I, *et al.* Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91**: 479–489.
2. Liston P, Roy N, Tamai K, *et al*. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 1996; **379**: 349–353.
3. Deveraux QL, Reed JC. IAP family proteins- suppressor of apoptosis. *Genes Dev* 1999; **13**: 239–252.
4. Miller LK. An exegesis of IAPs: Salvation and surprises from BIR motifs. *Trends Cell Biol* 1999; **9**: 323–328.
5. Deveraux QL, Roy N, Stennicke HR, *et al.* The IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 1998; **17**: 2215–2223.
6. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med* 1997; **3**: 917–921.
7. Li F, Ackermann EJ, Bennett CF, *et al.* Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol* 1999; **1**: 461–466.
8. Tamm L, Want Y, Sausville E, *et al.* The IAP family protein survivin inhibits caspase activity and apoptosis induced by Fas(CDC95), Bax, caspases and anticancer drugs. *Cancer Res* 1998; **59**: 5315–5320.
9. Verdicia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. *Nat Struct Biol* 2000; **7**: 602–608.
10. Song Z, Yao X, Wu M. Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during paclitaxel-induced apoptosis. *J Biol Chem* 2003; **278**: 23130–23140.
11. Du C, Fang M, Li Y, Li L, Wang X. SMAC, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000; **102**: 33–42.
12. Verhagen AM, Ekert PG, Pakusch M, *et al.* Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000; **102**: 43–53.
13. Li F, Altieri DC. The cancer antiapoptosis mouse survivin gene: Characterization of locus and transcriptional requirements of basal and cell cycle dependent expression. *Cancer Res* 1999; **59**: 3143–3151.
14. O'Connor DS, Grossman D, Plescia J, *et al.* Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc Natl Acad Sci USA* 2000; **97**: 13103–13107.
15. Lens SMA, Wolthuis RMF, Klompmaker R, *et al.* Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. *EMBO J* 2003; **22**: 2934–2947.
16. Carvalho A, Carmena M, Sambade C, Earnshaw WC, Wheatley SP. Survivin is required for stable checkpoint activation in paclitaxel-treated HeLa cells. *J Cell Sci* 2003; **116**: 2987–2998.
17. Blajeski AL, Kottke TJ, Kaufmann SH. A multistep model for paclitaxel-induced apoptosis in human breast cancer cell lines. *Exp. Cell Res* 2001; **270**: 277–228.
18. Castedo M, Perfettini J-L, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: A molecular definition. *Oncogene* 2004; **23**: 2825–2837.
19. Swanson PE, Carroll SB, Zhang XF, Mackey MA. Spontaneous premature chromosome condensation, micronucleus formation, and non-apoptotic cell death in heated HeLa S3 cells. Ultrastructural observations. *Am J Pathol* 1995; **146**: 963–997.
20. Iazini F, Mackey MA. Spontaneous premature chromosome condensation and mitotic catastrophe following irradiation of HeLa S3 cells. *Int J Radiat Biol* 1997; **72**: 409–421.
21. Roninson IB, Broude EV, Chang B-D. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resistance Updates* 2001; **4**: 303–313.
22. O'Connor DS, Wall NR, Porter AC, Altieri DC. A p34(cdc2) survival checkpoint in cancer. *Cancer Cell* 2002; **2**: 43–54.
23. Ling X, Bernacki RJ, Brattain MG, Li F. Induction of survivin expression by paclitaxel (paclitaxel) is an early event, which is independent of paclitaxel-mediated G2/M arrest. *J Biol Chem* 2004; **279**: 15196–15203.
24. Zhou J, O'Brate A, Zelnak A, Giannakakou P. Survivin deregulation in β-tubulin mutant ovarian cancer cells underlies their compromised mitotic response to paclitaxel. *Cancer Res* 2004; **64**: 8708–8714.
25. Glass CK, Rosenfeld MG, Rose DW, *et al.* Mechanisms of transcriptional activation by retinoic acid receptors. *Biochem Soc Trans* 1997; **25**: 602–605.
26. Nagy L, Thomazy VA, Heyman RA, Davies PJ. Retinoid-induced apoptosis in normal and neoplastic tissues. *Cell Death Differ* 1998; **5**: 11–19.
27. Mangiarotti R, Danova M, Alberici R, Pillicciari C. All-trans retinoic acid (ATRA)-induced apoptosis is preceded by G1 arrest in human MCF-7 breast cancer cells. *Br J Cancer* 1998; **77**: 186–191.
28. Pratt MAC, Niu MY, White D. Differential regulation of protein expression, growth and apoptosis by natural and synthetic retinoids. *J Cell Biochem* 2003; **90**: 692–708.
29. Elstner E, Muller C, Koshizuka K, *et al.* Ligands for peroxisome proliferator-activated receptor gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *Proc Natl Acad Sci USA* 1998; **95**: 8806–8811.
30. Wang Q, Yang W, Uytingco MS, Christakos S, Wieder R. 1,25-Dihydroxyvitamin D3 and all-trans retinoic acid sensitizes cancer cells to chemotherapy-induced cell death. *Cancer Res* 2000; **60**: 2040–2048.
31. Vivat-Hannah V, You D, Rizzo C, *et al.* Synergistic cytotoxicity exhibited by combination treatment of selective retinoid ligands with paclitaxel (Paclitaxel). *Cancer Res* 2001; **61**: 8703–8711.
32. Nehme A, Varadarajan P, Sellakumar G, *et al.* Modulation of docetaxel-induced apoptosis and cell cycle arrest by all-tran

retinoid acid in prostate cancer cells. *Br J Cancer* 2001; **84**: 1571–1576.

33. Pratt MAC, Niu M-Y. Bcl-2 controls caspase activation following a p53-dependent cyclin D1-induced death signal. *J Biol Chem* 2003; **278**: 14219–14229.
34. Li F, Ambrosini G, Chu EY, *et al.* Cell cycle control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 1998; **396**: 580–584.
35. Hu S, Yang X. Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. *J Biol Chem* 2003; **278**: 10055–10060.
36. Dahlseid JN, Lill R, Green JM, Xu X, Qui Y, Pierce SK. PBP74, a new member of the mammalian 70-kDa heat shock protein family is a mitochondrial protein. *Mol Cell Biol* 1994; **5**: 1265–1275.
37. Teixeira C, Pratt MAC. Cdk2 is a target for retinoic acid-mediated growth inhibition in MCF-7 human breast cancer cells. *Mol Endocrinol* 1997; **11**: 1191–1202.
38. Niu M.-Y, Menard M, Reed JC, Krajewski S, Pratt MAC. Ectopic expression of cyclin D1 amplifies a retinoic acid-induced mitochondrial death pathway in breast cancer cells. *Oncogene* 2001; **20**: 3506–3518.
39. Storchova Z, Pellman D. From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* 2004; **5**: 45–54.
40. Susin SA, Lorenzo HK, Zamzami N, *et al.* Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999; **397**: 441–446.
41. Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzani N, Kroemer G. Apoptosis-inducing factor (AIF): A ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett* 2000; **476**: 118–123.
42. Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem* 1998; **273**: 11177–11182.
43. Chen J, Wu W, Tahir SK, *et al.* Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-dependent growth. *Neoplasia* 2000; **2**: 235–241.
44. Lew DJ, Burke DJ. The spindle assembly and spindle position checkpoints. *Annu Rev Genet* 2003; **37**: 251–282.
45. Shah JV, Botvinick E, Bonday Z, Furnari F, Berns M, Cleveland DW. Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. *Curr Biol* 2004; **14**: 942–952.
46. Shankar SL, Mani S, O'Guin KN, Kandimalla ER, Agrawal S,, Shafit-Zagardo B. Survivin inhibition induces human neural tumor cell death through caspase-independent and -dependent pathways. *J Neurochem* 2001; **79**: 426–436.
47. Ahn HJ, Kim YS, Kim J-U, Han SM, Yang HO. Mechanism of paclitaxel-induced apoptosis in human SKOV3 ovarian carcinoma cells. *J Cell Biochem* 2004; **91**: 1043–1052.
48. Liao PC, Lieu CH. Cell cycle specific induction of apoptosis and necrosis by paclitaxel in leukemic U937 cells. *Life Sci* 2005; **76**: 1623–1639.
49. von Haefen C, Weider T, Essmann F, Schulze-Osthoff K, Dorken B, Daniel PT. Paclitaxel-induced apoptosis in BJAB cell proceeds via a death receptor-independent, caspases-3/-8-driven mitochondrial amplification loop. *Oncogene* 2003; **22**: 236–2247.
50. McNeish IA, Bell S, McKay T, Tenev T, Marani M, Lemoine NR. Expression of Smac/DIABLO in ovarian carcinoma cells induces apoptosis via a caspase-9-mediated pathway. *Exp Cell Res* 2003; **286**: 186–198.
51. Ofir R, Seidman R, Rabinski T, *et al.* Paclitaxel-induced apoptosis in human SKOV3 ovarian and MCF-7 breast cancinoma cells is caspase-3-and caspase-9 independent. *Cell Death Differ* 2002; **9**: 636–642.
52. Huisman C, Ferreira CG, Broker LE, *et al.* Paclitaxel triggers cell death primarily via caspase-independent routes in non-small cell lung cancer cell line NCI-H460. *Clin Cancer Res* 2002; **8**: 596–606.
53. Budman DR, Calabro A. *in vitro* search for synergy and antagonism: Evaluation of docetaxel combinations in breast cancer cell lines. *Breast Cancer Res Treat* 2002; **74**: 41
54. Caliaro MJ, Vitaux P, Lafon C, *et al.* Multifactorial mechanism for the potentiation of cisplatin (CDDP) cytotoxicity by all-trans retinoic acid (ATRA) in human ovarian carcinoma cell lines. *Br J Cancer* 1997; **75**: 333–340.
55. Lippman SM, Lotan R. Advances in the development of retinoids as chemopreventive agents. *J Nutr* 2000; **130**: 479S–482S.