Differential effects of doxorubicin treatment on cell cycle arrest and Skp2 expression in breast cancer cells

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Overexpression of Skp2, the ubiquitin ligase subunit that targets p27 for degradation, is often observed in cancers, and is associated with aggressive tumor proliferation and poor prognosis. As there is no drug at present that specifically targets Skp2, studies were undertaken to examine the effects of commonly used drugs on Skp2 regulation. Doxorubicin is among the most effective antitumor agents used for the management of breast cancer, but its effect on Skp2 expression is unknown. The objective of this study was to examine the effect of doxorubicin on Skp2 expression regulation in breast cancer cell lines. The expression of Skp2 mRNA and the protein levels of Skp2, p27, p21 and cyclin B were examined in doxorubicin-treated MCF-7 and MDA-MB-231 breast cancer cells. The effect of doxorubicin on the cell cycle profile was assessed by fluorescence-activated cell sorting analysis. Doxorubicin decreased Skp2 mRNA and protein levels in MCF-7 cells, but had the opposite effect in MDA-MB-231 cells. p27 levels were slightly decreased, whereas p53 and p21 levels were significantly upregulated in doxorubicin-treated MCF-7 cells. In contrast, p27 levels were unaffected by doxorubicin treatment in MDA-MB-231 cells, but cyclin B levels were markedly increased. Doxorubicin arrested MCF-7 cells at G_1/S and G_2/M checkpoints, whereas MDA-MB-231 cells were arrested at G_2/M only. The differential effects of doxorubicin on Skp2 expression in breast cancer cells depend upon the specific cell cycle checkpoints activated by the drug. These changes induced by doxorubicin, however, do not significantly affect p27 expression in these cell lines, suggesting that the potential of a given drug to alter p27 expression through Skp2 modulation might depend on its specific action on cell cycle arrest. *Anti-Cancer Drugs* 18:1113–1121 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The prognosis and clinical management of patients with breast cancer is commonly determined by traditional clinicopathological factors such as tumor size, grade, lymph node status, and the expression of receptors to estrogen (ER), progesterone and Her-2-*neu* [1]. Patients can, nevertheless, present with significantly different clinical outcomes despite similar clinicopathological features and treatments. Some of these differences can be attributed to alterations in cell cycle regulation, which ultimately lead to rapid tumor proliferation and aggressive tumor behavior. Among the various cell cycle proteins, deregulation of p27 was found to have a particularly important role in the development and progression of cancer [2]. Loss of p27 might contribute to the uncontrolled proliferation of malignant cells, as p27 is a negative regulator of protein kinases, Cdk2/cyclin E and Cdk2/cyclin A, which drive cells to the S phase of the cell division cycle. Numerous studies have shown that downregulation of p27 is associated with poor prognosis in breast cancer [2–5]. The decrease in p27 levels in these cancers was found to be the result of its rapid degradation by the ubiquitin–proteasome pathway, rather than from decreased protein synthesis or gene mutation [6,7]. The main rate-limiting regulator for p27 degradation was identified as an SCF-type ubiquitin ligase complex that contains Skp2 (S phase kinase protein 2) as the specific substrate-recognition subunit [8,9]. Skp2 specifically binds p27 and targets it for degradation by the ubiquitin proteolytic system. The important role of Skp2 in controlling p27 levels in human cancers, including breast cancer, has recently been emphasized [10–14]. It was found that overexpression of Skp2 was associated with low p27 levels, aggressive tumor behavior, and poor disease-free and overall survival. Moreover, the expression of Skp2 was found to be an independent prognostic marker, suggesting that it might have other oncogenic properties. Recent studies suggest that Skp2 also regulates the expression of other important cell cycle factors such as the Cdk inhibitors p21 and p57, c-Myc, and cyclin E, which can in turn have an impact on cancer progression [15–17]. Thus, Skp2 may be a novel target for molecular intervention in breast cancer and the ability to downregulate its expression can potentially be of great benefit in arresting tumor growth. Unfortunately, specific drugs that target Skp2 are unavailable at present and therefore, it is important to explore whether commonly used chemotherapies may downregulate Skp2 expression.

The regulatory effects of many of these drugs on the expression of Skp2, however, are currently unknown.

The anthracycline antibiotic doxorubicin (adriamycin) is among the most effective antitumor drugs used for the management of breast cancer [18]. Despite its common use, its exact mechanisms of action remain obscure. Doxorubicin blocks the activity of topoisomerase II, a DNA unwinding protein, causing a variety of anti-proliferative responses, such as cell cycle arrest, apoptosis and cell death [18–20]. Recent studies have shown that doxorubicin had differential effects on the transcription of several cell cycle-related genes, depending on cell type [21–24]. For example, in MCF-7 cells (p53 wild-type/ER-positive cell line) [25], *cyclin A2*, *Ki67* and *CDC2* genes were repressed in doxorubicin-treated cells, whereas cell cycle inhibitors such as p18 and p21 were induced. In contrast, MDA-MB-231 cells (p53 mutant/ER-negative cell line) [26] were found to initiate a transcriptional response that is distinct from that of MCF-7 cells. Cyclin A1 and p18 were upregulated, but no effect was seen on cyclin A2, Ki67 or CDC2 expression. These differences were suggested to have an important impact on the regulatory effects of doxorubicin on drug susceptibility, cell proliferation and mode of cell death.

Other studies have shown that the expression of p27 may be an important determinant for the efficacy of chemotherapy in breast cancer [27–29]. For example, overexpression of p27 was found to be associated with resistance to doxorubicin in breast cancer patients, whereas low levels of p27 were associated with resistance to docotaxel and trastuzumab *in vivo* in breast cancer cells. The mechanisms associated with p27-related resistance and the involvement of alterations in Skp2 regulation are, however, unknown. Therefore, it is important to examine the effects of chemotherapies on p27 regulation and in particular on the expression of its main regulatory protein Skp2. In this study, we examined the effects of doxorubicin treatment on the expression of Skp2 and other related cell cycle proteins in MCF-7 and MDA-MB-231 breast cancer cell lines, and their association with cell cycle arrest.

Material and methods

Cell cultures

Human breast cancer cell lines MCF-7 and MDA-MB-231 were provided by Dr H. Degani (Weizmann Institute of Science, Rehovot, Israel). As Skp2 levels change during the cell cycle (being normally highest in the S phase and lowest in G_1 phase), we cultured the cells under conditions of similar proliferation rates. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beth Ha'emek, Israel) supplemented with 10% fetal calf serum, 4.5 g/l glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mmol/l glutamine and 1 mmol/l sodium pyruvate at 37°C at 5% CO_2. Under these conditions, the proliferation rate of both cell lines were similar (21–22 h doubling time). All cell culture reagents were purchased from Biological Industries.

Proliferation assays

Cells were seeded in 24-well plates at a concentration of 2×10^4 cells/well for 24 h and then treated with different concentrations of doxorubicin hydrochloride (Alexis Corporation, San Diego, California, USA). Cells were detached from the wells using trypsin at different time points (24–72 h) and counted in duplicate with a hemocytometer. Trypan blue dye (Sigma, St Louis, Missouri, USA) exclusion was used to determine cell viability.

Cell synchronization

To arrest cells in S phase, cells were treated with thymidine (6 mmol/l) for 22 h. To release from thymidine block, cells were washed four times with 5 ml of preheated DMEM and 10 ml of DMEM were added for various time points. For mitotic checkpoint arrest, cells were treated with nocodazole (100 ng/ml) for 16 h and cells were then released by two washes with phosphate-buffered saline (PBS) followed by mitotic shake off to a new plate. Cells were harvested at different time points from 2 to 10 h for either fluorescence-activated cell sorting (FACS) analysis or immunoblot analysis.

Protein extract preparation

Cells were grown in 10-cm dishes until 80% confluence was reached before use. Cells were harvested into ice-cold PBS and pelleted by centrifugation (200*g* for 5 min). Cells were then suspended in one packed-cell volume of lysis buffer [containing 50 mmol/l Tris–HCl pH 7.6, 250 mmol/l NaCl, 10 mmol/l EDTA, 0.5% nonidet P-40, 50 mmol/l NaF, 10 μg/ml N_α-Tosyl-L-lysine chloromethyl ketone hydrochloride, N-*p*-Tosyl-L-phenylalanine chloromethyl ketone and soybean trypsin inhibitor, 1 μg/ml aprotinin and leupeptin, 1 mmol/l DL-dithiothreitol, and 0.1 mmol/l phenylmethanesulfonyl fluoride and sodium vanadate], incubated on ice for 30 min and centrifuged again at 20 000*g* for 15 min. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, California, USA) using bovine albumin as the standard.

Immunoblotting

Aliquots containing 50 μg protein were resolved by electrophoresis on a 12.5% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were probed with mouse monoclonal antibody directed against either Skp2 (1 : 500; Zymed, San Francisco, California, USA), $p27^{Kip1}$ (1 : 500; BD Pharmigen, San Jose, California, USA), cyclin B (1 : 500; BD Pharmigen), $p21^{waf1}$ (1 : 500; BD Pharmigen), Skp1 (1 : 1000; BD

Pharmigen) or p53 (1 : 1000; Santa-Cruz Biotechnology, Santa Cruz, California, USA). As levels of Skp1 do not change in the cell cycle, this protein served as an internal control for normalization with respect to the loading of cellular protein. After washing with Tris-buffered saline Tween-20, the immunoreactive proteins were visualized with horseradish-conjugated antimouse secondary antibody (1 : 10 000 dilution; Pierce Biotechnology, Rockford, Illinois, USA) and an enhanced chemiluminescence system (SuperSignal West Pico; Pierce Biotechnology). All immunoblot experiments were repeated at least three times.

RNA extraction and real-time RT-PCR experiments

Total RNA was extracted by a modification of the acid guanidinium thiocyanate–phenol–chloroform method using Tri-Reagent solution (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturer's instructions. Final pellets were dissolved in 40 µl RNase-free water with 1U/µl RNasin (Promega, Madison, Wisconsin, USA). RNA quantification was performed using spectrophotometry and samples were diluted up to 0.5 µg/µl concentration. The quality of RNA was determined by loading 2 µg of it on RNA-agarose gel (1.2%) and fine concentration corrections were made using UVIgelstarMw software (UVItec, Cambridge, UK). Only intact RNA was used for further experiments. Quantitative real-time RT-PCR analyses for mRNA were performed using a Rotor-Gene 2000 real-time cycler instrument and software (Corbett, Sydney, Australia) with QuantiTec SYBR Green RT-PCR kit (Qiagen, Valencia, California, USA). Phosphoglycerate kinase (PGK), a housekeeping gene, was chosen as an internal standard to control for variability in amplification. For each condition, duplicate test tubes containing 100 ng of total RNA and 400 nmol/l *Skp2* or *PGK* gene primers in a total volume of 25 µl were used. The primers used were Skp2: sense primer GCTGCTAAAGGTCTCTGGTGT and antisense primer AGGCTTAGATTCTGCAACTTG; PGK: sense primer TTTAAGGGTTCCTGGCACTG, antisense primer CAGTTTGGAGCTCCTGGAAG, resulting in one product of either 292 or 200 bp with T_m of 81 and 83°C for *Skp2* and *PGK* genes, respectively. Reaction profiles used were 35 cycles of 95°C for 5 s, 60°C for 20 s and 72°C for 15 s, followed by melting of 72–90°C. The number of copies was drawn from a standard curve of 10^3–10^7 copies/µl for each gene separately, and levels of expression were calculated as the ratio between Skp2 and PGK copies in each RNA sample.

Flow cytometry

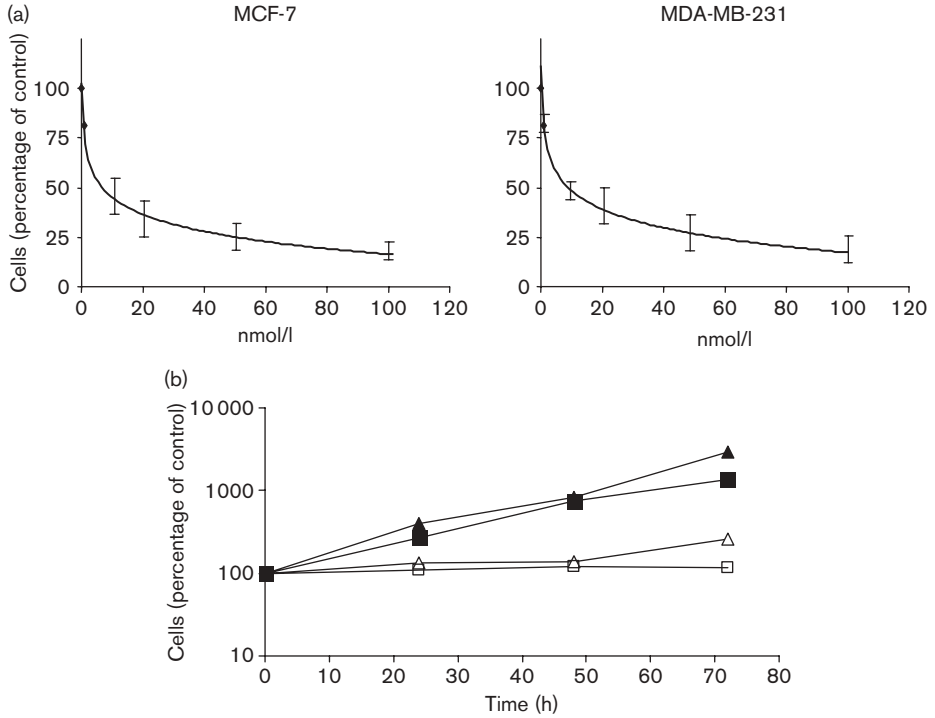
Cells were treated with different concentrations of doxorubicin nocodazole, thymidine or DMSO (0.02%) for 24 h, then trypsinized, resuspended in media and spun down for 5 min at 200*g*. Cells were then washed with PBS, and fixed at a final concentration of 10^6–10^7 cells/ml in 70% ethanol. Samples were kept at 4°C until staining. Fixed cells were incubated with 100 µl of RNase 1 mg/ml for 30 min at 37°C, followed by 20-min incubation with 100 µg/ml propidium iodide in PBS at room temperature. Cells were counted on a FACSCalibur cell sorter using CellQuest software (Beckton Dickinson, Mountain View, California, USA), and the percentages of cells in the G_1, S, and G_2/M phases of the cell cycle were determined.

Results

To examine the dose–effect response of doxorubicin on cellular growth rate in breast carcinoma cells, MDA-MB-231 and MCF-7 cells were exposed to different concentrations of doxorubicin for 72 h. A significant decrease in cell growth rate was observed after exposure to 10 nmol/l of doxorubicin and this effect was maximal at 100 nmol/l in both cell lines (Fig. 1a). Treatment with doxorubicin at a concentration of 1000 nmol/l, however, induced massive cell death in both cell lines (data not shown). To examine the time dependence effect of doxorubicin on cell proliferation, cells were treated with doxorubicin (100 nmol/l) for different time periods and the effect on growth rate was assessed. The inhibitory effect of doxorubicin was evident in both cell lines at 24 h and continued for up to 72 h (Fig. 1b).

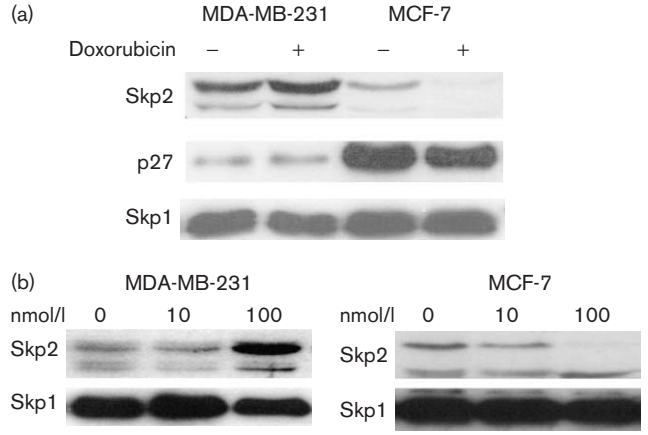
We have previously examined the relationship between the expression of Skp2 and p27 in MCF-7, T47D and MDA-MB-231 breast cancer cell lines [11]. We found that the expression of these proteins was inversely related in all cell lines. Although basal levels of Skp2 were high and p27 levels were low in MDA-MB-231 cells, the opposite condition was, however, found in MCF-7 cells (i.e. low Skp2 levels with high p27 levels). Moreover, we found that treatment with tamoxifen downregulated Skp2 levels in the ER-positive MCF-7 cell line, whereas estrogen upregulated Skp2 levels in a dose-dependent fashion. To determine the effect of doxorubicin treatment on protein expression of Skp2 and p27 in breast cancer cell lines that express different basal Skp2 and p27 protein levels, MCF-7 and MDA-MB-231 cells were both treated with doxorubicin (100 nmol/l) for 24 h and subjected to Western blot analysis. A significant decrease (73.8% ± 9.5) in Skp2 levels was observed in MCF-7-treated cells, but this was associated with a slight decrease in p27 levels (14% ± 4.9) (Fig. 2a). In contrast, we found that Skp2 levels were upregulated (52.0% ± 4.4) in MDA-MB-231-treated cells, whereas p27 levels were unaffected (Fig. 2a). As shown in Fig. 2b, we found that these effects of doxorubicin on Skp2 levels were dose dependent, starting at a concentration of 10 nmol/l and being most significant at a concentration of 100 nmol/l. The changes in Skp2 protein levels were associated with a direct change in Skp2 mRNA levels (Fig. 3a); Skp2 mRNA levels were increased by 30% at 4 h and by 61% at 8 h in MDA-MB-231-treated cells, whereas

Fig. 1



The dose–response curve of doxorubicin on cell growth of breast cancer cells. MCF-7 (a) and MDA-MB-231 (b) cells were treated with different concentrations of doxorubicin for 72 h and the decrease in growth rate compared with control cells was assessed. (b) Time dependence effect of doxorubicin on cell growth. Cells were treated with doxorubicin (100 nmol/l) for different time periods and the decrease in growth rate compared with control cells was assessed. MCF-7 (▲) and MDA-MB-231 (■); black, control cells; white, doxorubicin-treated cells.

Fig. 2

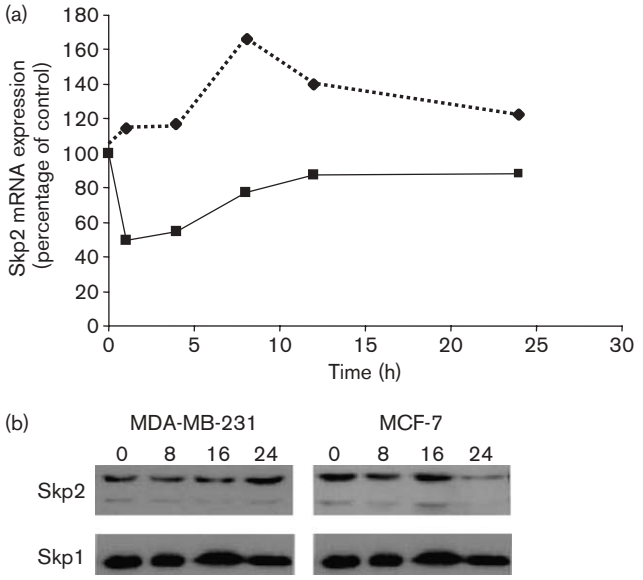


(a) The effect of doxorubicin treatment on the expression of Skp2 and p27 protein levels in MDA-MB-231 and MCF-7 cells. Cells were treated with doxorubicin (100 nmol/l) for 24 h and the expression of the proteins was determined by immunoblot analysis. Skp1 levels were determined to confirm equal protein loading. (b) The dose–response analysis of doxorubicin treatment on the expression of Skp2 protein levels in MDA-MB-231 and MCF-7 cells. Cells were treated with different concentrations of doxorubicin for 24 h and the expression of the proteins was determined by immunoblot analysis. Skp1 levels were determined to confirm equal protein loading.

Skp2 mRNA levels were downregulated in MCF-7-treated cells (by 45 and 25% at 4 h and 8 h, respectively). As shown in Fig. 3b, we did not detect significant changes in Skp2 protein levels before 24 h, suggesting that the observed early changes in Skp2 mRNA levels may not be reflected in protein levels owing to additional translational or posttranslational control mechanisms.

The differential effects of doxorubicin on the expression of Skp2 led us to speculate that doxorubicin arrests the two cell lines at different checkpoints of the cell cycle. To examine the effects of doxorubicin on the cell cycle profile, doxorubicin-treated and vehicle-treated cells were subjected to FACS analysis. The cell cycle distribution in vehicle-treated MCF-7 cells was 48% in G_1 phase, 29% in S phase and 22% in G_2/M phase (Fig. 4). Cell cycle distribution in doxorubicin-treated cells was 54% in G_1 phase, 4% in S phase and 42% in G_2/M phase. Taken together with our observations on the marked inhibition of cell proliferation under these conditions, these results indicate that doxorubicin induced cell cycle arrest of MCF-7 cells at both G_1 and G_2/M checkpoints. We next examined the effect of doxorubicin on the cell cycle profile in MDA-MB-231 cells. Cell cycle distribution in vehicle-treated MDA-MB-231 cells was 46% in G_1

Fig. 3



(a) The effect of doxorubicin on Skp2 mRNA levels in MDA-MB-231 and MCF-7 cells. Cells were treated with doxorubicin (100 nmol/l) for different time points up to 24 h and the change in Skp2 mRNA levels was determined by real-time RT-PCR. MCF-7 (■, continuous line); MDA-MB-231 (●, dashed line). (b) The time–response analysis of doxorubicin treatment on the expression of Skp2 protein levels in MDA-MB-231 and MCF-7 cells. Cells were treated with 100 nmol/l of doxorubicin for 8,16 and 24 h, and the expression of the proteins was determined by immunoblot analysis. Skp1 levels were determined to confirm equal protein loading.

phase, 31% in S phase and 23% in G_2/M phase (Fig. 4). After doxorubicin treatment, 6% of cells were in G_1 phase, 14% in S phase and 80% in G_2/M phase. Thus, in contrast to MCF-7 cells, MDA-MB-231 cells were arrested only in G_2/M, and not in G_1. To determine whether the differences in Skp2 levels between the two cell lines were due to changes in the cell cycle profile, we examined the expression of Skp2 levels in synchronized populations of cells from both cell lines using thymidine arrest and release experiments. Treatment with thymidine (6 mmol/l) for 22 h resulted in cell cycle arrest in S phase (Fig. 5, lane 2). As expected, Skp2 levels were high in both cell lines. After release from thymidine arrest (8 h), nearly 80% of MDA-MB-231 cells and 60% of MCF-7 cells were synchronized in G_2/M, and Skp2 levels were still high in both cell lines, although not as high as in S phase (Fig. 5, lane 3). After release from nocodazole arrest, 60–70% of cells were in G_1 and the rest were in G_2/M (Fig. 5, lane 4). Here, Spk2 levels were lower than in asynchronous (random) cells, presumably because there are practically no cells in the S phase 4 h after nocodazole release. Taken together, the cell cycle synchronization experiments are consistent with the interpretation that part of the observed changes in doxorubicin-treated cells are due to arrests at different stages of the cell cycle.

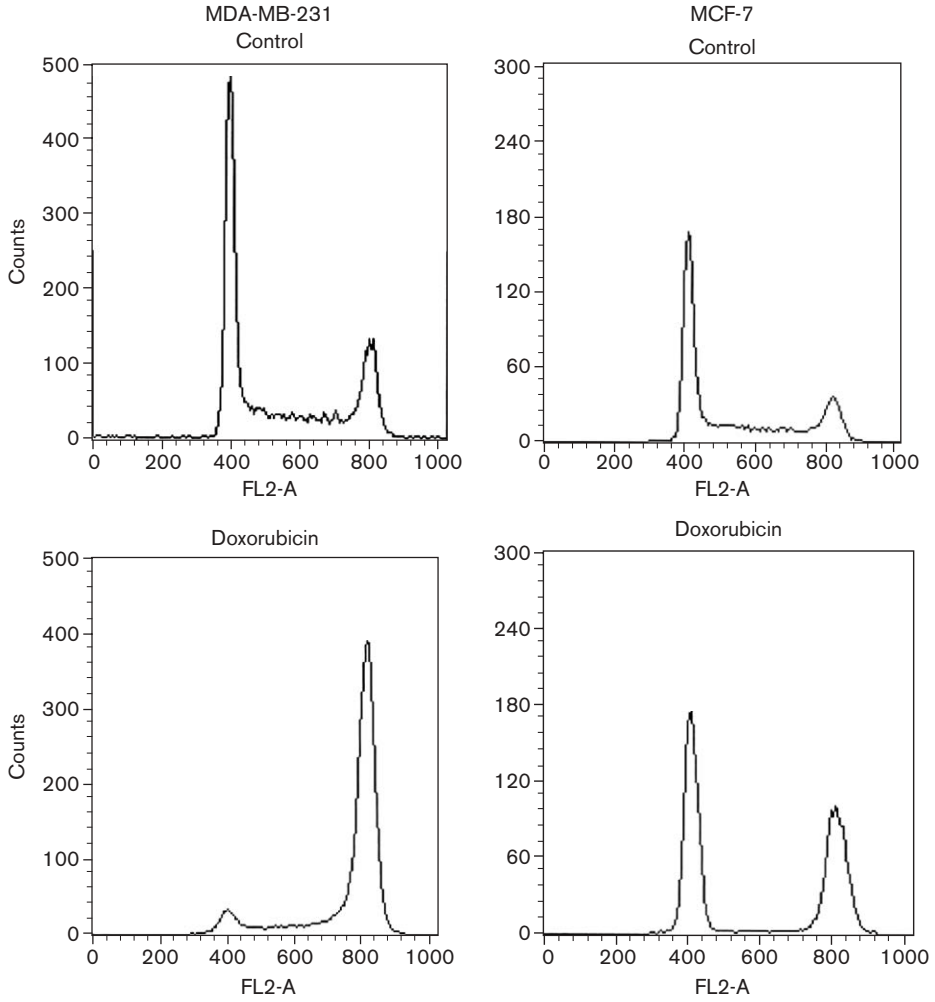
To further elucidate the differential effects of doxorubicin on cell cycle progression, we examined the effect of the drug on cyclin B expression. Cyclin B is a key regulator of the G_2/M transition in the cell cycle. In the normal cell cycle, levels of cyclin B are low at G_1/S and increase significantly at G_2/M. Cyclin B levels were strongly elevated in MDA-MB-231-treated cells, whereas minimal changes in cyclin B levels were observed in MCF-7-treated cells, supporting the findings of differential effects of doxorubicin on cell cycle arrest in the two cell lines (Fig. 6).

MCF-7 is an estrogen-dependent cell line, whereas MDA-MB-231 is estrogen-independent. These cell lines, however, also differ with respect to their *p53* gene status. Whereas MCF-7 cells have wild-type *p53*, MDA-MB-231 cells possess a nonfunctional *p53* mutant [21,22]. Previous studies have shown that doxorubicin induces p53 after DNA damage, which in turn induces the expression of p21. p21 interacts with cyclin D/Cdk4/6 and cyclin E/Cdk2 complexes to inhibit their protein kinase activity, resulting in hypophosphorylated retinoblastoma protein and cell cycle arrest in G_1 phase. To determine the effect of doxorubicin on p21 protein expression in both cell lines, cells were treated with doxorubicin (100 nmol/l) for 24 h and subjected to immunoblot analysis. A drastic increase in p21 levels was found in MCF-7-treated cells, whereas minimal induction of p21 was observed in MDA-MB-231 cells (Fig. 5). These effects of doxorubicin were accompanied by a significant increase in p53 levels in MCF-7 cells. As expected, levels of p53 were very high in MDA-MB-231 cells owing to decreased degradation of the mutant protein and were not significantly affected by doxorubicin treatment. Taken together, these results suggest that the induction of p21 through activation of p53 might have an important role in arresting cells at G_1 in doxorubicin-treated MCF-7 cells, whereas MDA-MB-231 cells were arrested only by the G_2/M checkpoint mechanism activated by doxorubicin.

Discussion

Recent studies have provided evidence that alterations in the expression of different cell cycle regulatory proteins can have a significant impact on the progression and outcome of cancer in general, and of breast cancer in particular. Among these cell cycle regulatory proteins, the oncogenic role of Skp2 in breast cancer has been clearly demonstrated [10,11]. Through mechanisms that have not yet been completely understood, Skp2 is overexpressed in some cancers, and conducts rapid ubiquitin-mediated degradation of the cell cycle inhibitor p27 and is associated with poor disease-free and overall survival. Moreover, Skp2 was found to be involved in the degradation of p21 and other cell cycle regulatory proteins, including c-Myc, cyclin E, p130 and E2F1,

Fig. 4

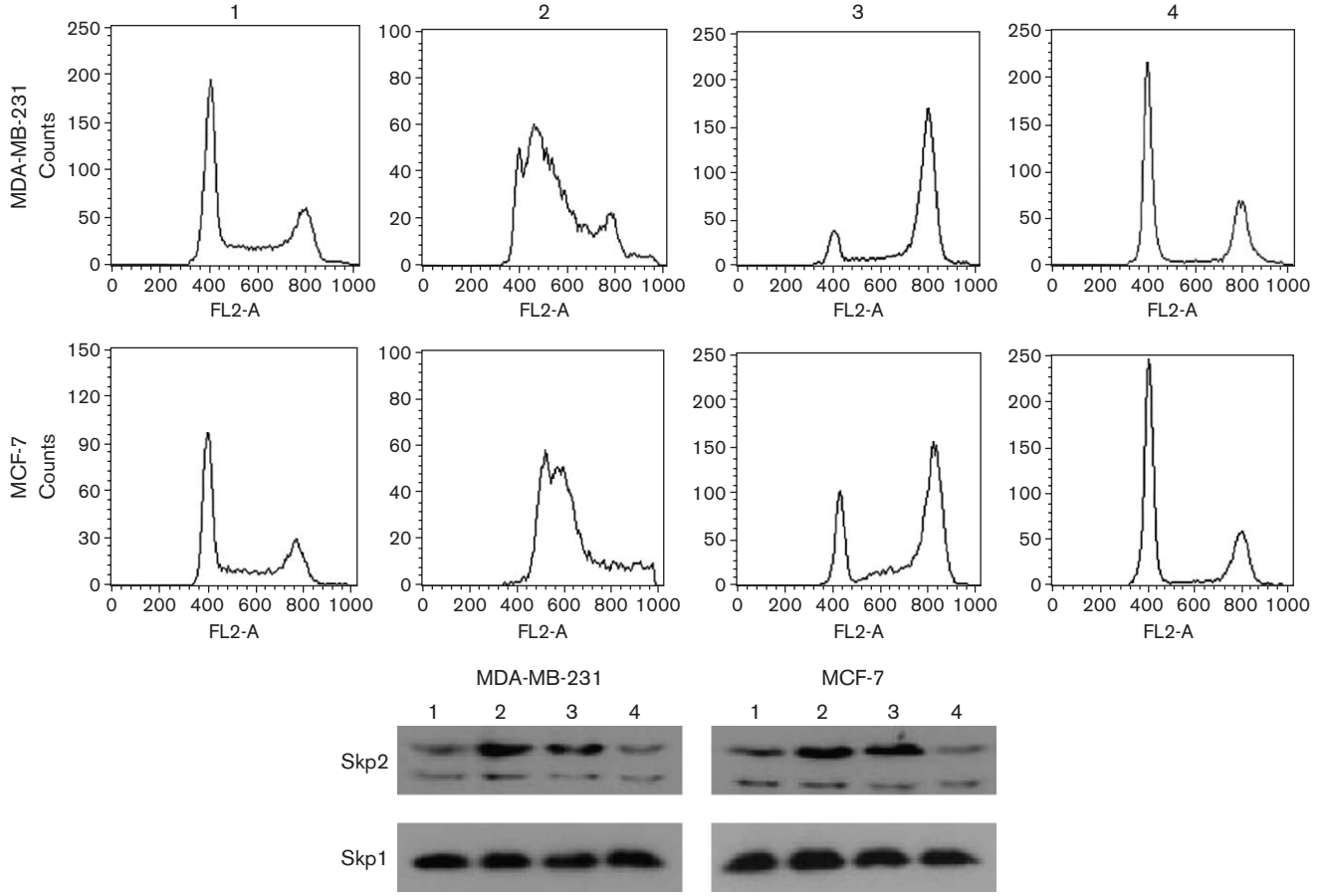


The effect of doxorubicin on cell cycle profile in MDA-MB-231 and MCF-7 cells. Cells were treated with doxorubicin (100 nmol/l) or vehicle (double-distilled water) control for 24 h and subjected to FACS analysis to determine the profile of the cell cycle. FACS, fluorescence-activated cell sorting.

which can also promote cancer progression [15–17]. Thus, the identification of therapeutic interventions that can downregulate the expression of Skp2 in cancer might potentially lead to a significant decrease in cancer progression in subsets of tumors expressing high Skp2 levels. At present there are no specific drugs that can target Skp2 *in vivo*. Small interfering RNA, which can effectively downregulate Skp2 levels *in vitro*, are not yet applicable for clinical use. Thus, studies are now being undertaken to examine the effects of different chemotherapies on Skp2 expression. In a recent study from our laboratory, we examined the effect of rapamycin, an inhibitor of mammalian target of rapamycin, which is being examined now in breast cancer in phase II and III trials [30]. Rapamycin downregulated Skp2 in both MCF-7 and MDA-MB-231 breast cancer cell lines in a dose-dependent fashion leading to increased expression of p27 levels. In this study we found that rapamycin arrested both cell types in G_1 phase.

In this study, we examined the effects of doxorubicin on the expression of Skp2 in MCF-7 and MDA-MB-231 breast cancer cell lines. We found that doxorubicin had completely opposite effects on Skp2 regulation in the different cell lines. In MCF-7 cells, doxorubicin inhibited both Skp2 mRNA and protein expression in a dose-dependent fashion. In contrast, in MDA-MB-231, a cell line that expresses high basal Skp2 levels, we found that doxorubicin upregulated Skp2 mRNA and protein levels. We, however, believe that the rapid changes in levels of Skp2 mRNA after doxorubicin treatment might result from a direct influence of the drug on Skp2 expression, rather than as a secondary outcome of cell cycle arrest. Our results suggest that these differences can be the

Fig. 5

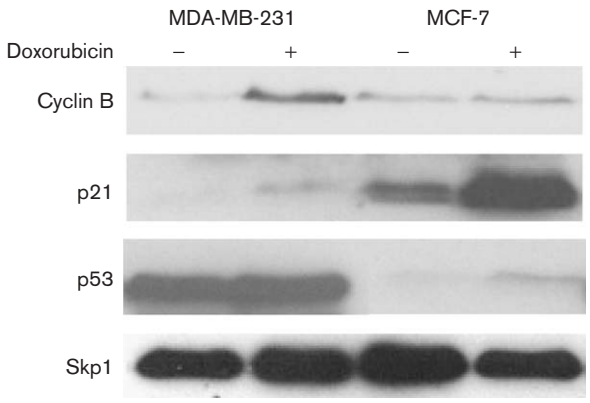


The expression of Skp2 protein levels in relation to the cell cycle profile of synchronized cells. Analysis was performed using FACS and immunoblotting. MDA-MB-231 and MCF-7 cells were synchronized by thymidine arrest and nocodazole arrest as described in Material and methods. Lane 1, random cells; lane 2, cells blocked with thymidine (22 h); lane 3, cells released from thymidine (8 h); lane 4, cells released from nocodazole block (4 h). FACS, fluorescence-activated cell sorting.

result of doxorubicin-induced arrest at different checkpoints of the cell cycle. In both cell lines, doxorubicin effectively inhibited cell proliferation; but whereas MCF-7 cells were arrested in both G_1 and G_2/M, MDA-MB-231 cells were arrested in G_2/M only.

We postulate that these findings might be associated with differential activity of p53 in the two cell lines. MCF-7 cell possess a wild-type *p53* gene, whereas in MDA-MB-231 cells this gene is mutated and is functionally inactive [23,24]. After doxorubicin-mediated DNA damage, p53 is thus activated in MCF-7 cells and induces massive p21 upregulation, which leads to cell cycle arrest in G_1. The increase in p21 can also be attributed, at least in part, to the decrease in Skp2 levels, as Skp2 is also the ubiquitin ligase that targets p21 for degradation. Moreover, the dramatic increase in levels of p21, rather than the slight increase in the percentage of cells in G_1, can explain why MCF-7 cells are not blocked only at the G_2/M checkpoint. As p53 is inactive in MDA-MB-231 cells, p21 levels do not change significantly but the G_2/M checkpoint mechanism is effectively activated, explaining the massive arrest in G_2/M. These observations can explain the differential effects of doxorubicin on Skp2 expression in these cell lines; Skp2 levels are highest in the S phase and are relatively high in G_2/M, but decrease significantly in G_1 phase. In MCF-7 cells, doxorubicin arrested cells also in G_1 leading to the decrease in Skp2 levels, whereas in MDA-MB-231 cells, cells were arrested only in G_2/M, resulting in minimal cells at G_1 and thus leading to upregulation of overall Skp2 levels in immunoblots. Moreover, although MCF-7 cells might be expected to show some increase in the levels of Skp2 and cyclin B as they are arrested in G_2/M, doxorubicin caused a more drastic decrease of the S phase (during which levels of Skp2 are highest) in these cells compared with the MDA-MB-231 cells (Fig. 4). This phenomenon, in addition to the less prominent increase of cells blocked in

Fig. 6



The effect of doxorubicin-treatment on the expression of cyclin B, p21 and p53 in MDA-MB-231 and MCF-7 cells. Cells were treated with doxorubicin [(100 nmol/l) for 24 h] and the expression of the proteins was determined by immunoblot analysis. Skp1 levels were used to determine protein loading.

G_2/M in MCF-7 cells, contributes to the overall decrease in the levels of Skp2 in immunoblots, which reflects the average levels of this protein. Levels of cyclin B are actually slightly increased in doxorubicin-treated MCF-7 cells, although this is not as dramatic as in MDA-MB-231 cells (Fig. 6).

Another important finding is the observation that the changes in p27 levels in doxorubicin-treated cells were not associated with the expected changes in Skp2 levels. Despite an increase in Skp2 levels, p27 levels were thus not downregulated in MDA-MB-231 cells, whereas the decrease in Skp2 levels in MCF-7 cells did not translate to p27 upregulation. In fact, p27 levels were slightly decreased in this cell line. These findings are in contrast to our previous observations in estrogen-modulated breast cancer cells, whereby estrogen treatment increased Skp2 levels resulting in p27 downregualtion, whereas blockage of the receptor by tamoxifen repressed Skp2 levels leading to p27 upregulation [11]. A number of potential explanations exist for these observations. First, in MDA-MB-231 cells, basal levels of Skp2 are very high and, hence, further upregulation of Skp2 might not affect p27 levels, as Skp2 might not be rate limiting for p27 degradation in these cells due to its excessive levels. In MCF-7 cells, the slight decrease in p27 levels can be explained by recent studies suggesting that p27 might also be downregulated by Skp2-independent mechanisms at the G_1 phase of the cell cycle. For example, it was shown that Kip1 ubiquitylation-promoting complex 2 (KPC2) together with KPC1 forms the ubiquitin ligase complex KPC, which regulates the degradation of p27 in G_1 [31]. Thus, because MCF-7 cells are arrested by doxorubicin in G_1 for a prolonged time, the time for KPC action is also prolonged and, therefore, it is possible that in this case p27 is degraded through this Skp2-independent mechanism.

The results of this study are important for several reasons. First, we show that doxorubicin can selectively downregulate Skp2 levels in some breast cancer cells, but in other cell types the opposite results might occur. It should, however, be noted that Skp2 status by itself does not seem to account for the inhibitory action of doxorubicin on cell proliferation. Whether upregulation of Skp2 adversely affects treatments and clinical outcomes is at present unknown, but owing to its known oncogenic properties, this consideration should be taken into account and investigated further. Thus, it might be of interest to examine whether lack of response to doxorubicin treatment can be associated, at least in part, to upregulation of Skp2 levels. This may be of clinical importance in patients with locally advanced breast cancers as many of these patients are empirically treated in the neoadjuvant setting with doxorubicin-based protocols, yet a considerable number of these patients fail to respond to this regimen and are at the same time exposed to the toxic side effects of the drug. Second, we suggest that the differential effects of doxorubicin on Skp2 expression are largely dependent on the presence of intact p53, which induces p21 and cell cycle arrest at G_1. Thus, effects on Skp2 expression by themselves are thus not always sufficient to predict outcome of drug treatments and other factors, such as p21 or p53 induction, might be important determinants. Finally, the results of this and previous studies suggest that drugs that arrest the cell cycle at only G_1 might have the most effective inhibitory effect on Skp2 regulation.

In conclusion, the results of this study provide additional insights into the mechanisms of action of doxorubicin on cell cycle arrest in breast cancer cells and the differential effects of the drug on Skp2 expression. Doxorubicin induces cell cycle arrest at different checkpoints of the cell cycle, largely depending on its ability to induce p53 activity. As both Skp2 and p53 play important roles in tumor progression in breast cancer and clinical outcomes, these results suggest that the functions of both important regulatory proteins should be taken into account in the treatment of breast cancer by this drug.

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