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Activated Cell Cycle Checkpoints in Epirubicin-Treated Breast Cancer Cells Studied by BrdUrd-Flow Cytometry

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Genetic alterations, such as *p53* mutations, may affect a tumour's response to chemotherapy. We have treated two human breast cancer cell lines that differ in *p53* status with epirubicin in order to study if there are differences in cell cycle kinetic response. MCF-7 cells express wild-type *p53*, while SK-BR-3 cells express only a mutated form of *p53*. The transition of cells from one cell cycle stage to another was studied by a bromodeoxyuridine (BrdUrd)-flow cytometry (FCM) method. MCF-7 cells showed a block in the G_1 phase after treatment with 50 nM epirubicin for 24 hours, in agreement with the actions of p53 at the G_1 checkpoint. SK-BR-3 cells, on the other hand, progressed through the G_1 checkpoint and were blocked in late S and G_2 phases, presumably due to the activation of a later checkpoint. In addition, studies of the mRNA levels of *p53* and its effector gene *p21* revealed that although both cell lines expressed *p53* mRNA, a marked difference in the mRNA levels of *p21* was seen. A dramatic increase in the level of *p21* mRNA was seen in epirubicin-treated MCF-7 cells, while no such increase was seen in SK-BR-3 cells. Cytometry 29:321–327, 1997. © 1997 Wiley-Liss, Inc.

Key terms: cell cycle kinetics; flow cytometry; bromodeoxyuridine; RNA; *p53*; *p21*; breast cancer; epirubicin

It is widely known that tumours of the same origin may exhibit different sensitivity towards chemotherapeutic drugs. Altered gene expression and variations in growth kinetics could partly explain these differences in tumour response. Little is known about the specific mechanisms underlying these observations.

At a molecular level, the development of human tumours is a complex multistep process involving multiple genetic alterations that abrogate the normal mechanisms of control of cell proliferation. A characteristic of most tumour cells is their ability to enter and progress through the cell cycle under conditions in which normal cells would be growth arrested or destined to die; for instance, under circumstances when the tumour suppressor gene *p53* is mutated. Mutations in *p53* are among the most commonly found genetic alterations in human malignancies, as *p53* is mutated in approximately 50% of all breast cancers.

The anthracycline antibiotic epirubicin (Farmorubicin®, Farmitalia Carlo Erba, Milan, Italy) is used in the treatment of a variety of solid tumours, among them breast cancer. The cytotoxic effect of epirubicin and other anthracyclines has been reported to be mediated through multiple mechanisms. These include intercalation into DNA with inhibition of DNA synthesis, interference with topoisomerase II activity, interference with DNA unwinding, the induction of DNA double-strand breaks, presumably through the generation of oxygen free radicals, and alterations of membrane function (9). These are potentially lethal insults toward which the normal cell has developed defense mechanisms; these mechanisms are usually altered in cancer cells. Defects in the ability to detect or repair DNA damage, or to induce growth arrest or apoptosis, can lead to drug resistance.

A multitude of cell cycle kinetic parameters can be analyzed in individual cells using the established bromodeoxyuridine (BrdUrd)-flow cytometry (FCM) method, e.g., the lengths of the G_1, S, and G_2 phases and the rate of the G_1/S transition (10). These parameters are presumably controlled by genes that regulate cell proliferation, and during drug treatment they may be affected in different ways, depending on variations in tumour genetic expression.

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Many DNA damaging agents, such as epirubicin, induce *p53* expression, which causes a cellular G_1 block (13). This block may be caused by p53 specific upregulation of the *p21* gene, which encodes an inhibitor of cyclin-dependent kinases (7, 19). This G_1 arrest allows the cells to repair their DNA prior to entry into the S phase. MCF-7 breast cancer cells contain wild-type *p53* (wt *p53*), whereas SK-BR-3 cells contain a mutated form of *p53*. We have studied the effect of epirubicin treatment on the cell cycle kinetics of the two human breast cancer cell lines MCF-7 and SK-BR-3, with different genetic profiles. In addition, we have investigated the mRNA levels of *p53* and *p21* in the two cell lines, before and after epirubicin treatment.

This study was performed in order to evaluate if mutations in the *p53* gene in human breast cancer cells affect the response, with respect to cell cycle kinetics, to epirubicin. In an attempt to elucidate the possible mechanism(s) underlying this response, we have also investigated the mRNA expression of the *p53* and *p21* genes. We report that epirubicin treatment caused a G_1 block in MCF-7 cells, while it caused an accumulation of cells in late S and G_2 phases in SK-BR-3 cells. This difference in response is presumably due to the difference in *p21* induction in the two cell lines, as an elevated amount of *p21* mRNA was seen in epirubicin-treated MCF-7 cells, but not in epirubicin-treated SK-BR-3 cells.

MATERIALS AND METHODS

Materials

Growth medium components were purchased from Biochrom (Berlin, Germany) and tissue culture plastics from Nunc (Roskilde, Denmark). Epirubicin (Farmorubicin®) was obtained from Farmitalia Carlo Erba (Milan, Italy). 5-Bromo-2′-deoxyuridine (BrdUrd), propidium iodide (PI), and standard analytical grade laboratory reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal mouse anti-BrdUrd antibodies (M744) and secondary fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibodies (F313) were obtained from Dakopatts (Glostrup, Denmark). Hybond-N membrane and [α-^{32}P]UTP (800 Ci/mmol) were purchased from Amersham AB (Solna, Sweden). RNA polymerases and restriction enzymes were purchased from SDS Promega (Falkenberg, Sweden). The p53BSK- and pZL-WAF1 cDNA clones were kind gifts from Dr. Urban Gullberg (6) and Dr. Bert Vogelstein (7), res pectively.

Cell Cultures

MCF-7 and SK-BR-3 human breast adenocarcinoma cell lines originally came from the American Type Culture Collection (Rockville, MD). Both cell lines are DNA aneuploid and manifest several different characterised genomic changes. They differ with respect to *p53* gene mutation status, MCF-7 cells containing a wt *p53* gene and SK-BR-3 cells carrying a point-mutated *p53* allele (Arg175His). The cell lines were maintained in serial passages in Eagle's MEM (MCF-7) and McCoy's 5a (SK-BR-3) medium supplemented with 10% foetal calf serum (FCS) and antibiotics (penicillin and streptomycin). Insulin (10 µg/ml) was also added to the MCF-7 growth medium. The cells were subcultured once weekly and the growth medium was exchanged twice in between. The cell doubling times were 35 h and 41 h for MCF-7 and SK-BR-3 cells, respectively. The cultures were incubated at 37°C in a water-saturated atmosphere containing 5% CO_2 in air.

Drug Treatment and BrdUrd Labelling

Three days after seeding of 2.5×10^6 (MCF-7) or 1.5×10^6 (SK-BR-3) cells in fresh medium in Petri dishes (9 cm diameter), a number of cultures were treated with epirubicin (50 nM) for 24 hours, while untreated ones served as controls (5 samples for each data point). The cell cultures (both control and epirubicin-treated) were then rinsed twice with RPMI 1640 medium containing 0.5% FCS and fresh culture medium (E-MEM or McCoy's, 37°C) was added. BrdUrd [1 mM dissolved in a Ca^{2+} and Mg^{2+} free phosphate-buffered NaCl solution (PBS), and then sterilised by filtration] was added to a final concentration of 5 µM and the cultures were further incubated at 37°C. Labelling and subsequent cell handling were carried out under subdued light. After 30 min of incubation, the BrdUrd containing medium was aspirated and the cells were rinsed twice with RPMI 1640 medium containing 0.5% FCS (37°C). Finally, fresh culture medium (37°C), specific for each cell line, was added and the cultures were further incubated at 37°C. At various times after BrdUrd labelling (post-labelling times, t: 0, 4, 5, 6, 7, 8, 9, and 10 h), cultures were trypsinised (day 4 after seeding), cell numbers were determined by counting in a haemocytometer, and the cells were pelleted at 700 g for 10 min at 4°C. The pelleted cells were fixed by resuspension in ice cold 70% ethanol (1×10^6 cells/ml) and the samples were stored at −20°C until analysis. After epirubicin treatment, a number of control and epirubicin-treated cultures were allowed to progress in drug-free medium for two days before labelling with BrdUrd (day 6 after seeding), as described above.

Staining Procedure

The method was originally described by Dolbeare et al. (4) and has been further developed and modified by Schutte et al. (16) and van Erp et al. (18). Ethanol-fixed cells (1×10^6) were washed twice in 10 ml PBS and were pelleted at 700 g for 5 min. All centrifugations were carried out at 700 g at room temperature. A freshly made pepsin-HCl solution (2 ml of 0.2 mg pepsin/ml in 0.1 M HCl) was added to partially digest proteins, and the cells were incubated for 30 min at 37°C. All incubations at 37°C were carried out in a shaking water bath. Concentrated HCl was then added to a final concentration of 2 M and the nuclei were further incubated at 37°C for 20 min. DNA denaturation was terminated by immersion of the test tubes in an ice bath and by the addition of 9 ml of 0.1 M $Na_2B_4O_7$ (pH 9.3). After pelleting the nuclei and washing them in PBS, 100 µl primary anti-BrdUrd [diluted 1:10 in PBS containing 0.5% Tween 20 and 1% bovine serum albumin (PBT)] was added and the incubation proceeded for 45 min at 37°C. PBS was added and the nuclei were pelleted. The samples

were then incubated with 100 µl secondary FITC-conjugated antibodies (diluted 1:20 in PBT) for 45 min at 37°C. Thereafter, PBS was added and the samples were pelleted. PI-nuclear isolation medium (1 ml), PI-NIM [PBS containing 10 µg/ml PI, 0.12% Nonidet P-40 and 20 µg/ml RNase A (17)], was added during vigorous agitation, and the samples were stored in the dark for 30 min at 4°C before analysis.

Flow Cytometry

Immediately prior to FCM analysis, the nuclear suspension was suctioned three times through a cannula (0.7 mm diameter) and filtered through a 50 µm nylon mesh in order to remove nuclear aggregates.

Simultaneous analysis of DNA and BrdUrd contents was performed in an Ortho Cytoron Absolute flow cytometer (Ortho, Raritan, NJ) equipped with a 15 mW air-cooled argon-ion laser. The laser line at 488 nm was used for the excitation of both fluorochromes (i.e., PI and FITC). Ten thousand nuclei per sample were analysed at a rate of approximately 100–200 nuclei per second. PI fluorescence (DNA content) was separated from FITC fluorescence (BrdUrd content) with a 560 nm dichroic mirror. The FITC fluorescence intensity was quantified in the interval 515–545 nm, and the PI fluorescence was quantified beyond 620 nm. The fluorescence signals were digitised by a multichannel analyser and stored as list data files according to the flow cytometry standard FCS 1.0 by acquisition software, running on a PC, onto a data file server (VAX/VMS, Digital Equipment), for further computerised analyses. Nuclear doublets and triplets were excluded by electronic threshold settings (1). For the computerised analysis, MultiPlus® (Phoenix Flow Systems, CA) was used.

Data Analysis

First, a region was set around the total cell population in DNA versus BrdUrd cytograms, according to Begg et al. (2). Second, two new regions were set within the first region, separating BrdUrd-labelled cells (FITC fluorescence) from BrdUrd-non-labelled cells. Regions were set manually and the cut-off between BrdUrd-labelled and BrdUrd-non-labelled cells was determined from samples incubated with the secondary antibody only, or from BrdUrd-non-labelled cells treated according to the staining procedure. In DNA histograms of BrdUrd-labelled cells from the various post-labelling times (t), non-divided and divided cells were distinguishable. The number of BrdUrd-labelled divided cells was determined, and their percentage out of the total cell number was calculated for each post-labelling time. BrdUrd-non-labelled cells were also displayed in DNA histograms at the different post-labelling times (t), in order to determine their distributions in the G_1, S, and G_2 phases.

Northern Blot Analysis

Cells intended for Northern blot analysis were pelleted at 700 g for 10 min at 4°C, the medium was decanted, 500 µl of a buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.3 M β-mercaptoethanol was added, and the samples were stored at −80°C. Total cellular RNA was isolated as described by Chomczynski and Sacchi (3). The quality and quantity were determined spectrophotometrically. Aliquots containing 10 µg RNA were size-fractioned by gel electrophoresis in a 1% agarose gel containing formaldehyde. The ethidium bromide-stained gel was then photographed on a UV-transilluminator. After capillary transfer to a Hybond-N membrane, the RNA was hybridised to ^{32}P-labelled *p53* or *p21* antisense probes. The *p53* probe was synthesised using XbaI-linearised p53BSK-, T7 RNA polymerase and [α-^{32}P]UTP. The *p21* probe was synthesised using StuI-linearised pZL-WAF1, SP6 RNA polymerase and [α-^{32}P]UTP. After hybridisation at 63°C, the membranes were washed in 0.1% SSC and 0.5% SDS at 68°C overnight, before exposure to autoradiographic film at −70°C.

Statistical Analysis

Multiple regression analysis was used for statistical evaluation of the data in Figures 1 and 2. An F-test was used to test the hypothesis of equal slopes, and $P < 0.05$ was considered statistically significant.

RESULTS

The G_1/S transition was studied by investigating the progression of BrdUrd-non-labelled cells from G_1 into the S phase (Fig. 1). The percentage of BrdUrd-non-labelled cells in G_1 in relation to the total number of BrdUrd-non-labelled cells was plotted against the post-labelling time. The slope of the line indicates the rate of the progression of cells through the G_1/S transition; a steep line with a negative slope implies rapid cell movement through the G_1/S transition, while a horizontal line reveals that the cells have been blocked prior to the G_1/S transition. Directly after the 24 hours of epirubicin treatment (day 4 after seeding), the MCF-7 cells were blocked before the G_1/S transition, whereas control cells progressed into the S phase, as can be seen from the negative slope of the line (Fig. 1A). Similar results were found two days after epirubicin treatment (day 6 after seeding; Fig. 1B). The slopes of the lines for control versus epirubicin-treated MCF-7 cells were significantly different ($P < 0.05$) on both days. Figures 1C and 1D show that the rate of progression of both control and epirubicin-treated SK-BR-3 cells through the G_1 phase and into the S phase was similar on days 4 and 6. The lines are parallel, indicating similar progression rates, although the line representing the epirubicin-treated cells is shifted downward, which can be explained by the fact that these cells were blocked mainly in the S and G_2 phases, and therefore fewer cells were in the G_1 phase (see below).

The progression of BrdUrd-labelled cells from the S phase, through G_2 and M and into G_1, was followed by determining the percentage of BrdUrd-labelled divided cells in relation to the total number of cells (Fig. 2). The BrdUrd-labelled cells that appear in the G_1 phase have divided since the BrdUrd labelling. The slope of the line indicates the rate at which these cells appear in G_1, and the

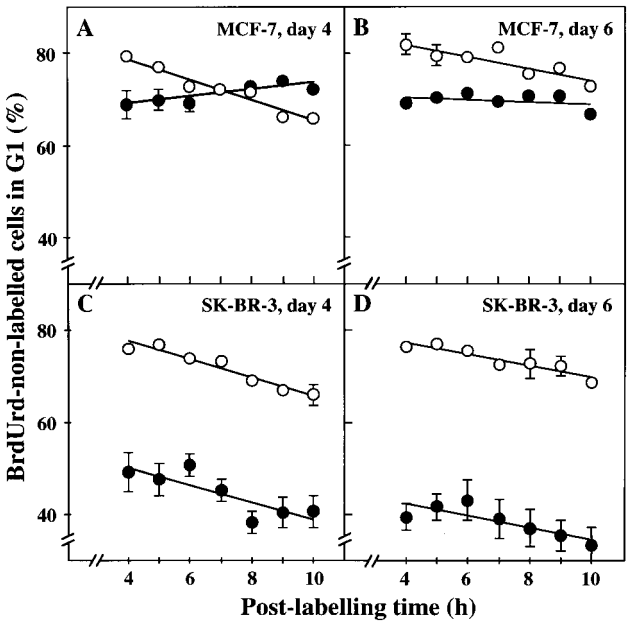

FIG. 1. The effect of epirubicin treatment on the G_1/S transition in MCF-7 (**A** and **B**) and SK-BR-3 breast adenocarcinoma cells (**C** and **D**). Epirubicin (50 nM) was added 3 days after seeding when the cultures were growing exponentially. The cells were treated for 24 hours. Cultures were labelled and samples collected directly after drug treatment [day 4; panels A (MCF-7) and C (SK-BR-3)] or two days after treatment [day 6; panels B (MCF-7) and D (SK-BR-3)] in the following way. The cells were labelled with 5 μM bromodeoxyuridine for 30 minutes and the cultures were rinsed and incubated as described in Materials and Methods. Cultures were harvested by trypsinisation at 4, 5, 6, 7, 8, 9, and 10 hours post-labelling. The cultures were finally prepared for the flow cytometric determination of BrdUrd and DNA contents. The symbols are the means of 5 samples from 2 experiments, and the bars represent ± SEM. Control cells, ○; epirubicin-treated cells, ●. The differences in the slopes of the lines for control versus epirubicin-treated cells were statistically significant ($P < 0.05$) for MCF-7 cells and non-significant ($P > 0.05$) for SK-BR-3 cells.

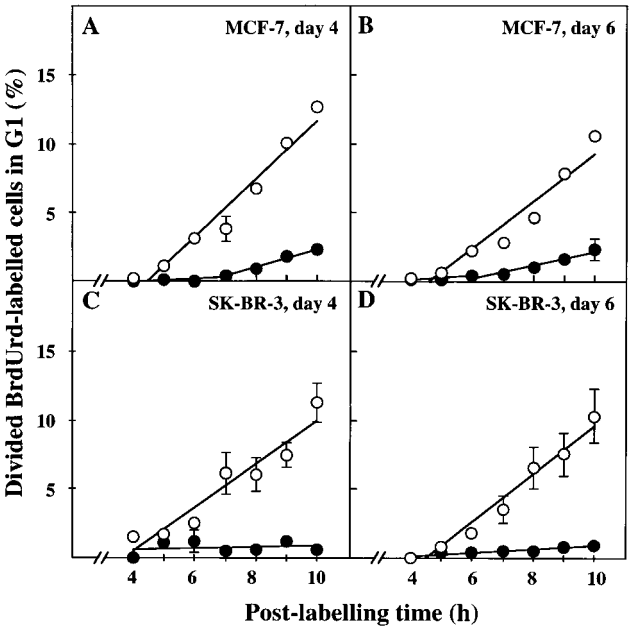

FIG. 2. The effect of epirubicin treatment on the progression of bromodeoxyuridine-labelled cells from the S phase, through the G_2 and M phases, and into the G_1 phase in MCF-7 and SK-BR-3 breast adenocarcinoma cells. The cells were treated as described in the legend to Figure 1. The symbols are the means of 5 samples from 2 experiments, and the bars represent ± SEM. Control cells, ○; epirubicin-treated cells, ●. The differences in the slopes of the lines for control versus epirubicin-treated cells were statistically significant ($P < 0.05$) for both cell lines.

intercept at the x-axis reveals the time the cells need to progress through the G_2 and M phases (T_{G2+M}). BrdUrd-labelled MCF-7 control cells appeared in the G_1 phase at approximately 5 hours post-labelling, while epirubicin-treated BrdUrd-labelled divided cells appeared 7 to 8 hours post-labelling, pointing toward an increased T_{G2+M} in MCF-7 cells after epirubicin treatment (Figs. 2A and 2B). The lower slope of the lines in epirubicin-treated MCF-7 cells indicates that fewer cells were entering the G_1 phase. In SK-BR-3 control cultures BrdUrd-labelled divided cells appeared at 6 hours post-labelling. In epirubicin-treated cultures, however, there was no clear increase in the number of BrdUrd-labelled divided cells in G_1 (Figs. 2C and 2D), as can be seen from the horizontal line. The slopes of the lines for control versus epirubicin-treated cells were significantly different ($P < 0.05$) for both MCF-7 cells and SK-BR-3 cells.

It can be seen from Figure 1 that the epirubicin-induced G_1 block in MCF-7 cells was apparent immediately after drug treatment (day 4). The G_2 block in epirubicin-treated SK-BR-3 cells, although evident directly after drug treatment, became more prominent during the time course of the experiment (see Fig. 4). In epirubicin-treated SK-BR-3 cultures, 17% of the cells were found in G_2, compared to 10% in the control cultures at t = 0. Ten hours later, 26% of the cells had accumulated in G_2 in epirubicin-treated cultures, as compared to 13% in control cultures.

A comparison of DNA histograms from control and epirubicin-treated MCF-7 cells immediately after epirubicin treatment revealed an accumulation of cells in the G_1 phase of the cell cycle in the latter case (Figs. 3A and 3B). This accumulation remained for 10 hours (Figs. 3C and 3D). DNA histograms of SK-BR-3 cells showed that the cell cycle phase distribution remained constant in control cells during the 10 hours that the cells were studied (Figs. 4A and 4C). Epirubicin-treated SK-BR-3 cells were found mainly in the late S phase immediately after epirubicin treatment (Fig. 4B), and 10 hours later the majority of cells was found in both S and G_2 phases (Fig. 4D).

MCF-7 cells manifested a discernible level of *p53* mRNA expression that was uniform at days 1, 3, 4, and 6 in control experiments and was unaffected by epirubicin treatment (Fig. 5B). SK-BR-3 cells expressed a considerably lower level of *p53* mRNA, which was slightly elevated in epirubicin-treated cells as compared to untreated cells at day 4 (Fig. 5B). MCF-7 cells demonstrated a dramatic increase in the expression of *p21* mRNA after epirubicin treatment at day 4, and, even more pronounced, at day 6 of the experiment (Fig. 5C). Epirubicin treatment of SK-BR-3 cells, on the other hand, did not elicit any such response,

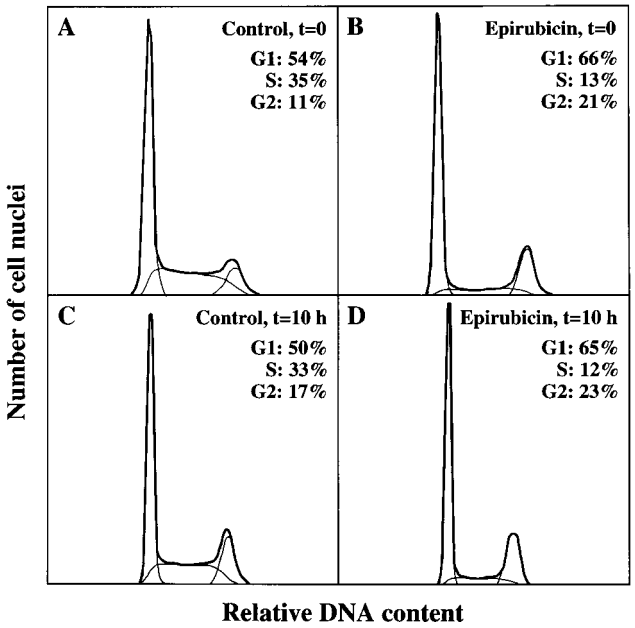

FIG. 3. DNA histograms showing the cell cycle phase distribution of MCF-7 control and epirubicin-treated cells (day 4). The cells were treated as described in the legend to Figure 1. Panels **A** and **C** show control cells at t = 0 (directly after labelling) and t = 10 h (10 hours post-labelling), respectively. Epirubicin-treated cells are displayed in panels **B** and **D** (t = 0 and t = 10 hours, respectively). The figures are from representative experiments performed twice.

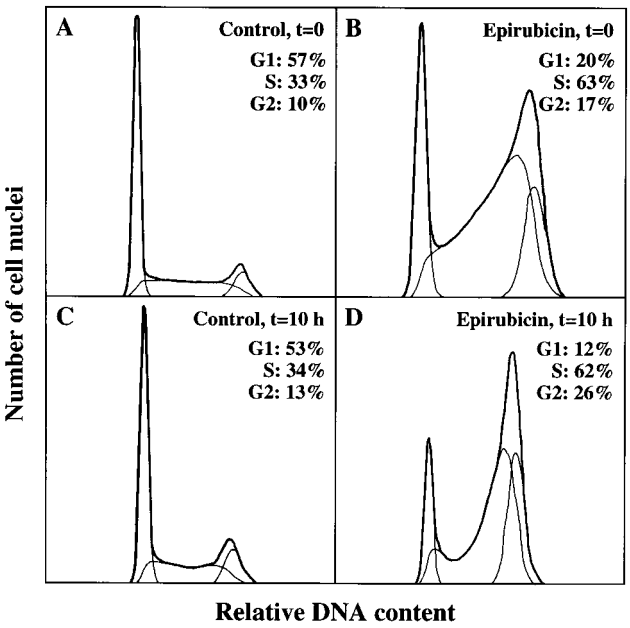

FIG. 4. DNA histograms showing the cell cycle phase distribution of SK-BR-3 control and epirubicin-treated cells (day 4). The cells were treated as described in the legend to Figure 1. Panels **A** and **C** show control cells at t = 0 (directly after labelling) and t = 10 h (10 hours post-labelling), respectively. Epirubicin-treated cells are displayed in panels **B** and **D** (t = 0 and t = 10 hours, respectively). The figures are from representative experiments performed twice.

as the level of *p21* mRNA expression remained barely detectable throughout the experiment (Fig. 5C).

DISCUSSION

This study was carried out in an attempt to investigate whether the varying sensitivity of breast tumours to cytotoxic drugs, in this case epirubicin (Farmorubicin®), can be illustrated by differences in growth kinetics and genetic alterations of breast cancer cells grown and treated in vitro. Within the near future, it is possible that these parameters can be used in the development of individual cancer treatment regimens. An improved understanding of the cellular and molecular mechanisms controlling cell proliferation and cell death during different stress conditions can be of value in designing new and more individualised treatment strategies, as well as in the development of more efficient drugs. This is of particular concern in the case of drugs, such as epirubicin, displaying a wide range of cellular toxicity.

The best known, and perhaps most important, cellular checkpoint is present in the G_1 phase, where DNA damage gives rise to an elevated expression of the p53 tumour suppressor protein, which, depending on the cell type, induces growth arrest (by induction of the *p21* cyclin dependent kinase inhibitor), DNA replication block (by titrating the level of PCNA), DNA repair (by induction of *gadd45*), apoptosis (by regulating the expression of the *bcl-2* and *bax* genes), or other, less well known effects (14). Confirming its role in tumour suppression, mutation and inactivation of the *p53* gene are among the most commonly found genetic alterations in human cancer (11). In many forms of cancer, among them breast cancer, there is a correlation between the occurrence of *p53* mutations and poor prognosis, as well as poor response to radiation and chemotherapeutic treatment.

Another major cell cycle checkpoint is present in the G_2 phase, and has so far been studied mainly in yeast cells. The *S. cerevisiae MEC1* and *S. pombe rad3* genes have been implicated in recognition of DNA damage and not fully replicated chromosomes (15). A similar function has been implicated for the related human ataxia telangiectasia (*ATM*) gene (15), and other members of the family of phosphatidylinositol-3-kinases, such as the DNA dependent protein kinase catalytic subunit (20).

In this report we demonstrate that epirubicin has different effects, with regard to cell cycle kinetics, in MCF-7 and SK-BR-3 human breast cancer cells. MCF-7 breast cancer cells, which have wt p53 activity, manifested no, or insignificant, G_1 to S transition after epirubicin treatment, in keeping with a functional G_1 checkpoint. The absence of a G_1 to S transition in MCF-7 cells is presumably due to a G_1 arrest, as we saw no evidence of extensive apoptosis (data not shown). This G_1 arrest is probably caused by an accumulation of p53 protein due to increased translation or post-translational stabilisation, as we saw no increase in *p53* transcript levels in epirubicin-treated MCF-7 cells. Activation of *p53* transcriptional activity is further supported by the observation that epirubicin treatment caused an upregulation of *p21* mRNA in these cells. Interestingly, a high level of *p21* mRNA and

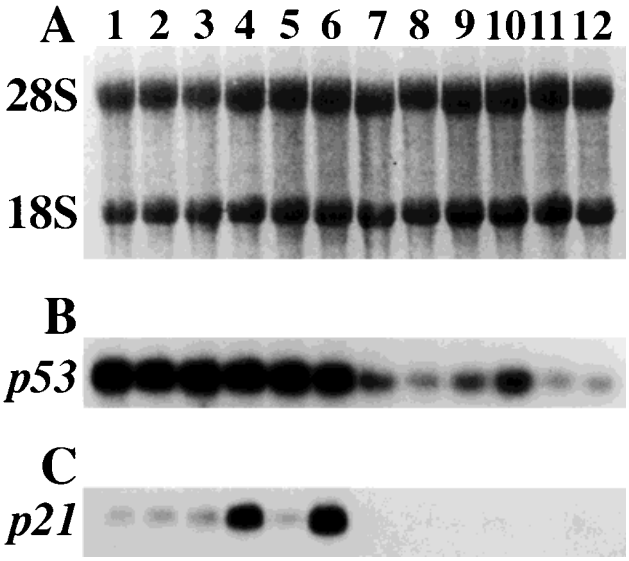

FIG. 5. Relative mRNA levels of *p53* and *p21* in MCF-7 and SK-BR-3 breast adenocarcinoma cells before and after epirubicin treatment. The cells were treated as described in the legend to Figure 1. Cells were harvested for the analysis of mRNA by Northern blotting directly after drug treatment (day 4) and two days after treatment (day 6). Panel **A** shows a photograph of the ethidium bromide-stained agarose gel that was used for the Northern blots. Panels **B** and **C** show autoradiograms of *p53* and *p21* mRNA, respectively, in the two cell lines before and after drug treatment. **Lanes 1–6** are MCF-7 cells. Lane 1, control day 0. Lane 2, control day 3. Lane 3, control day 4. Lane 4, epirubicin treatment day 4. Lane 5, control day 6. Lane 6, epirubicin treatment day 6. **Lanes 7–12** are SK-BR-3 cells. Lane 7, control day 0. Lane 8, control day 3. Lane 9, control day 4. Lane 10, epirubicin treatment day 4. Lane 11, control day 6. Lane 12, epirubicin treatment day 6.

a G_1 block persisted also at day 6 of the experiments, two days after drug withdrawal, unlike the normally transient block induced by *p53* in response to DNA damage. In contrast, epirubicin-treated SK-BR-3 cells were not arrested in the G_1 phase but manifested a G_1 to S transition similar to untreated cells. Apparently the G_1 checkpoint present in MCF-7 cells is not functional in SK-BR-3 cells. This is highly likely, since these cells express a mutant *p53* and since no stimulation of *p21* mRNA expression was observed after epirubicin treatment. This implies a causality between *p53* status and the different responses of the two cell lines, and also that epirubicin indeed does invoke DNA damage detected by the *p53* checkpoint in MCF-7 cells.

However, the present study does not exclude the importance of other genetic alterations in the different responses to drug treatment, as both cell lines contain a multitude of genomic changes. For instance, SK-BR-3 cells manifest amplification of the chromosomal regions 17q12 and 8q24, regions known to harbour potent oncogenes such as *erbb2* and *myc,* respectively. On the other hand, MCF-7 cells have also been shown to carry amplifications in putative oncogenes, most notably in the chromosomal regions 17q22–q24 and 20q13 (12). We are presently analysing the expression of additional factors in these cell lines, including those involved in the p53 signalling pathway and the apoptosis pathway, which might also illuminate the prolonged G_1 block observed in MCF-7 cells.

Our data suggest the presence of a functional G_2 checkpoint in both SK-BR-3 and MCF-7 breast cancer cell lines, since a more or less prominent accumulation of cells in G_2 was found in both cell lines. According to Fan et al. (8), loss of *p53* function might be partially compensated by activation of the G_2 checkpoint. This G_2 checkpoint would delay entry of DNA-damaged cells into mitosis, thereby allowing more time for DNA repair processes to remove DNA lesions before mitosis. Epirubicin-treated SK-BR-3 cells, which showed no decrease in the rate of G_1/S transition, manifested an accentuated accumulation of cells in late S and G_2 phases, as shown by BrdUrd-FCM. In contrast, SK-BR-3 control cells were seen to progress through the G_2 and M phases, and reappear in the G_1 phase. This suggests that epirubicin-induced DNA damage, acquired during G_1 and possibly fortified during DNA replication, is recognised at the G_2 checkpoint, leading to a prolonged block, evident also at day 6 of the experiments. Epirubicin-treated MCF-7 cells were also blocked in the G_2 phase, but to a much lesser extent, possibly explained by the observation that cells with DNA damage were arrested in the G_1 phase. This experimental design allows us to further investigate the components of the G_2 checkpoint by analysing the differential expression of candidate genes involved in DNA damage recognition and repair, as well as in the execution of growth arrest. The activity of the maturation promoting factor (MPF) is of considerable interest.

An alternative and likely mechanism of epirubicin-induced S and G_2 arrest may be found in the known interaction between the drug and topoisomerase II. This indispensable enzyme is activated by sites on chromosomes where two double helices are crossed, preventing DNA tangling that may restrain DNA transcription, replication, recombination, and condensation. The normal activity of topoisomerase II peaks during the G_2 phase, when the DNA is condensated in preparation for mitosis (5). Inactivation of topoisomerase II may result in repairable DNA cleavage, and activation of double-strand break specific detection systems, but also in irreversible damage. If this inactivation of topoisomerase II is the major cause of G_2 arrest in the breast cancer cell lines used in the present study, it is more pronounced in SK-BR-3 cells, which showed a permanent block. Epirubicin-treated MCF-7 cells were found to progress through G_2 and reappear in the G_1 phase, although at a reduced rate, and with a two-hour delay. Again, our model allows further investigations in the varying effects of the drug on enzyme activity.

The objective of the present study was to create a working model for the study of the drug epirubicin and its cytotoxic effects on cell cycle kinetics of human breast cancer cells. The cell lines used differ widely in their cell cycle kinetic responses to the drug; one is arrested in G_1 and the other in S/G_2. We are now extending our studies to include a normal human breast epithelial cell line (MCF-10A), which should provide good opportunities for further studies of gene expression in relation to growth

arrest, DNA damage detection and repair, apoptosis, and drug resistance in normal versus cancer cells.

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