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Genistein Blocks Breast Cancer Cells in the G_2M Phase of the Cell Cycle

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Abstract Genistein, a natural isoflavone phytoestrogen present in soybeans, caused a dose-dependent growth inhibition of the two hormone-sensitive cell lines T47D and ZR75.1 and of the two hormone-independent cell lines MDAMB-231 and BT20. Flow cytometric analysis of cells treated for 4 days with 15 and 30 μM genistein showed a dose-dependent accumulation in the G_2M phase of the cell cycle. At the highest tested concentration, there was a sevenfold increase in the percentage of cells in G_2M (63%) with respect to the control (9%) in the case of T47D cells and a 2.4-fold increase in the case of BT20. An intermediate fourfold accumulation was observed in the case of MDAMB-231 and ZR75.1. The G_2M arrest was coupled with a parallel depletion of the G_0/G_1 phase. To understand the mechanism of action underlying the block in G_2M induced by genistein, we investigated the expression and the activity of cyclins and of cyclin-dependent kinases specifically involved in the $G_2 \rightarrow M$ transition. As expected, $p34^{cdc-2}$ expression, monitored by Western blotting, was unaffected by genistein treatment in all cell lines. With exception of the T47D cell line, we revealed an increase in the tyrosine phosphorylated form of p34, suggesting an inactivation of the $p34^{cdc-2}$ catalytic activity consequent to treatment of cells with genistein. In fact, immunoprecipitates from genistein-treated MDAMB-231 and BT20 cells displayed a fourfold decrease in kinase activity evaluated using the histone H1 as substrate. Conversely, no variation in kinase activity was observed between treated and untreated ZR75.1 cells despite the increase in p34 phosphorylation. In cells treated with 30 μM genistein, cyclin B_1 (p62) increased 2.8-,8-and 103-fold, respectively, in BT20, MDAMB-231, and ZR75.1 cells, suggesting an accumulation of the p62, which is instead rapidly degraded in cycling cells. No effects were observed on cyclin expression in T47D cells. We therefore conclude that genistein causes a G_2M arrest in breast cancer cell lines, but that such growth arrest is not necessarily coupled with deregulation of the $p34^{cdc-2}$/cyclin B_1 complex only in all of the studied cell lines. J. Cell. Biochem. 79:594–600, 2000.

Key words: genistein; breast cancer; G_2M phase

The phytoestrogen genistein has been extensively studied in relation to its anticarcinogenic activity [Barnes, 1995] in animal models and to its antiproliferative activity observed in various types of cell lines. Genistein displays structural similarities with estradiol and, in fact, acts as a weak agonist or antagonist of estradiol, depending on the dose [Fioravanti et al., 1998]. Furthermore, genistein is a tyrosine kinase inhibitor and a kinase DNA topoisomerase II inhibitor [Akiyama et al., 1987; Markovits et al., 1989]. Altogether, such effects may account for the growth-inhibitory activity of the molecule.

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In a previous study [Fioravanti et al., 1998], we showed that in MCF-7 cells, genistein interfered with estradiol- and growth factor–induced proliferation, inhibited paracrine stimulation promoted by coculture with estrogen-negative cells, and decreased tyrosine phosphorylation induced by transforming growth factor-α. Such growth-inhibitory effects were also accompanied by an accumulation of cells in late S- and G_2M-phase of the cell cycle.

G_2M block is often observed in cells after treatment with radiation or cytotoxic agents [O'Connor and Kohn, 1992]. In such cells, the DNA damage caused by physical or chemical agents is coupled with inhibition of cell division through deregulation of the $p34^{cdc-2}$/cyclin B_1 complex [Draetta and Beach, 1988]. $p34^{cdc-2}$ is a serine/threonine kinase whose activity is regulated by phosphorylation and by association

in a complex with cyclin B. $p34^{cdc-2}$ levels are constant throughout the cell cycle, but the phosphorylation state changes and the catalytic activity increases during the $G_2 \rightarrow M$ transition because of dephosphorylation of tyrosine 15 and threonine 14 [Draetta and Beach, 1988]. Therefore, the observation of tyrosine phosphorylation suggests an inhibition of $p34^{cdc-2}$ activity. Cyclin B is instead rapidly degraded after $G_2 \rightarrow M$ transition and tends to accumulate if cells are blocked in the G_2M phase of the cell cycle [Muschel et al., 1991].

In the present study, we evaluated the effect of genistein on growth and cell cycle distribution of the two hormone-dependent breast cancer cell lines T47D and ZR75.1 and the two hormone-independent cell lines, MDAMB-231 and BT20. We also investigated the interference of the phytoestrogen on the functional activity of cyclin B and $p34^{cdc-2}$ with special reference to changes induced in the phosphorylation state and catalytic activity of the cyclin-dependent kinase.

MATERIAL AND METHODS

Cell Lines

Human breast cancer cell lines were kindly provided by K. Horwitz, University of Colorado at Denver (T47D), by M. Siciliano, MD Anderson Cancer Center at Houston (MDAMB-231), by G. Daxenbichler, University of Innsbruck (BT20), and by F. Fox, UCLA, Los Angeles (ZR75.1). Cells were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Sigma Chemical Co., St. Louis, MO) without phenol red and supplemented with 2% fetal calf serum (FCS) and 4 g/l glucose.

Cell Growth Experiments

Experiments were run in triplicate in 24-well plates in DMEM/F12 supplemented with 2% FCS. After 24 h of attachment, medium containing genistein (0.1–30 μM) or its diluent was added and changed after 3 days. Experiments were stopped at day 7 when cells were still in the exponential phase of growth. Cell growth was determined by total cell DNA evaluated directly in the 24 wells with the diphenylamine assay [Burton, 1956]. Linearity between cell number variations and DNA content of the wells was preliminarily checked for each cell line. For flow cytometry, immunoblot analysis, and $p34^{cdc-2}$ kinase assay, cells were grown in 75 cm^2 flasks in medium supplemented with 5% FCS and treated for 4 days with the indicated concentrations of genistein.

Flow Cytometric Analysis: Cell Cycle Perturbations

Cell samples (1×10^6) were stained in a solution containing 50 μg/ml propidium iodide, RNase (100 kU/ml), and 0.05% of the non ionic detergent Nonidet P40 for 30 min at room temperature in the dark. The fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser at 488-nm wavelength excitation and 610-nm filter for propidium iodide fluorescence detection. A minimum of 10^4 cells was measured for each sample, and the data were processed with LYSIS II software (Becton Dickinson). Cell cycle analysis was performed on linear DNA plots by CellFit software according to the SOBR model (Becton Dickinson).

Immunoblot Analysis

Cells were lysed on ice with RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P40, 50 mM NaF) containing the protease inhibitors aprotinin, leupeptin, and pepstatin-A at a concentration of 10 μg/ml and 1 mM phenylmethylsulphonylfluoride (PMSF) and centrifuged at 20,000*g* for 30 min. The protein content of each supernatant was quantified by the BioRad protein assay. Sodium dodecyl sulfate (SDS)-denaturated proteins (50 μg) were electrophoresed in a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Nonspecific binding was blocked by a 1-h incubation with phosphate-buffered saline (PBS) containing 0.1% Tween20 and 5% skim milk, and membranes were incubated overnight with the primary antibodies (anti-$p34^{cdc-2}$ and anti-cyclin B_1 murine monoclonal antibodies, Santa Cruz, CA, antiphosphotyrosine murine monoclonal antibody, Upstate Biotechnology Inc., Lake Placid, NY). Monoclonal murine anti-PCNA IgG (Oncogene Research Products, Calbiochem, San Diego, CA) was used as a control for protein loading. Unbound antibody was removed, and filters were incubated with an antimouse Ig horseradish peroxidase–linked whole antibody (Amersham International, Buckinghamshire, UK). Bound antibody was detected using the enhanced chemiluminescence Western blotting detection system (ECL, Amersham).

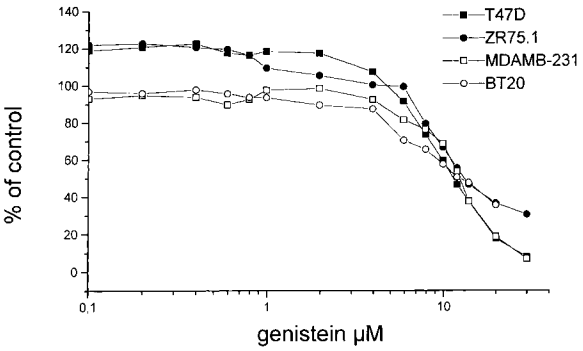

Fig. 1. Effect of various doses (0.1–30 μM) of genistein on the growth of 747D, ZR75.1, MDAMB-231, and BT20 cells. Cells were plated in 24-well culture dishes at a cell density of 20,000 cells/well. Each point represents the average of three independent experiments performed in quadruplicate (Latin square).

P34^{cdc-2} Kinase Assay

Total cellular proteins (100 μg) obtained treating cells with lysis buffer (PBS containing 1% Nonidet P-40, 1% bovine serum albumin, 10 μg/ml leupeptin, and 1 mM PMSF), were immunoprecipitated with anti-p34^{cdc-2} in the presence of protein A-Sepharose slurry (Protein-A Sepharose CL4B, Pharmacia, Uppsala, Sweden) for 3 h at 4°C under rotation. Immunoprecipitates, washed four times with lysis buffer, were resuspended in 20 μl of kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing 5 μM cold ATP and 3 μg histone H1 (Boehringer, Mannheim, FRG) and incubated for 30 min at 37°C in the presence of 0.37 Bq of [γ-^{32}P] ATP (specific activity 111 TBq/mmol). Reactions were stopped by addition of an equal volume of 2× loading buffer. After denaturation, samples were loaded on 12% SDS–polyacrylamide gel, and bands were detected by autoradiography.

RESULTS

Effect of Genistein on Cell Growth

The effect of genistein on cell growth was tested over a range of concentrations of 0.1 to 20 μM. As shown in Figure 1, in the hormone-responsive cell lines T47D and ZR75.1, lower concentrations of genistein were growth stimulatory (up to +20%, $P < 0.01$ at genistein concentrations ranging from 0.1 μM to 0.8 μM in the case of ZR75.1 and ranging from 0.1 to 2.0 μM in the case of T47D cells), whereas a dose-dependent growth inhibition was observed between 6 and 20 μM ($P < 0.001$). In the case of the hormone-independent cell line MDAMB-231, lower concentrations (<2 μM) did not modify cell growth. At higher concentrations, growth inhibition was similar to that observed in the T47D cell line. BT20 were inhibited around 2 μM and a dose dependency in growth inhibition was observed up to the highest tested dose, i.e. 30 μM.

Flow Cytometric Studies

Cells treated with 15 or 30 μM genistein for 4 days were collected and divided into aliquots to be used for flow cytometric studies and for immunoblots and kinetic studies.

Figure 2 shows the distribution of the cells in the G_0/G_1, S, and G_2M phases, respectively, of the cell cycle as a function of treatment. Genistein treatment induced an accumulation of cells in the G_2M phase of the cell cycle, mainly evident at the highest dose, with a parallel depletion of the percentage of cells in G_0/G_1.

The increase of the percentage of cells in the G_2M phase of the cell cycle after treatment with 30 μM genistein ranged from 2.4-fold in the case of BT20 cells ($P < 0.01$) to a 7-fold increase in the case of T47D cells ($P < 0.01$). MDAMB-231 and ZR75.1 showed an intermediate 4-fold increase ($P < 0.01$). The decrease in cells in the G_0/G_1 phase of the cell cycle induced by the treatment with 30 μM genistein ranged from 1.3- to 2.6-fold ($P < 0.01$). Lower doses (15 μM) of genistein caused a statistically significant accumulation in G_2M only in the case of BT20 and ZR75.1 cells ($P < 0.01$).

Effect of Genistein on the Mitosis-Promoting Factor Catalytic and Regulatory Subunits

To investigate whether the mechanism of genistein-induced accumulation of cells in the G_2M phase of the cell cycle was mediated through alterations of the mitosis-promoting factor (MPF), immunoblots were run to evaluate the catalytic subunit p34^{cdc-2} expression level and phosphorylation state, and the regulatory subunit cyclin B_1 (p62) expression levels. Aliquots of cells from the same samples used for flow cytometry were used for such studies.

In the case of the T47D cells no remarkable difference was observed in the expression levels of the investigated proteins between untreated cells and cells treated with 15 or 30 μM genistein (data not shown).

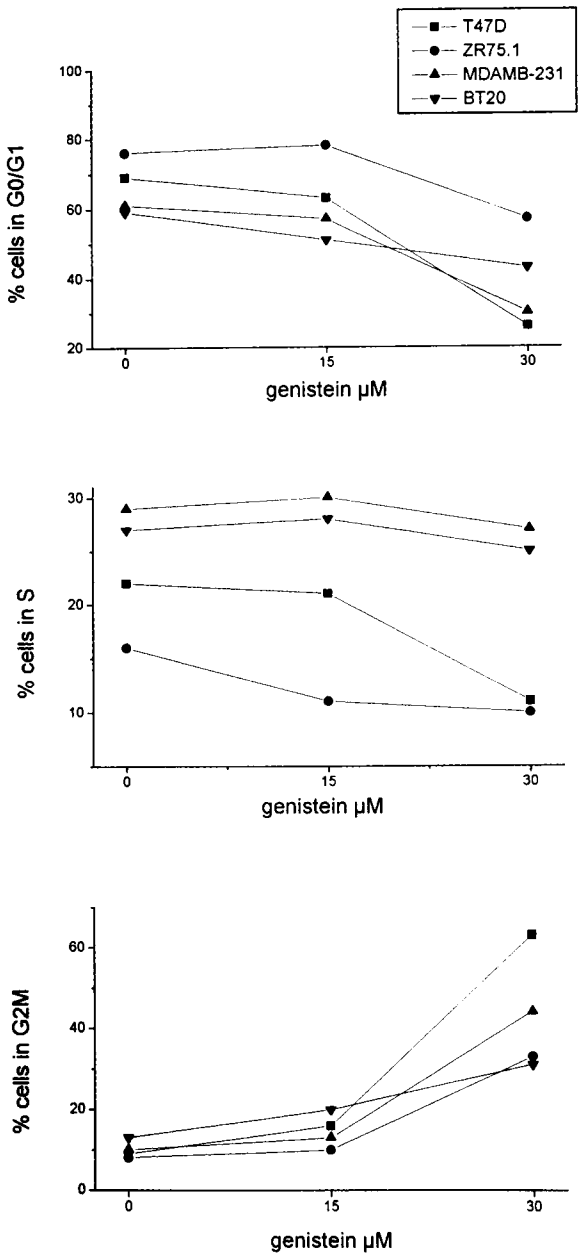

Fig. 2. Effect of treatment with 15 μM and 30 μM genistein on the distribution of cells in the various phases of the cell cycle. Each point represents the average of three separate experiments. Statistical significance of the reported variations are given in the Results section.

The same type of experiments for the other estrogen-sensitive cell line investigated in our study, ZR75.1 (Fig. 3), showed that the expression of the $p34^{cdc-2}$ protein was unaffected by the treatment, whereas its phosphorylation state was modified. In fact, there was a 1.3-fold and a 2.6-fold increase on treatment with 15 μM and 30 μM genistein. The accumulation

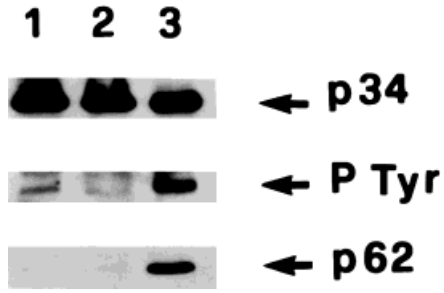

Fig. 3. A representative experiment illustrating the effect of a 4-day treatment of ZR75.1 cells with genistein on the expression of $p34^{cdc-2}$, its phosphorylated form (P-Tyr), and cyclin B_1 (p62). **Lane 1**, control; **lane 2**, 15 μM genistein; **lane 3**, 30 μM genistein.

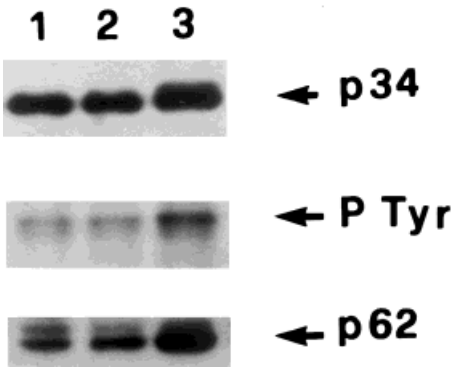

Fig. 4. A representative experiment illustrating the effect of a 4-day treatment of MDAMB-231 cells with genistein on the expression of $p34^{cdc-2}$, its phosphorylated form (P-Tyr), and cyclin B_1 (p62). **Lane 1**, control; **lane 2**, 15 μM genistein; **lane 3**, 30 μM genistein.

of cyclin B was markedly affected by treatment, with a moderate (1.5-fold) increase at the low-dose treatment and a remarkable increase (103-fold) at the higher genistein dose.

Figure 4 shows the results obtained with the hormone-insensitive MDAMB-231 cell lines. The intensity of the band corresponding to p34 did not vary between control and cells treated with 15 or 30 μM genistein. The p34 protein migrated as a dimer because of the different electrophoretic mobility of the variously phosphorylated forms, and the slowly migrating band, which represented the phosphorylated form, was increased by the treatment. When the same samples were probed in parallel with the anti-phosphotyrosine antibody, a clear increase in the phosphorylated form was observed on treatment with 30 μM genistein (relative intensity 1.00 in controls vs 1.5 for 15 μM and 2.65 for 30 μM genistein). Genistein also

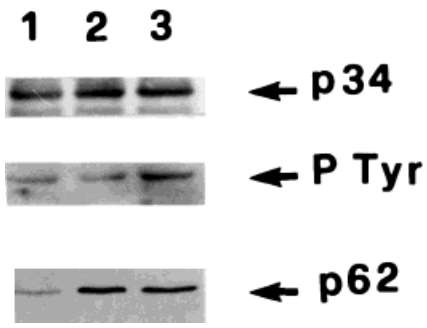

Fig. 5. A representative experiment illustrating the effect of a 4-day treatment of BT20 cells with genistein on the expression of $p34^{cdc-2}$, its phosphorylated form (P-Tyr), and cyclin B_1 (p62). **Lane 1**, control; **lane 2**, 15 μM genistein; **lane 3**, 30 μM genistein.

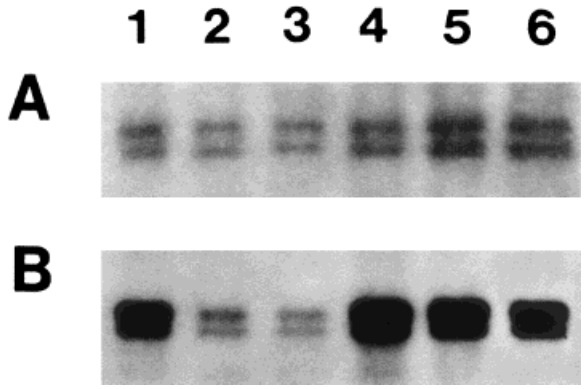

Fig. 6. A representative experiment showing the effect of a 4-day treatment of cells with genistein on the catalytic activity of $p34^{cdc-2}$ kinase. Aliquots of 100 μg of total cellular proteins were immunoprecipitated with anti-$p34^{cdc-2}$, and histone H1 kinase activity of immunoprecipitates was evaluated as described. **Lanes 1 and 4**, control; **lanes 2 and 5**, 15 μM genistein; **lanes 3 and 6**, 30 μM genistein, respectively, for MDAMB-231 and T47 cells **(A)** and for BT20 and ZR75.1 cells **(B)**.

caused an increase in cyclin B_1 expression. Quantification by image scanning indicated a 1.4-fold increase in cells treated with the lowest dose, and an 8-fold increase in cells treated with the highest dose.

In the case of the other hormone insensitive cell line BT20 (Fig. 5), $p34^{cdc-2}$ was almost constant in treated and untreated cells, but the phosphorylation state was slightly increased after treatment (1.5-fold increase with respect to the control, at the highest dose). The expression of the cyclin B_1 increased after administration of genistein in a relatively independent way (2.5- and 2.8-fold after 15 and 30 μM genistein, respectively).

Effect of Genistein on $p34^{cdc-2}$ Kinase Activity

After the evaluation of the expression of the catalytic and regulatory subunits of the MPF, we investigated the kinase activity of the complex using histone H1 as a substrate. The kinase assays were run on whole-cell immunoprecipitates of treated and untreated cells. Results are shown in Figure 6 for all four cell lines. As expected, in the T47D cell line, no variation in kinase activity was observed as a function of the treatment. The same type of result was unexpectedly obtained in the ZR75.1 cells.

Conversely, in the two hormone-independent cell lines, genistein treatment caused a strong inhibition of kinase activity. In the MDAMB-231 cells, kinase activity dropped to levels corresponding to 34% and 25% of the control, respectively, after 15 and 30 μM genistein treatment. Similarly, the decrease of kinase activity in the BT20 cells lines was quantified as 22% and 23% of the control.

DISCUSSION

The study was designed to gain insight into the antiproliferative activity of genistein in human breast cancer. We chose two hormone-dependent cell lines, T47D and ZR75.1, and two hormone-independent cell lines, MDAMB-231 and BT20, because in a previous study we found that genistein exerted its antiproliferative activity independently of the steroid receptor status of the cell. Cells were treated with high concentrations of genistein to avoid the agonistic activity of lower concentrations consistently observed in hormone-sensitive cell lines. Such concentrations are, in fact, approximately 6 to 30 times higher than genistein blood levels achieved in subjects with a high dietary soy intake [Barnes, 1995]. However, it should be mentioned that similarly to what occurs with estrogens, genistein is much more concentrated in breast fluid than in serum [Zava and Duwe, 1997].

Experiments were performed without previous synchronization of cells, deliberately to reproduce a situation closer to the physiological one and to allow genistein to exert its pleiotropic activity on the various aspects of cell metabolism (inhibition of DNA topoisomerase II, inhibition of tyrosine kinase, estrogenic effects). Treatment with genistein for 4 days (ap-

proximately two cell-doubling times) induced a dose-dependent accumulation of cells in the G_2M phase of the cell cycle, which comprised up to 63% of T47D cells, 44% of MDAMB-231 cells, 31% of BT20 cells, and 33% of ZR75.1 cells and was accompanied by a depletion of cells in the G_0/G_1 phase of the cycle. We therefore attempted to further investigate the molecular basis for such a block. Surprisingly, despite a similar block, the molecular mechanism appears to be different. In fact, in all investigated cell lines, except T47D cells, the G_2M block was accompanied as expected by an inactivation of the catalytic activity of $p34^{cdc-2}$. Although fewer than 50% of such cells were apparently blocked in the G_2M phase of the cell cycle, a clear increase in the tyrosine-phosphorylated form of the $p34^{cdc-2}$ protein could be documented in Western blots with a specific anti-phosphotyrosine antibody. In the same cell extracts, we were able to show an increase in cyclin B_1 expression, which we interpreted as an accumulation consequent to inhibition of cyclin B_1 degradation. In the MDAMB-231 and BT20 cells, the observed increase in the proportion of tyrosine-phosphorylated p34 was paralleled by an inhibition of the kinase activity measured in immunoprecipitates from genistein-treated cells. Our findings therefore suggest that accumulation of MDAMB-231 and BT20 cells in G_2M results from inability to activate the MPF, whose catalytic subunit is $p34^{cdc-2}$ and whose regulatory subunit is cyclin B_1.

As regards T47D, after 4 days of treatment with genistein, almost two thirds of the cells were in G_2M. Despite such a result, no alteration of cdc-2 phosphorylation state could be demonstrated, and cyclin B_1 levels were comparable between treated and untreated cells. Also, the ZR75.1 cells had an abnormal behavior, and despite phosphorylation of $p34^{cdc-2}$ and accumulation of cyclin B_1, no inhibition of kinase activity was observed. Nonetheless, 33% of cells were in the G_2M phase of the cycle after treatment with 30 μM genistein.

The latter finding is unexpected, in the presence of such a massive G_2M accumulation of cells (especially in the case of T47D cells). However, it cannot be excluded that additional mechanisms not involving $p34^{cdc-2}$ may contribute to the G_2 arrest that follows DNA damage. In fact, others [Jin et al., 1996] have reported that cells transfected with a mutant p34 that cannot be phosphorylated on Thr14 or Tyr15 still show a significant G_2 delay after irradiation. In such cells, no entry into mitosis was observed despite the high $p34^{cdc-2}$-associated kinase levels. This could be a possible explanation for the T47D cells, but not for the ZR75.1 cells. Another possible explanation could be linked to the experimental design. In fact, our observations were made at a single experimental time. However, it cannot be excluded that such a G_2M block is a transient accumulation that, for some unknown reasons, is later overcome.

Furthermore, a recent study [Kao et al., 1999] suggested that in HeLa cells, radiation-induced G_2M blockade is associated with an increase of histone H1 in the cytoplasmic fraction, which is followed by an increase of kinase activity in the nuclear fraction as soon as cells exit the G_2 delay. Because our results refer to whole cell extracts, it can be hypothesized that selective regulation by cell subcompartimentalization could paradoxically result in apparent absence of regulation.

Despite the uncertainty about the role of MPF inhibition in T47D and ZR75.1 cells, our results confirm and extend previous observations reported in breast cancer cells [Shao et al., 1998] and gastric cancer cells [Matsukawa et al., 1993]. Both studies reported the G_2M block but did not investigate the role of MPF in such premitotic arrest. Shao et al. [1998] reported a p53-independent increase in $p21^{WAF1/CIP1}$, whereas Matsukawa et al. [1993] stressed the different behavior of genistein compared to the phytoestrogen quercetin, which caused a G_0/G_1 arrest.

Another study [Choi et al., 1998] on the two breast cancer cell lines MCF-7 and MDAMB-231 reported a time-dependent inhibition of cyclin B_1 and induction of $p21^{WAF}$ in human breast carcinoma cells. Indeed, our study did confirm the G_2M block and also gave some evidence about the role of MPF in the G_2M block observed in MDAMB-231 and BT20 cells. Our results, together with results reported by others, suggest that in human breast cancer cells, arrest in the G_2M phase of the cycle is not necessarily associated with a deregulation of the MPF. Further studies are needed to understand the molecular mechanism underlying such block.

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