Distinct Chk2 Activation Pathways Are Triggered by Genistein and DNA-damaging Agents in Human Melanoma Cells*

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Genistein, a natural isoflavone found in soybeans, exerts a number of biological actions suggesting that it may have a role in cancer prevention. We have previously shown that it potently inhibits OCM-1 melanoma cell proliferation by inducing a G2 cell cycle arrest. Here we show that genistein exerts this effect by impairing the Cdc25C-dependent Tyr-15 dephosphorylation of Cdk1, as the overexpression of this phosphatase allows the cells to escape G2 arrest and enter an abnormal chromatin condensation stage. Caffeine totally overcomes the G2 arrest, whereas the block caused by etoposide is not bypassed and that caused by adriamycin is only partially abolished. We also report that genistein activates the checkpoint kinase Chk2 as efficiently as the two genotoxic agents and that caffeine may counteract the activation of Chk2 by genistein but not by etoposide. In contrast, caffeine abolishes the accumulation of p53 caused by all the compounds. Wortmannin does not suppress the Chk2 activation in any situation, suggesting that the ataxia telangiectasia-mutated kinase is not involved in this regulation. Finally, unlike etoposide and adriamycin, genistein induces only a weak response in terms of DNA damage in OCM-1 cells. Taken together, these results suggest that the G2 checkpoints activated by genistein and the two genotoxic agents involve different pathways.

Genistein, the major isoflavonoid contained in soybeans, is believed to exert a pleiotropic effect including anti-angiogenic, anti-oxidant, and anti-carcinogenic actions (1–3). Epidemiological studies, as well as work performed on animal models (4, 5), suggest that it is responsible for a chemopreventive effect on breast, colon, and skin tumors. Genistein potently inhibits cell proliferation and may also induce cell differentiation or apoptosis (1, 3, 6). It has been shown to inhibit several tyrosine-specific protein kinases, including pp60v-src and epidermal growth factor receptor tyrosine kinases (7). However, it remains unknown whether the anti-proliferative effect of genistein is dependent on these inhibitions.

Genistein has also been reported to be a non-intercalative inhibitor of DNA topoisomerase II, an enzyme that interacts with DNA and catalyzes the concerted breaking and rejoining of the two DNA strands. Indeed, genistein has been shown to inhibit the relaxation activity of topoisomerase II in vitro and to stabilize topoisomerase II-DNA cleavage complexes in vitro as well as in cellulo (8–11).

Topoisomerase II has been reported to be the primary cellular target for a series of clinically important antitumor agents, including intercalating (i.e. the anthracycine antibiotic, adriamycin, also termed doxorubicin) and non-intercalating (i.e. the epipodophyllotoxin etoposide) compounds. Inhibition of the strand passing activity of topoisomerase II by these compounds is accompanied by stabilization of the topoisomerase II-DNA cleavable complexes, which prevents the rejoining of the two DNA strands (12–14). Inhibitors of DNA topoisomerase II have been shown to arrest mammalian cells in the G2 phase of the cell cycle (15, 16), even when they do not cause direct DNA damage (17).

Cell cycle checkpoints are biochemical pathways that ensure the orderly and timely progression and completion of critical events such as DNA replication and chromosome segregation. Activation of checkpoints in G1 and G2 in response to DNA damage, in S phase upon inhibition of DNA replication, or in mitosis after disruption of the spindle leads to cell cycle arrest. Such delays provide time for repair processes or, in case of severe damage, for the activation of programmed cell death. Defects in the checkpoint regulatory network result in increased sensitivity to damaging agents and to the genomic instability that is often observed in cancer. Paradoxically, checkpoint-evading agents, such as the methylxanthine caffeine, are used in cancer therapy to sensitize cells to killing by genotoxic agents as they override the drug-induced G2 checkpoint (18, 19).

The ultimate target of the G2 checkpoint signaling pathway is the cyclin-dependent kinase (Cdk) complex Cdk1-cyclin B1 (Cdc2 in Schizosaccharomyces pombe), whose activation depends on the phosphorylation of Thr-14 and Tyr-15 residues by the dual specificity phosphatase Cdc25C (20, 21). This dephosphorylation/activation has been demonstrated to be an absolute requirement for the onset of mitosis. Recently, it has been shown that Cdc25C can be negatively regulated by phosphorylation on its Ser-216 residue, during interphase or in response to DNA damage or incomplete DNA replication (22). Phosphorylation at this residue creates a binding site for 14-3-3 proteins that is believed to be responsible for the nuclear export of Cdc25C and the subsequent impairment of nuclear Cdk1 dephosphorylation. Consistent with this model, expression of a

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Cdc25C mutant in which Ala was substituted for Ser-216 abrogated the interaction between Cdc25C and 14-3-3 proteins and induced premature entry in mitosis by overriding both replication and DNA damage checkpoints (22). Two checkpoint kinases have been recently identified in humans; these phosphatase Cdc25C on Ser-216. Chk1 has been proposed to be activated in γ-irradiated HeLa cells (23), whereas Chk2 (Cds1 in S. pombe) has been reported to be involved both in the replication and DNA damage checkpoints (24). The response to DNA damage occurred in an ataxia telangiectasia-mutated (ATM)-dependent manner (24–26).

We have recently reported that genistein arrests human melanoma cells in G2, as has been shown in a number of other cell types (6, 27–30). Furthermore, we have demonstrated that genistein impairs the Tyr-15 dephosphorylation of Cdk1 and that Cdk1-cyclin B1 complexes from genistein-arrested cells can be reactivated in vitro by Cdc25 phosphatase. In this paper, we address the question of whether genistein exerts these effects by activating the signaling cascade involving the kinases Chk1 or Chk2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human choroidal melanoma cells (spindle-shaped OCM-1) were kindly provided by Dr. Maria A. Saornil, Valladolid Institute, Spain, and cultured in RPMI 1640 medium, pH 7.3, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM l-glutamine, and 5% fetal calf serum. All culture reagents and media were from Life Technologies, Inc. Culture media were changed every 2–3 days. When they reached confluence, cells were dissociated by 0.05% trypsin, 0.02% EDTA and replated at 1:30 dilution.

**Cell Cycle Progression Analysis**—Cells were plated at a density of 4 × 10⁵ cells per 100-mm Petri dish. After 24 h, 60 μM genistein, 0.32 μM adriamycin, 30 μM etoposide, or 1 mM hydroxyurea were added in the absence or presence of 2 mM caffeine for a further 24 h (except for etoposide, which was removed after 1 h). Genistein and etoposide were dosed in MeSO (final concentration 0.1%). After 24 h, approximately 10³ cells were harvested by brief trypsinization and centrifuged at 500 x g for 5 min. The cell pellet was washed in PBS and fixed by the gradual addition of ice-cold 70% ethanol. Cells were then labeled with propidium iodide, and the cell cycle distribution was determined by flow cytometry analysis using a Coulter Elite.

**Immunoblotting**—Whole cell lysates were prepared by directly lysing cells in sample buffer (0.5 M Tris, pH 6.8, containing 10% glycerol, 1% SDS-polyacrylamide gel electrophoresis). Gels were either stained with Coomassie Blue to control for balanced loading or electroblotted to nitrocellulose membranes (BA85 from Schleicher and Schuell) for 1 h at 200 V using a semi-dry transfer system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Mouse monoclonal anti-Cdk1 (SC54) and anti-p53 (clone DO1) antibodies were obtained respectively from Santa Cruz Biotechnology and PharMingen. Rabbit polyclonal anti-phospho-Cdk1 (Tyr-15) and anti-phospho-p53 (Ser-15) antibodies were from Biolabs. The anti-hemagglutinin (HA11) antibody was purchased from Babco.

**Overexpression of Cdc25C and Abnormal Chromatin Condonation**—HeLa cells were grown on glass coverslips in Dulbecco's modified Eagle's medium supplemented with glutamine, antibiotics, and 10% fetal calf serum. After treatment with 60 μM genistein for 24 h, the cells were transfected with the HA-tagged Cdc25C pcDNA using linear ethylenimine polymers (Exgen 500, Euromex), according to the instructions of the manufacturer. The transfection efficiency was usually around 10%. Twenty four hours later, the cells were recovered, washed once with PBS, fixed in 3.7% formaldehyde in PBS at 4 °C for 20 min, and then washed 3 times with PBS. They were then permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature and with 100% cold methanol for 10 min at −20 °C. The cells were then washed 3 times in PBS, 1% bovine serum albumin (fraction V, Euromex) for 15 min at room temperature, and then incubated for 1 h at 37 °C with polyclonal anti-14-3-3 proteins Chk1 or Chk2. After 3 washes with PBS, the cells were incubated with secondary antibodies (Alexa 594, 1:400 dilution in PBS, 1% bovine serum albumin) for 45 min at room temperature and then in PBS/Hoechst 33342 (1 μM/ml) for 10 min at room temperature. After 3 washes with PBS and a 4th wash with water, coverslips were mounted for microscopic observation.

**DNA Damage Measurement Using the Alkaline Single Cell Gel Electrophoresis (Comet) Assay**—Comet assays were performed according to Singh et al. (31) with slight modifications. After treatment with the different compounds for 1–4 or 24 h, OCM-1 cells were trypsinized. Immediately, 3.5 × 10⁶ cells were embedded in 0.35 ml of low melting agarose (1% in distilled water) that was layered onto microscope slides. After solidification of agarose, the slides were immersed in cold lysing buffer (1% sodium sarcosinate, 2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, pH 10, and 1% Triton X-100 added fresh) for 1–2 h at 4 °C. The slides were then removed from the lysis solution, gently rinsed with distilled water, and placed on a horizontal gel electrophoresis unit, which was filled with freshly made alkaline buffer (1 mM Na₂EDTA, 300 mM NaOH, pH >12.5). After immersion of the slides for 20 min, electrophoresis was performed in the same buffer for the next 20 min at 2 V/cm. The slides were then gently washed with 0.4 M Tris, pH 7.4, and DNA was stained by immersion in 2.5 μg/ml propidium iodide for 10 min at room temperature in the dark. After 4 gentle rinses in distilled water, the slides were mounted for observation with an epifluorescence microscope (LSM 410 invert laser scan microscope, Zeiss).

The DNA damage in each cell was quantified using an image analysis system (Triton OptiSoft). Twenty five to 76 comets were analyzed for each sample. The “tail moment” accounted for DNA damage and was defined as the product of the percentage of DNA in the comet tail and of the distance between the means of the head and tail DNA distributions (32).

**RESULTS**

**Caffeine Overrides the G2 Cell Cycle Checkpoint Caused by Genistein but Not by Etoposide**—We first compared the effects of genistein and the DNA-damaging agents adriamycin and etoposide on cell cycle distribution in human melanoma cells. As shown in Fig. 1, treatment with genistein, adriamycin, and etoposide led to a clear-cut G2 cell cycle arrest in OCM-1 cells. Interestingly, the G2 block induced by genistein was bypassed by a concomitant treatment of the cells with 2 mM caffeine, whereas the arrest caused by etoposide was not. An intermediate sensitivity to caffeine was observed with adriamycin. These cell cycle events mirrored fairly well the effects of the drugs on cell proliferation (data not shown).

As the status of Cdk1 phosphorylation on Tyr-15 has been shown to be a key event in the control of the G2/M transition (the kinase remaining inactive until it is dephosphorylated), we investigated the effects of the different compounds on the phosphorylation of this residue. As shown in Fig. 2, all three agents resulted in an increase in the amount of the slower electrophoretic mobility form of Cdk1 (upper panel). That this band corresponded to the Tyr-15-phosphorylated form was confirmed by using a specific anti-phospho-Cdk1(Tyr-15) antibody (lower panel). Remarkably, the phosphorylation of Cdk1 induced by genistein and DNA-damaging agents was abolished by caffeine (Fig. 2).

**Overexpression of Cdc25C Bypasses the Genistein-induced G2 Arrest**—We have previously shown that Cdk1-cyclin B1 complexes from genistein-treated OCM-1 cells can be reactivated in vitro by recombinant Cdc25 phosphatase.² We also reported (33) similar results with Cdk1-cyclin B1 complexes isolated from HeLa cells exposed to etoposide. To confirm that the inhibition of Cdk1 was due to the inactivation of the phosphatase in treated cells, we performed transient overexpression of Cdc25C in genistein-arrested HeLa cells. As shown in Fig. 3, when the cells were forced to overexpress Cdc25C (56% of them) left the G2 block and entered an abnormal chromatin condensation stage. In contrast, under our experimental conditions, cells that were not exposed to genistein but that were transfected with Cdc25C did not display any significant sign of chromatin condensation (not shown). These in vivo results strongly suggest that the genistein-induced G2 block

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² F. Casagrande and J. M. Darbon, submitted for publication.
was due to an impairment of Cdc25C activity in genistein-treated cells.

Caffeine Suppresses the Activation of Chk2 by Genistein but Not by Etoposide—Activation of the Chk1 and/or Chk2 kinases has been proposed to be a key event in the DNA damage G2 checkpoint pathway. Phosphorylation/activation of these kinases can be revealed by the appearance of a slower electrophoretic mobility form (23, 24). As shown in Fig. 4a, none of the drugs tested induced any modification of Chk1 electrophoretic mobility. In contrast, genistein activated the checkpoint kinase Chk2, as did etoposide and adriamycin. Indeed, all these agents caused a discrete but significant shift in the electrophoretic mobility of Chk2. Caffeine abolished the genistein-induced and, to some extent, the adriamycin-induced Chk2 activation, but it was unable to counteract the etoposide effect. Whereas caffeine was able to suppress the activation of Chk2 resulting from short term adriamycin treatment (2–4 h, Fig. 4b), it was less efficient in long term treatment (24 h, Fig. 4a).

Genistein and DNA-damaging agents induced p53 accumulation in a somewhat different manner, with adriamycin having a much greater effect, followed by etoposide; genistein treatment led to only a slightly increased level of p53 (Fig. 4a). Similar results were obtained with respect to p21 induction, except that the differences were attenuated (not shown). Caffeine counteracted the accumulation of both p53 (Fig. 4a) and p21 (not shown) induced by all the drugs tested.

As shown in Fig. 4a, the effect of genistein on Chk2 activation was rather rapid, as a 1–2-h cell stimulation led to a significant shift of the protein. The maximal response was reached at 4 h and was maintained at least until 24 h. A dose-response study indicated that Chk2 activation could already be observed with 15–30 μM genistein and that the max-
mammals. Genistein caused only slight DNA damage in melanoma cells. DNA damage was measured using the alkaline single cell gel electrophoresis (Comet) assay as indicated under "Experimental Procedures." Shown here are the typical DNA migration patterns of OCM-1 cells that were untreated (Cont) or treated with 60 μM genistein (Gen), 0.32 μM adriamycin (Adr), or 30 μM etoposide (Eto) for 1–2 h. Similar results were obtained when cells were exposed to genistein or adriamycin for 24 h (not shown). Magnification was × 400.

**DISCUSSION**

Is the G2 Checkpoint Activated by Genistein Distinct from Those Induced by DNA-damaging Agents?—We demonstrate here that genistein exerts its effects on cell cycle somewhat differently from those of the DNA-damaging agents adriamycin and etoposide. (i) Although genistein induced only a weak response in terms of DNA damage in OCM-1 melanoma cells, it caused a complete G2 cell cycle arrest and was as efficient as the two DNA-damaging agents in activating Chk2. (ii) Caffeine completely overrode the genistein-induced G2 cell cycle arrest. In contrast, the blocks caused by etoposide and adriamycin were either not bypassed (etoposide) or only partially abolished (adriamycin) in the presence of caffeine.

These results suggest that the G2 checkpoints activated by genistein and the two DNA-damaging agents are likely to be different in nature. They are compatible with the existence of two different G2 checkpoint pathways in mammals, as proposed by Downes et al. (17) as follows: one sensitive to DNA damage and the other to a decatenation state of DNA. Indeed, these authors reported that the bisdioxopiperazine ICRF-193, a potent topoisomerase II inhibitor, induced a G2 cell cycle block without inducing DNA damage. Unlike other inhibitors, which arrest the enzyme at its transition state after DNA-strand breakage, bisdioxopiperazines trap DNA topoisomerase II as a closed protein clamp, with no associated DNA break.
pho-p53 (Ser-15) antibody. measured by blotting the same membrane with the specific anti-phos-

was evaluated as before. The phosphorylation of p53 on Ser-15 was

HU  

a

6

b 24h

HU (mM) - - - 1 1 5 5

Caff (mM) - - 2 5 - 2 - 5

Chk2

p53

(Wort)

(Eto)

(HU)

FIG. 7. Activation of Chk2 by the DNA replication inhibitor hydroxyurea. OCM-1 cells were treated with 1–5 mM hydroxyurea (HU) for 4 h (a) or 24 h (b) in the absence or in the presence of 2–5 mM caffeine (Caff). Chk2 activation was evaluated as before.

FIG. 8. Lack of inhibition by wortmannin of Chk2 activation in response to G2 and replication block inducers. OCM-1 cells were untreated (Cont) or treated for 4 h with 60 μM genistein (Gen), 0.32 μM adriamycin (Adr), 30 μM etoposide (Eto), or 1 mM hydroxyurea (HU) in the absence or presence of 20 μM wortmannin (Wort). Chk2 activation was evaluated as before. The phosphorylation of p53 on Ser-15 was measured by blotting the same membrane with the specific anti-phospho-p53 (Ser-15) antibody.

(8–10), we show here that genistein is a very weak inducer of DNA damage in OCM-1 cells. However, treatment of other cell types with genistein has been shown to result in the occurrence of protein-linked DNA strand breaks (9, 11).

Activation of Chk2, but Not Chk1, Occurs during S and G2 Cell Cycle Checkpoints in Melanoma Cells—Our data demonstrate a close correlation between the activation of Chk2 by genistein or DNA-damaging agents and the induced cell cycle arrest in G2. When caffeine was able to override completely the G2 checkpoint (in genistein-treated cells), it was also found to abolish Chk2 activation. In contrast, in etoposide-treated cells, caffeine was inefficient both in counteracting Chk2 activation and in bypassing G2 arrest. This close correlation between the two events is further emphasized by the fact that caffeine had an intermediate effect both on Chk2 and G2 block in adriamycin-treated cells. Moreover, when DNA replication was inhibited by hydroxyurea treatment, caffeine was shown to be unable to counteract either the Chk2 activation or the replication block. At first glance, this is surprising, because caffeine has been shown to cause premature mitosis in hamster fibroblasts arrested in early S phase (35). However, in contrast to hamster cells, it has been shown that the S phase block induced by hydroxyurea in murine and human cells is not released by caffeine (36).

Cdk1 Phosphorylation Status Does Not Systematically Correlate with G2 Arrest and Chk2 Activation—We show that Cdk1 is phosphorylated on Tyr-15 in OCM-1 cells treated either with genistein or the DNA-damaging agents adriamycin and etopo-

side. Surprisingly, caffeine treatment led to Cdk1 dephosphorylation in all cases, regardless of its ability to bypass the G2 block. In particular, caffeine caused dephosphorylation/activation of Cdk1 in etoposide-treated cells, although the block was maintained. Such an inability of caffeine to bypass the G2 arrest caused by etoposide has been already reported (17). Recently, Toyoshima et al. (37) demonstrated that, in etoposide-treated HeLa cells, Cdk1 was dephosphorylated (and activated) in the presence of caffeine but that cyclin B1 remained cytoplasmic. When cyclin B1 was forced to accumulate in the nucleus either by treatment with leptomycin B, a specific inhibitor of the nuclear export signal-dependent intracellular transport, or by expressing a nuclear export signal-disrupted cyclin B1, caffeine became able to override the G2 block (37). These results suggest that the etoposide-induced G2 arrest is under the control of at least two different mechanisms, a caffeine-sensitive mechanism (the inhibition of Cdk1 dephosphorylation) and a caffeine-insensitive, nuclear export signal-mediated transport mechanism (leading to the nuclear exclusion of cyclin B1).

In any case, we have shown that caffeine is unable to disrupt not only the G2 block but also the Chk2 activation pathway in etoposide-treated cells. Cdk1 was dephosphorylated in these cells, although Chk2 was still activated. This could suggest that Chk2 is not really involved in the regulation of Cdk1. One can rather speculate that Cdk1 is mainly cytoplasmic in these cells due to the impairment of nuclear localization of cyclin B1. As a consequence, the kinase might be dephosphorylated in the cytoplasm by Cdc25C that is itself prevented from localizing in the nucleus because of the maintained Chk2 activation.

Is the Activation of Chk2 ATM-independent?—In this study we show that wortmannin does not inhibit the Chk2 activation caused by all the drugs we have tested. This is rather surprising with regard to the agents causing important DNA damage like adriamycin and etoposide, since the PI 3-kinase inhibitor has been previously reported to abolish the activation of Chk2 caused by γ-irradiation in HeLa cells (34). This inhibition was believed to reflect the ATM dependence of Chk2 activation (24–26). As the concentration of wortmannin used in our experiments was shown to inhibit the phosphorylation of p53 on Ser-15, the Chk2 activation we observed was very likely ATM-independent. Indeed, the phosphorylation at that specific site has been reported to reflect cellular ATM activity (38–40).

Chk2 Activation Pathways

TABLE I

Quantification of DNA damage caused by genistein, adriamycin, and etoposide in OCM-1 cells

OCM-1 were either untreated (Cont) or treated with 60 μM genistein (Gen), 0.34 μM adriamycin (Adr), or 30 μM etoposide (Eto) for short term (1–4 h) or long term (24 h) periods. Twenty-five to 76 comets were analyzed for each sample. The tail moment accounted for DNA damage and was defined as the product of the percentage of DNA in the comet tail and of the distance between the means of the head and tail DNA distributions.

Data for each of the four experiments are given as means ± S.D. of the different tail moment parameters. To compare further the increase in the tail moment caused by genistein, means ± S.D. of the four experiments are also indicated (right column).

|          | Exp 1 |          | Exp 2 |          | Exp 3 |          | Exp 4 |          | Mean ± S.D. |
|----------|-------|----------|-------|----------|-------|----------|-------|----------|-------------|
| Short term (1–4 h) |       |          |       |          |       |          |       |          |             |
| Cont     | 3.63 ± 3.44 | (n = 46) | 3.13 ± 2.15 | (n = 66) |       |          |       |          |             |
| Gen      | 4.61 ± 3.38 | (n = 53) | 4.17 ± 2.80 | (n = 60) |       |          |       |          |             |
| Adr      | 18.78 ± 7.70 | (n = 74) |          |          |       |          |       |          |             |
| Long term (24 h) |       |          |       |          |       |          |       |          |             |
| Cont     | 3.50 ± 1.83 | (n = 25) | 3.12 ± 2.29 | (n = 4) | 3.34 ± 0.25 | (n = 4) |       |          |             |
| Gen      | 4.61 ± 1.64 | (n = 25) | 5.50 ± 3.76 | (n = 4) | 4.77 ± 0.55 | (n = 4) |       |          |             |
| Adr      | 26.64 ± 9.58 | (n = 25) |          |          |       |          |       |          |             |
kinase has been recently shown to be caffeine-sensitive (38), and it does for p53. In contrast with distinct effects on Chk2, we have shown that caffeine strongly decreases the accumulation of p53 induced by the compounds tested. Consistent with this observation, caffeine was found to inhibit the p53 phosphorylation on Ser-15 (not shown). These results and those obtained with wortmannin suggest a dissociation between Chk2 activation and p53 phosphorylation/acumulation. As discussed above, ATM is very likely the main kinase that phosphorylates p53 on Ser-15 in response to DNA damage. As a consequence, we propose that the different compounds we have studied induced phosphorylation/stabilization of p53 via ATM (Fig. 9). Since we have also demonstrated the ATM independence of the Chk2 activation caused by these agents, this suggests that ATM is unable to activate Chk2 in cellulo by direct phosphorylation.

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Distinct Chk2 Activation Pathways Are Triggered by Genistein and DNA-damaging Agents in Human Melanoma Cells

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