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The Plant Isoflavenoid Genistein Activates p53 and Chk2 in an ATM-dependent Manner*

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Genistein is an isoflavenoid that is abundant in soy beans. Genistein has been reported to have a wide range of biological activities and to play a role in the diminished incidence of breast cancer in populations that consume a soy-rich diet. Genistein was originally identified as an inhibitor of tyrosine kinases; however, it also inhibits topoisomerase II by stabilizing the covalent DNA cleavage complex, an event predicted to cause DNA damage. The topoisomerase II inhibitor etoposide acts in a similar manner. Here we show that genistein induces the up-regulation of p53 protein, phosphorylation of p53 at serine 15, activation of the sequence-specific DNA binding properties of p53, and phosphorylation of the hCds1/Chk2 protein kinase at threonine 68. Phosphorylation and activation of p53 and phosphorylation of Chk2 were not observed in ATM-deficient cells. In contrast, the topoisomerase II inhibitor etoposide induced phosphorylation of p53 and Chk2 in ATM-positive and ATM-deficient cells. In addition, genistein-treated ATM-deficient cells were significantly more susceptible to genistein-induced killing than were ATM-positive cells. Together our data suggest that ATM is required for activation of a DNA damage-induced pathway that activates p53 and Chk2 in response to genistein.

Genistein is a naturally occurring isoflavenoid that is abundant in soy beans and has been reported to have anti-carcinogenic effects (1–3). Soy-based diets have been reported to reduce the risk of a variety of cancers including, breast, prostate, and colon cancers (4–6). Moreover, genistein has been reported to be responsible for low rates of breast cancer in Asian women (7). However, the mechanism of action of genistein on cells remains unclear. Genistein acts as a broad specificity tyrosine kinase inhibitor (8) but also inhibits topoisomerase II and generates DNA damage by stabilizing the covalent topoisomerase II-DNA cleavage complex (9–11). Genistein has been reported to induce DNA laddering and apoptosis in breast cancer cell lines (10–14), suggesting that in some cells it initiates a DNA damage response that leads to apoptosis. Other topoisomerase II poisons, such as etoposide, induce apoptosis in a variety of cell lines (15–18). Treatment of HL60 cells with etoposide induces caspase-mediated cleavage of ATM (ataxia-telangiectasia mutated protein; Ref. 19), and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (16–18, 20).[1]

DNA-PKcs and ATM are both members of a family of phosphatidylinositol 3-kinase-like proteins (reviewed in Refs. 21 and 22). ATM is mutated in the human autosomal disorder, ataxia-telangiectasia (A-T), which is characterized by ataxia, abnormal vasodilation, radiosensitivity, predisposition to cancer, and immune defects (reviewed in Ref. 23). ATM encodes a polypeptide of ~360 kDa that, like DNA-PKcs, contains a C-terminal phosphatidyl inositol 3-kinase-like catalytic domain (24–26). Another characteristic of A-T cells is failure to arrest at G_2/M following exposure to ionizing radiation (27, 28). Indeed, ATM is required for activation of p53 and Chk2, resulting in cell cycle arrest at G_1/S and G_2/M respectively (reviewed in Ref. 29). Cells that lack ATM show delayed up-regulation and phosphorylation of p53 (reviewed in Ref. 30) and defective phosphorylation of Chk2 in response to ionizing radiation (31, 32). *In vitro*, ATM is a manganese-dependent serine threonine protein kinase that phosphorylates serine 15 of p53 (33–35) and N-terminal sites in Chk2 (35). ATM is therefore a key player in signaling DNA damage to cell cycle checkpoints.

Because etoposide and genistein have both been reported to induce apoptosis by initiating topoisomerase II-induced DNA damage, we were interested to determine whether they activated an ATM-dependent pathway. Indeed, genistein has been shown to induce up-regulation of p53 protein levels in ATM-positive cells (36). Here we show that treatment of ATM-positive cells with genistein activates the DNA-binding function of p53, induces phosphorylation of p53 at serine 15, and induces phosphorylation of Chk2 at threonine 68. Genistein did not induce activation of p53 or phosphorylation of Chk2 in two ATM-deficient human cell lines, suggesting that genistein activates a pathway that requires ATM or is mediated by ATM. Moreover, we show that the topoisomerase inhibitor, etoposide, but not the tyrosine kinase inhibitor herbimycin A, also induces phosphorylation of p53 at serine 15 and phosphorylation of Chk2, supporting the idea that genistein induces DNA damage that is recognized by an ATM-dependent pathway. In contrast, etoposide induced phosphorylation of both p53 and Chk2 in ATM-deficient cells as well as in normal cells, suggesting that etoposide activates protein kinases other than ATM *in vivo*.

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[1] The abbreviations used are: DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; A-T, ataxia-telangiectasia; GST, glutathione *S*-transferase; ATR, ATM-Rad3-related protein; Gy, gray.

MATERIALS AND METHODS

Reagents—Genistein was obtained from Calbiochem. Etoposide was purchased from Sigma. Herbimycin was purchased from Life Technologies, Inc.

Cells—ATM-positive cells (BT and C3ABR) and ATM-deficient cells (L3 and AT1ABR) (37) were grown at 37 °C, 5% CO_2 in RPMI containing 10% fetal calf serum (Hyclone). Genistein, etoposide, and herbimycin A were dissolved in Me_2SO and stored as stock solutions at −20 °C. Where indicated genistein, etoposide, or herbimycin A was added directly to the cell medium in which the cells were suspended. Control cells were treated with an equivalent volume of Me_2SO. Where indicated, cells were irradiated, in the presence of serum and medium, with 10 Gy ionizing radiation using a Gammacell 1000 Cesium 137 source (MDS Nordion), at a dose rate of 250 cGy/min. After irradiation or addition of genistein, etoposide, or herbimycin A, cells were returned to a humidified incubator at 37 °C under 5% CO_2.

Protein Extracts—Crude cytoplasmic (S10) and nuclear (P10) extracts were made as described previously (38). Briefly, cells were harvested and washed in phosphate buffered saline, followed by hypotonic buffer (low salt buffer; Ref. 39). Cell pellets were lysed by a single freeze thaw and centrifuged at 10,000 × *g* for 10 min to produce the S10 supernatant. The resulting pellet was extracted with low salt buffer containing 0.5 M NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and the supernatant was recovered after centrifugation at 10,000 × *g* for 10 min to produce the P10. Protease inhibitors (aprotinin, leupeptin, and phenylmethysulfonyl fluoride, 5 μg/ml, 5 μg/ml, and 0.2 mM, respectively) and phosphatase inhibitors (sodium vanadate, β-glycerol phosphate and sodium fluoride at 0.04 mM, 2 mM, and 10 mM, respectively) were added to cell extracts immediately after freeze-thaw and to the 0.5 M salt buffer used to prepare the P10 extracts. Protein concentrations in extracts were determined using the Bio-Rad microprotein assay using bovine serum albumin as standard.

Electrophoretic Mobility Shift Assays—A synthetic oligonucleotide corresponding to the consensus for p53 binding was as described previously (40). DNA binding extracts contained 9 μg of total protein in 25 mM Hepes, pH 7.5, 75 mM KCl, 10% glycerol, 1 mM dithiothreitol plus 1 μg of poly(dI-dC) as competitor DNA, 4 μl of the p53 monoclonal antibody, Pab 421, and ~10 femtomoles of p53 oligonucleotide that had been labeled at the 5′ position with [^{32}P]ATP (40). Samples were set up on ice to contain buffer, competitor DNA, and protein, and radiolabeled DNA was added last. Samples were incubated at room temperature for 15 min then loaded directly on prerun, nondenaturing polyacrylamide gels. Electrophoresis was as described previously (41).

Western Blots—SDS-polyacrylamide gel electrophoresis and Western blots for DNA-PKcs and ATM were as described previously (42). Chk2 and p53 were analyzed on 10% acrylamide SDS gels. A rabbit polyclonal antibody to the rad3-like domain of ATM (4BA) was used as described previously (35). Antibodies to p53 (Pab421 and DO-1) and p21 (Ab-4) were purchased from Oncogene Scientific. Phosphospecific antibodies to serine 15 of p53 and threonine 68 of Chk2 were as described previously (37, 43). A rabbit polyclonal antibody to Chk2 was as previously described (32). Western blots were developed using ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunoprecipitation Assays—ATM was immunoprecipitated from cells as described by Canman *et al.* (34). Briefly, ~10^6 cells were pelleted and washed two times in phosphate-buffered saline. Cells were lysed by sonication in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 50 mM β−glycerol phosphate, 150 mM NaCl, 10% glycerol, 1% Tween 20, 1 mM NaF, 1 mM $NaVO_4$, 1 mM phenylmethysulfonyl fluoride, 2 μg/ml pepstatin, 5 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM dithiothreitol), and ATM was immunoprecipitated by a ATM rabbit polyclonal antibody (Ab-3, Oncogene Scientific). Immunoprecipitates were washed two times with 500 μl of immunoprecipitation buffer, twice with 100 mM Tris-HCl, pH 7.5, containing 0.5 M LiCl, followed by two washes in pre-kinase buffer (10 mM Hepes, pH 7.5, 50 mM β-glycerophosphate, 50 mM NaCl, and 1 mM dithiothreitol). ATM kinase activity was assayed in pre-kinase buffer containing 10 mM $MnCl_2$, 10 μM ATP containing 5 μCi of [γ-^{32}P]ATP, and 0.5 μg of protein substrate. Substrates used were PHAS-I (Stratagene), GST-tagged recombinant Chk2 (amino acids 1–222), or recombinant GST-tagged p53 (amino acids 1–40) as described previously (35). Samples were analyzed on 16% polyacrylamide SDS gels and exposed to Fuji x-ray film at −80 °C with intensifying screens for 12–16 h.

DNA Fragmentation Assays—Genomic DNA was extracted from ~10^6 cells using DNAzol reagent (Life Technologies, Inc.) exactly as described in the manufacturer's instructions. 20 μg of DNA was run on 1.5% agarose submarine gels in Tris-borate-EDTA buffer at 100 V for 1.5 h. Gels were stained with ethidium bromide for 1 h, and DNA was imaged under UV light using a BioGel scanner (Bio-Rad).

Cell Survival Assays—2 × 10^5 BT and L3 cells were cultured overnight in RPMI medium containing penicillin/streptomycin and 10% fetal calf serum (Hyclone). Genistein and etoposide were then added to the media to final concentrations of 0, 25, 50, 100, 200, and 300 μM and 6.8, 13.6, 34, 68, and 136 μM, respectively. After 2 h the drug was removed, and the cells were washed twice in 1 ml RPMI/10% fetal calf serum medium and allowed to grow for 5–7 days. Following incubation with 5 μCi/ml tritiated thymidine (Amersham Pharmacia Biotech) for 90 min, cells were centrifuged at 1,500 × *g* for 5 min and washed twice in phosphate-buffered saline. The cell pellet was lysed in 1 N NaOH and added to 1 ml of ReadySafe (Beckman) scintillation fluid for counting on the Beckman LS6500 scintillation counter.

RESULTS

Effects of Genistein on ATM-dependent Signaling—Exposure of cells to ionizing radiation results in activation of ATM-dependent signaling pathways that leads to activation of p53 and the protein kinase Chk2 (31). In the case of p53, ionizing radiation results in an increase in total protein levels and phosphorylation of p53 at N-terminal sites, including serine 15. Cells that lack ATM show delayed up-regulation of p53 protein levels in response to ionizing radiation. Consequently, phosphorylation of p53 at serine 15, and DNA binding of p53 to its cognate DNA binding site is reduced in A-T cells (reviewed in Ref. 30). In addition, ionizing radiation results in ATM-dependent activation and phosphorylation of Chk2, which in turn leads to phosphorylation and inactivation of the dual specificity tyrosine phosphatase, cdc25 (32).

Genistein has been shown to induce stabilization of p53 protein levels in normal cells but not in A-T cells (36), suggesting that it may activate a pathway similar to that activated by ionizing radiation. To further understand the mode of action of genistein, we first examined whether genistein also induced phosphorylation of p53 at serine 15. A-T cells and normal cells were exposed to genistein (100 μM) for 2 h. Cells were then harvested, crude cytoplasmic (S10) and nuclear (P10) extracts were prepared, and extracts were analyzed for total p53 protein levels using DO-1 antibody. As shown previously, genistein resulted in an increase in p53 protein levels in ATM-positive cells, which were greatly reduced in A-T cells (Fig. 1*A*, *upper panel*). When the same samples were probed with a phosphospecific antibody to serine 15 of p53, p53 was seen to be highly phosphorylated in genistein-treated control, but not A-T cells (Fig. 1*A*, *lower panel*). We note that the reduced phosphorylation of p53 observed in A-T cells may reflect lack of protein induction rather than a decrease in the stoichiometry of p53 phosphorylation. The effects of genistein on p53 are therefore highly similar to those observed with ionizing radiation, in that both up-regulation of p53 protein and phosphorylation at serine 15 is greatly diminished in ATM-defective (A-T) cells.

We next examined whether genistein affected the ability of p53 to bind to a DNA oligonucleotide containing the p53 consensus sequence. Treatment of ATM-positive cells with genistein was found to dramatically increase the ability of p53 to bind to its specific DNA sequence (Fig. 1*B*, *lanes 1–4*). Binding of p53 to DNA was significantly reduced in A-T cells compared with normal cells (Fig. 1*B*, *lanes 5–8*), again indicating that the effect of genistein on p53 is very similar to that of ionizing radiation.

Activation of the sequence-specific DNA binding properties of p53 suggests that genistein might activate the transcriptional activation activity of p53 and result in induction of downstream genes such as p21. Western blotting with an antibody to p21 revealed that p21 protein levels were elevated in response to genistein in ATM-positive cells (Fig. 1*C*).

When ATM-positive cells are exposed to ionizing radiation, Chk2 undergoes a reduction in mobility on SDS-polyacryl-

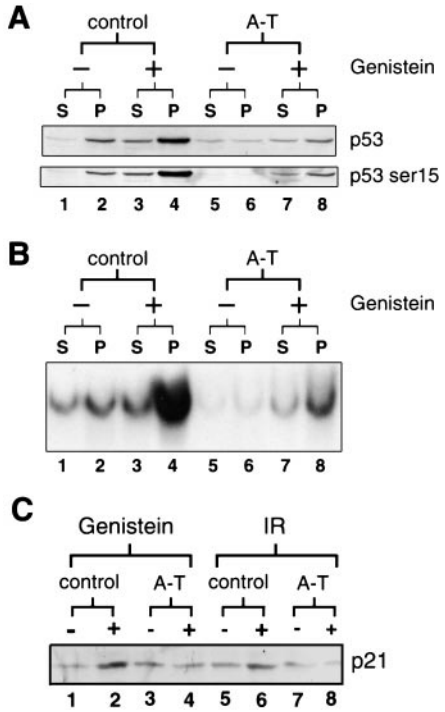

FIG. 1. **Genistein activates p53 and Chk2 in ATM-positive cells.** *A*, Western blot of p53 levels and serine 15 phosphorylation in genistein-treated cells. Control lymphoblastoid cells (BT, *lanes 1–4*) or A-T cells (L3, *lanes 5–8*) were incubated with genistein (100 μM, *lanes 3*, *4*, *7*, and *8*) or an equal volume of Me_2SO (*lanes 1*, *2*, *5*, and *6*) for 2 h at 37 °C under CO_2 and humidity. S10 (*S*) and P10 (*P*) extracts (cytoplasmic and nuclear respectively) were prepared in the presence of phosphatase and protease inhibitors as described under "Materials and Methods." 20 μg of total protein was analyzed by Western blot using a panspecific antibody (DO-1) to p53 (*upper panel*) or a phosphospecific antibody to serine 15 of p53 (*lower panel*). *B*, electrophoretic mobility shift assay of p53 DNA binding activity. The same extracts were analyzed by electrophoretic mobility shift assay for the ability of endogenous p53 to bind to DNA as described under "Materials and Methods." ATM-positive cells (*lanes 1–4*) or ATM-negative cells (*lanes 5–8*) were either treated with an equivalent volume of Me_2SO (*lanes 1*, *2*, *5*, and *6*) or genistein (100 μM, *lanes 3*, *4*, *7*, and *8*) for 2 h. Shown is an autoradiogram of an overnight exposure at −80 °C with intensifying screens. *C*, Western blot of genistein treated cells for p21 protein expression. ATM-positive (*lanes 1*, *2*, *5*, and *6*) or ATM-negative (*lanes 3*, *4*, *7*, and *8*) cells were exposed to 100 μM genistein for 2 h (*lanes 1–4*) or irradiated with 10 Gy ionizing radiation and allowed to recover for 2 h (*lanes 5–8*). 100 μg of protein extract was analyzed by Western blot using a monoclonal antibody to p21. IR, ionizing radiation.

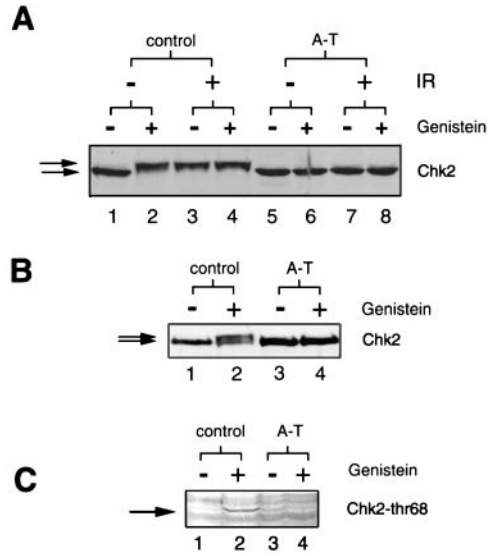

FIG. 2. **Western blot for Chk2 modification in normal and A-T cell lines.** *A*, a normal lymphoblastoid cell line (BT, *lanes 1–4*) or an A-T line (L3, *lanes 5–8*) were treated with genistein (100 μM) for 2 h. S10 samples were prepared as described in the legend to Fig. 1*A*, and 20 μg of protein was analyzed by Western blot using an antibody to Chk2. The phosphorylated form of Chk2 migrates slightly slower than the unphosphorylated form of the protein, as indicated by the *arrows* (32). Where indicated, cells were irradiated at 10 Gy either in the absence (*lanes 3* and *7*) or the presence of genistein (*lanes 4* and *8*). The majority of the Chk2 was found in the S10 fraction (shown), but similar results were observed in the P10 fraction (data not shown). *B*, extracts were prepared exactly as in *A* from either a normal cell line (C3ABR, *lanes 1* and *2*) or an A-T line containing mutant, inactive ATM (AT1ABR, *lanes 3* and *4*) either in the absence (*lanes 1* and *3*) or presence of genistein (*lanes 2* and *4*). *C*, extracts were prepared from BT or L3 cells (control and A-T, respectively) exactly as in *A*. 100 μg of protein from the S10 fraction was loaded onto a 10% SDS-polyacrylamide gel and blotted with an phosphospecific antibody to threonine 68 of Chk2. IR, ionizing radiation.

amide gel electrophoresis/Western blot that has been shown to be the result of phosphorylation (32). The same antibody was therefore used to probe for changes in Chk2 mobility in response to genistein. Slower migration of Chk2 was observed in control cells (ATM-positive cells) that had been treated with genistein (Fig. 2*A*, *lane 2*) but was completely absent in ATM-defective (A-T) cells (Fig. 2*A*, *lane 6*). The genistein-induced change in migration of Chk2 in control cells was indistinguishable from that observed by exposure to ionizing radiation (Fig. 2*A*, compare *lanes 2* and *3*).

The cell lines used for these experiments were lymphoblastoid cell lines from an A-T patient (L3) and a normal control (BT). This therefore raises the possibility that the lack of effect of genistein on Chk2 in the ATM-deficient cell line L3 could be due to some other defect in these cells. We therefore repeated these experiments on another pair of cell lines, C3ABR, which has normal levels of ATM, and AT1ABR, which contains mutant inactive ATM. Again, genistein treatment was found to result in modification of Chk2 only in the cell line containing normal ATM (Fig. 2*B*, *lanes 1* and *2*). Similarly, phosphorylation of p53 at serine 15 was also more pronounced in the normal cell compared with the A-T line (AT1ABR) (data not shown). Together, these data strongly support the idea that the effects of genistein on Chk2 are mediated through ATM.

It has recently been shown that ionizing radiation induces ATM-dependent phosphorylation of Chk2 on threonine 68 (43, 44). We therefore used a phosphospecific antibody to threonine 68 (43) to examine phosphorylation of Chk2 in response to genistein. Exposure to genistein was found to induce phosphorylation of Chk2 at threonine 68 in ATM-positive cells but not in A-T cells (Fig. 2*C*). We therefore conclude that ATM is essential for the phosphorylation of Chk2 in response to exposure to both ionizing radiation and genistein.

Genistein Activates ATM Kinase Activity—Exposure of cells to ionizing radiation or the radiomimetic drug neocarzinostatin, results in increased kinase activity of immunoprecipitated ATM (33, 34). We therefore assayed for ATM kinase activity toward known *in vitro* substrates, PHAS-I, Chk2, and p53 (33, 34, 35) in cells treated with genistein. Treatment with genistein induced a modest but reproducible increase in ATM kinase activity in ATM-positive cells (Fig. 3) but not A-T cells (data not shown), consistent with activation of a DNA damage response pathway.

Caffeine and Wortmannin Abrogate the Effects of Genistein on p53 and Chk2—The protein kinase activity of ATM and other phosphatidylinositol 3-kinase-like proteins such as DNA-PKcs and ATM-Rad3-related protein (ATR) is inhibited by treatment of cells with wortmannin or caffeine (45–47). ATM-positive cells were therefore treated with genistein plus either wortmannin or caffeine. As shown in Fig. 4, wortmannin re-

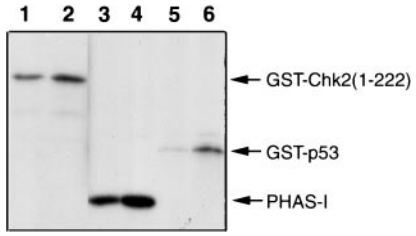

FIG. 3. **ATM-positive (BT) cells were either untreated (*odd-numbered lanes*) or treated with genistein (100 μM) (*even-numbered lanes*).** After 2 h, extracts were made for immunoprecipitation, and ATM kinase activity was assayed with protein substrates at 0.5 μg. Substrates used were: recombinant GST-tagged Chk2 (amino acids 1–222) (*lanes 1* and *2*), PHAS-1 (*lanes 3* and *4*), and recombinant GST tagged p53 (amino acids 1–40) (*lanes 5* and *6*).

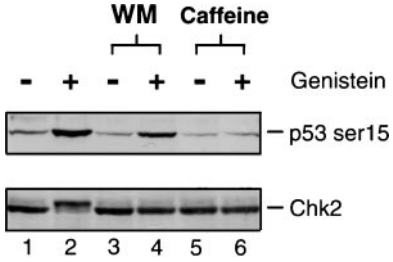

FIG. 4. **Wortmannin and caffeine abrogate the genistein induced activation of p53 and Chk2.** Normal (BT) cells were incubated with genistein (100 μM) (*lanes 2*, *4*, and *6*) in the presence of either 20 μM wortmannin (*WM*, *lanes 3* and *4*) or 10 mM caffeine (*lanes 5* and *6*) for 2 h. Extracts were prepared as previously and assayed by Western blot using antibodies to either serine 15 of p53 or Chk2.

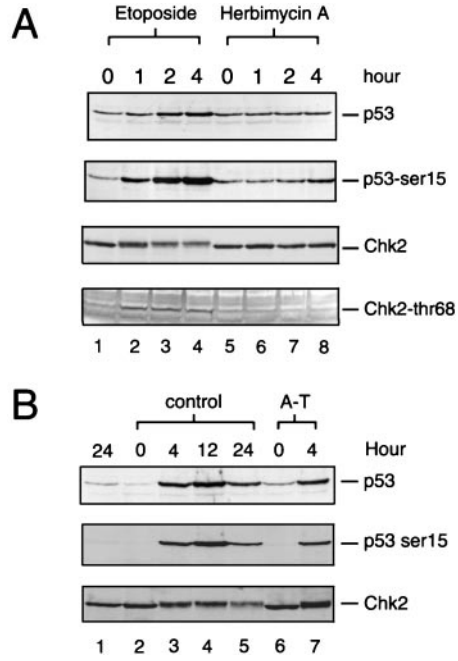

FIG. 5. **Etoposide but not herbimycin induces phosphorylation of p53 and Chk2.** *A*, control cells (BT) were untreated (*lanes 1* and *5*), incubated with etoposide (68 μM) (*lanes 2–4*), or herbimycin A (*lanes 6–8*). At the times indicated, cells were harvested, and 20 μg total protein was analyzed by Western blot using antibodies to p53 (DO-1), serine 15 of p53, or Chk2 as indicated. In the *lower panel*, 100 μg of protein was analyzed by Western blot using a phosphospecific antibody to threonine 68 of Chk2. *B*, control cells (BT, *lanes 1–5*) or A-T cells (L3, *lanes 6* and *7*) were incubated with herbimycin A at 1 μM (*lane 1*) or etoposide at 68 μM (*lanes 2–7*) for 0–24 h as indicated. Extracts were made and blots were analyzed with antibodies to DO-1, serine 15 of p53, and Chk2 as described for *A*.

duced genistein-induced phosphorylation of p53 (on serine 15) and completely abrogated the phosphorylation-dependent mobility shift of Chk2 (*lane 4*). Caffeine, on the other hand completely abrogated both phosphorylation of p53 and the phosphorylation dependent mobility shift of Chk2 (Fig. 4, *lane 6*).

Genistein and the Topoisomerase II Inhibitor Etoposide, but Not the Tyrosine Kinase Inhibitor Herbimycin A, Induce Phosphorylation of p53 and Chk2—Genistein has been reported to act as both a broad specificity inhibitor of protein-tyrosine kinases and an inhibitor of topoisomerase II. To determine whether the effects of genistein on p53 and Chk2 were due to ATM-dependent inhibition of a tyrosine kinase or of topoisomerase II, cells were treated with either herbimycin A, a tyrosine kinase inhibitor that does not induce apoptosis (48, 49), or etoposide. Like genistein, etoposide stabilizes the covalent topoisomerase II-DNA cleavage intermediate, a reaction that is known to cause DNA breaks (11) and induce apoptosis (15) *in vivo*. Etoposide induced phosphorylation of p53 on serine 15, a phosphorylation-dependent reduction in mobility of Chk2, and phosphorylation of Chk2 on threonine 68 (Fig. 5*A*). In contrast, herbimycin at concentrations known to inhibit tyrosine kinases (48–50) did not induce significant phosphorylation of p53 or Chk2 (Fig. 5*A*). From these data we conclude that genistein-induced activation of p53 and Chk2 is due to topoisomerase II induced damage rather than inhibition of a tyrosine kinase.

Our results clearly show that genistein activates p53 and Chk2 in an ATM-dependent manner. Because genistein and etoposide both act on topoisomerase II, we next asked whether, like genistein, the action of etoposide is also ATM-dependent. Treatment of cells with etoposide was found to induce phosphorylation of p53 at serine 15, the phosphorylation-dependent mobility shift of Chk2 (Fig. 5*B*), and phosphorylation of Chk2 on threonine 68 (data not shown). However, unlike genistein, none of these events was dependent on the presence of ATM (Fig. 5*B*, *lane 7*). These data suggest that although genistein and etoposide both act on topoisomerase II, they may act via different pathways, one that requires ATM and one that does not.

Etoposide but Not Genistein Induces Protein and DNA Fragmentation—Among the hallmarks of apoptosis are caspase-mediated cleavage of key protein substrates and fragmentation of chromatin to produce characteristic DNA ladders. We and others have shown that treatment of a variety of cell types with etoposide results in cleavage of the ATM-related protein, DNA-PKcs, to produce an N-terminal 240-kDa fragment and a 150-kDa C-terminal fragment (16–18). Similarly, the ATM protein is cleaved during apoptosis to produce a 240-kDa fragment (19). We therefore treated ATM-positive (BT) and ATM-deficient cells (L3) with genistein, etoposidem or the tyrosine kinase inhibitor herbimycin A. Because etoposide induces cleavage of both DNA-PKcs and ATM in HL60 cells, HL60 cells were used as a positive control.

Neither ATM nor DNA-PKcs proteins were cleaved when BT or L3 cells were treated with genistein, or etoposide for 2, 4, 8, or 24 h (Fig. 6*A* and data not shown). Even exposure of BT and L3 cells to 300 μM genistein for 24 h did not induce cleavage of DNA-PKcs or ATM (data not shown), suggesting that genistein is not activating a caspase-mediated pathway in these particular cells. Because etoposide also failed to induce cleavage of ATM and DNA-PKcs, our data suggest that these cells may be resistant to apoptosis. Treatment of HL60 cells with etoposide resulted in cleavage of both DNA-PKcs and ATM within 4 h of treatment (Fig. 6*B*); however, incubation with up to 300 μM genistein had no effect on either protein in HL60 cells (data not shown). Similarly, etoposide but not genistein induced DNA fragmentation in HL60 cells (Fig. 6*C*). Neither etoposide nor

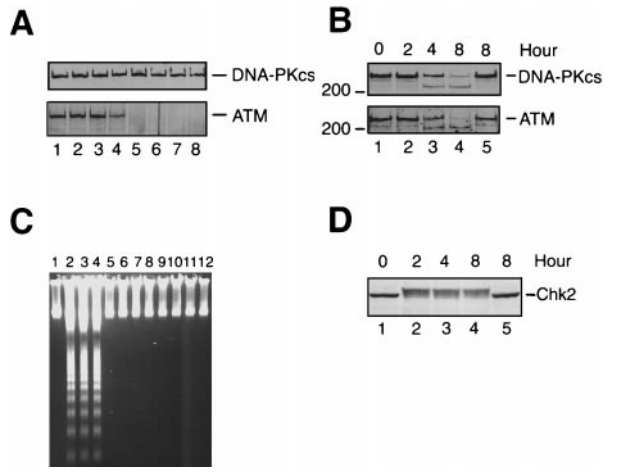

FIG. 6. **Etoposide but not genistein induces cleaved of DNA-PKcs and ATM.** *A*, control (BT) cells (*lanes 1–4*) or A-T cells (L3) (*lanes 5–8*) were incubated for 24 h with either etoposide (68 μM) (*lanes 2* and *6*), genistein (100 μM) (*lanes 3* and *7*), or herbimycin A (1 μM) (*lanes 4* and *8*), or were untreated (*lanes 1* and *5*). Extracts were made as described for Fig. 5*A* and assayed by Western blot using an antibody (DPK1) to amino acids 2018–2137 of DNA-PKcs or antibody 4BA to ATM as indicated. *B*, HL60 cells were incubated with etoposide (68 μM) (*lanes 1–4*) or with an equivalent volume of Me_2SO (*lane 5*) for 0–8 h as indicated. Samples were prepared for Western blot exactly as for Fig. 5*A*. *C*, total genomic DNA was extracted from cells using DNAzol reagent (as described under "Materials and Methods"), and 20 μg was analyzed on 1.5% agarose gels and stained with ethidium bromide as described. *Lane 1*, HL60 cells, untreated; *lanes 2–4*, HL60 cells treated with etoposide for 2, 4, or 8 h, respectively; *lanes 5–8*, ATM-positive (BT) cells either untreated (*lane 5*) or incubated with etoposide (*lane 6*), genistein (*lane 7*), or herbimycin (*lane 8*) at 68, 100, or 1 μM, respectively, for 24 h. Samples in *lanes 9–12* were derived from A-T cells (L3) that were treated with etoposide, genistein, or herbimycin exactly as in *lanes 5–8*. *D*, samples were prepared from etoposide treated HL60 cells exactly as described for *B*. Samples were assayed for Chk2 phosphorylation using the antibody previously described (32).

genistein induced cleavage of DNA in the BT or L3 cells under the conditions used in this study (Fig. 6*C*).

Incubation of HL60 cells (which do not contain p53 protein) with etoposide also resulted in phosphorylation of Chk2 (Fig. 6*D*), indicating that etoposide induced activation of Chk2 can occur in the absence of an intact p53 damage response pathway.

Although our data suggest that genistein does not induce an apoptotic pathway in BT and L3 cells, phosphorylation of p53 and Chk2 suggested that a DNA damage response pathway was being activated. Because our data clearly show that genistein preferentially acts on ATM-positive cells, we reasoned that ATM-positive and -negative cells might have different survival rates after exposure to genistein. Cells were treated with either genistein or etoposide for 2 h, and cell survival was determined as described under "Material and Methods." As predicted, ATM negative cells were more susceptible to genistein treatment than ATM-positive cells, particularly at higher doses (Fig. 7, *left panel*). In contrast, etoposide induced similar levels of cell death in both cell types at all doses used (Fig. 7, *right panel*).

DISCUSSION

Genistein belongs to a family of naturally occurring phytoestrogens that includes quercetin, biochanin A, kaempferol, and daidzein. These compounds have been reported to have a wide variety of biological functions, including both growth promoting and anti-tumorigenic effects. Genistein, in particular, has been reported to have a plethora of biological activities, including inhibition of tyrosine kinases, inhibition of topoisomerase II, anti-proliferative effects in breast cancer cells, induction of growth arrest, and apoptosis. Here we show that

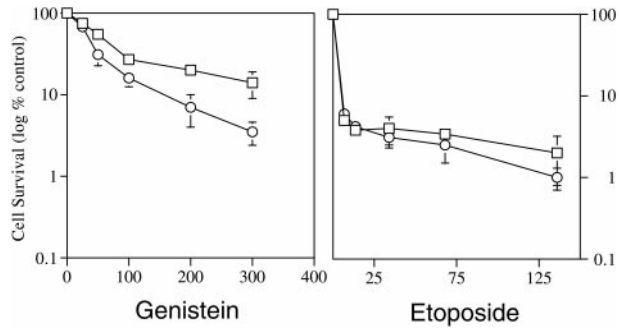

FIG. 7. **Cell survival analysis of BT and L3 cells treated with genistein or etoposide.** Cells were treated for 2 h with either genistein (*left panel*) or etoposide (*right panel*) at the concentrations indicated. Cell survival was measured as described under "Materials and Methods." *Squares* indicate BT cells (ATM-positive, control cells), and *circles* represent L3 cells (ATM-deficient cells). Results represent the average of three separate experiments.

genistein induces ATM-dependent up-regulation of p53, phosphorylation of p53 at serine 15, and activation of the sequence specific DNA binding properties and transcriptional activation of p53. These and previous data (36) suggest that genistein signals via ATM to p53 at the G_1/S cell cycle checkpoint.

Genistein also induced reduced mobility of Chk2 protein on Western blot. The reduced mobility of Chk2 was indistinguishable from that induced by exposure to ionizing radiation, an event that has previously been shown to be due to phosphorylation. Moreover, we show that, like ionizing radiation, genistein induces phosphorylation of Chk2 on threonine 68. Significantly, genistein-induced phosphorylation of Chk2 was completely absent in two lymphoblastoid A-T cell lines, suggesting that ATM is absolutely required for genistein-induced signaling through Chk2. Also, our data show that cells containing ATM display increased cell survival in the presence of genistein. We speculate that genistein may activate an ATM-dependent DNA damage response pathway that protects cells from induced DNA damage.

Recently, Darbon and colleagues (51) reported that genistein induced phosphorylation of serine 15 of p53 and activation of Chk2 in a human melanoma cell line and that the effects of genistein were abrogated by caffeine, a known inhibitor of ATM, and the related protein, ATR (45–47). In the study by Darbon *et al.* (51) ATM-deficient cell lines were not examined; however, the authors concluded that ATM was not involved in the genistein-induced activation of p53 and Chk2 because treatment of cells with wortmannin, a known inhibitor of phosphatidylinositol 3-kinase-like protein kinases, including ATM, ATR, and DNA-PK (24, 33–35), did not abrogate genistein-induced phosphorylation of p53 and Chk2. In our study, wortmannin reduced phosphorylation of serine 15 of p53 and completely abrogated phosphorylation of Chk2. Moreover, we show that in two separate pairs of lymphoblastoid cell lines, the effects of genistein on p53 and Chk2 are dependent on the presence of ATM. We speculate that these differences may be due to the different types of cell lines used in the two studies.

Although genistein is known to inhibit tyrosine kinases, the tyrosine kinase inhibitor herbimycin A did not induce phosphorylation of Chk2 or p53, suggesting that genistein is acting via a topoisomerase-dependent DNA damage response pathway rather than via inhibition of a tyrosine kinase. Genistein and etoposide have both been reported to induce DNA damage via stabilization of the topoisomerase II-DNA cleavage intermediate. Although both agents induce phosphorylation of p53 and Chk2, our results show that only genistein activates an ATM-dependent pathway and suggest that etoposide is activating a different DNA damage response pathway than genistein, that

is not mediated by ATM. This suggests that other protein kinases that phosphorylate p53 (at serine 15) and Chk2 at threonine 68 and possibly other sites, must be activated by etoposide treatment. Possible candidates include the related protein DNA-PK, which phosphorylates threonine 68 of Chk2 and serine 15 of p53 *in vitro* (Refs. 35 and 38 and data not shown) and ATR, which phosphorylates serine 15 of p53 and threonine 68 of Chk2 *in vitro* (44, 50) and plays a role in the ionizing radiation-induced stabilization of p53 *in vivo* (50–53).

Although genistein clearly activates a DNA damage response pathway, the type of damage induced by genistein remains unclear. Genistein did not induce DNA fragmentation in BT, L3, or HL60 cell lines used in this study. Similarly, genistein was only weakly effective in causing DNA damage in melanoma cells, as measured by the single cell alkaline electrophoresis (Comet) assay (51). However, these results do not preclude the possibility that genistein induces a subtle form of DNA damage that was not efficiently detected by either assay method.

Although genistein and etoposide both inhibit topoisomerase II, *in vitro* studies indicate that genistein and etoposide induce different types of DNA damage and that genistein but not etoposide also induces protein-DNA cross-links (9). It is therefore conceivable that ATM is required for repair of some types of DNA damage, breaks, or cross-links but not others. Another possibility is that the combined effects of genistein on tyrosine kinases and topoisomerase II selects for or causes a specific type of lesion, the repair of which requires ATM. Interestingly, the protein-tyrosine kinase, c-Abl, has been shown to interact with ATM and to play a role in signaling of DNA damage (54, 55) and, therefore, could play a role in the cellular response to genistein.

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The Plant Isoflavenoid Genistein Activates p53 and Chk2 in an ATM-dependent Manner

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