Epidermal Growth Factor Sensitizes Cells to Ionizing Radiation by Down-regulating Protein Mutated in Ataxia-Telangiectasia*

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Epidermal growth factor (EGF) has been reported to either sensitize or protect cells against ionizing radiation. We report here that EGF increases radiosensitivity in both human fibroblasts and lymphoblasts and down-regulates both ATM (mutated in ataxia-telangiectasia (A-T)) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs). No further radiosensitization was observed in A-T cells after pretreatment with EGF. The down-regulation of ATM occurs at the transcriptional level. Concomitant with the down-regulation of ATM, the DNA binding activity of the transcription factor Sp1 decreased. A causal relationship was established between these observations by demonstrating that up-regulation of Sp1 DNA binding activity by granulocyte/macrophage colony-stimulating factor rapidly reversed the EGF-induced decrease in ATM protein and restored radiosensitivity to normal levels. Failure to radiosensitize EGF-treated cells to the same extent as observed for A-T cells can be explained by induction of ATM protein and kinase activity with time post-irradiation. Although ionizing radiation damage to DNA rapidly activates ATM kinase and cell cycle checkpoints, we have provided evidence for the first time that alteration in the amount of ATM protein occurs in response to both EGF and radiation exposure. Taken together these data support complex control of ATM function that has important repercussions for targeting ATM to improve radiotherapeutic benefit.

Functional loss of the ATM1 protein, mutated in the human genetic disorder ataxia-telangiectasia (A-T) (1), leads to a pleiotropic phenotype including neurological abnormalities, immunodeficiency, and a predisposition to develop a number of malignancies, primarily leukemias and lymphomas (2). The characteristic most widely studied in A-T is hypersensitivity to ionizing radiation (3–5) that appears to be due to an inability of ATM to recognize and facilitate the repair of a subcategory of double strand breaks or a form of damage that is converted into a double strand break in DNA (6, 7). It is also likely that recognition of a specific form of DNA damage represents the trigger for ATM to activate a number of cell cycle checkpoints (8, 9). A-T cells are defective in the phosphorylation of p53 on serine 15 and serine 20 and dephosphorylation at serine 376 after exposure of cells to ionizing radiation (10–12). Evidence for a direct involvement of ATM with p53 was provided by the demonstration that these two molecules interact directly (13) and ATM phosphorylates p53 on serine 15 in response to DNA damage (14, 15). Pre-existing ATM protein is rapidly activated by ionizing radiation and radiomimetic agents by an undescribed mechanism (13–15). Exposure of cells to ionizing radiation fails to change either the subcellular distribution or the total amount of cellular ATM protein (16–18). Furthermore, ATM protein levels are relatively constant throughout the cell cycle in human fibroblasts (17). These data point to a post-translational mechanism of activation for ATM. However, there is evidence for alteration in the amount of ATM under certain conditions. Although ATM is not detectable by immunohistochemical staining in quiescent myoepithelial cells lining normal breast ducts, significant expression is observed in the proliferative myoepithelium of sclerosing adenosis (19). In addition the amount of ATM protein changes dramatically in quiescent lymphocytes (PBMCs) in response to mitogenic agents (20). In the latter study a 6–10-fold increase in ATM in PBMCs was observed in response to PHA, reaching a maximum by 3–4 days. As the amount of ATM protein increased, so did its protein kinase activity. Clearly this is a slow response compared with the rapid activation of ATM post-irradiation. Alteration in the amount of the ATM protein could occur by transcriptional control at the promoter. The housekeeping gene NPAT/E14/CAND3 lies ~0.55 kb from the 5′ end of the ATM gene (21–23). These genes share a bidirectional promoter that contains CCAAT boxes and 4 consensus sites for the Sp1 transcription factor (21, 22). Further delineation of the ATM promoter is required to assist in understanding the transcriptional regulation of ATM.

ATM is located predominantly in the nucleus of proliferating cells, which is in keeping with its role in DNA damage recognition and cell cycle control, but ATM has also been detected in cytoplasmic vesicles (16, 17, 24). Since ATM has been impli-
EGF Sensitizes Cells to IR by Down-regulating ATM

EGF Sensitizes Cells to Radiation—Engagement of the EGF receptor by EGF and other ligands initiates signal transduction pathways giving rise to mitogenesis and can also alter the radiosensitivity status of cells (25–29). We determined whether EGF might increase the sensitivity of human fibroblasts and lymphoblasts by incubating cells in EGF for 16 h prior to exposure to radiation (1–4 Gy). The results in Fig. 1A demonstrate that EGF-treated lymphoblastoid cells are more sensitive to radiation, and this is observed over the radiation dose range 1–4 Gy (Fig. 1B). The effect of EGF treatment was significant for both dose and time course experiments for C3ABR; p = 0.0023 (α) and p = 0.0053 (β) for dose and p = 0.0065 (α) and p = 0.07 (β) for time course. On the other hand treatment of A-T cells with EGF failed to increase the radiosensitivity of these cells (Fig. 1, A and B). A similar sensitization was observed in EGF-treated control fibroblasts, but again no further increase in sensitivity was revealed in EGF-treated A-T fibroblasts (Fig. 1C).

We observed that EGF receptor was activated under these conditions (results not shown).

EGF Treatment Reduces ATM Protein Expression—Since radiosensitivity and abnormalities in mitogenesis and other signaling pathways are characteristic of the A-T phenotype, we initially determined whether the radiosensitizing effect of EGF might be due to alteration in the amount of ATM protein in normal control cells. Immunoblotting with anti-ATM antibo-
ies showed that the amount of ATM protein was reduced significantly with time after incubation with EGF in normal fibroblasts (Fig. 2A). No ATM was detected in negative control AT3ABR cells, as expected (16). The same pattern of reduction was observed for the catalytic subunit of DNA-dependent protein kinase, DNA-PKcs, after EGF treatment, but Ku, the DNA damage recognition component of DNA-dependent protein kinase, remained largely unchanged (Fig. 2A). It is evident that the amount of DNA-PKcs is normal in the A-T cell line, AT3ABR, when compared with C3ABR. EGF treatment of C3ABR lymphoblastoid cells revealed a similar pattern of loss of expression of ATM, down to 30% by 9 h post-treatment (Fig. 2B). A similar pattern of decrease was also observed for DNA-PKcs (Fig. 1A). To test the universality of these observations we employed several control lymphoblastoid cell lines. In all four control lines investigated, ATM protein decreased significantly after EGF treatment (Fig. 2C). It is evident from the positive control (100 μg of extract from the lymphoblastoid cell line, C3ABR) that the amount of ATM protein in HSF7 fibroblasts (at equal loading) is considerably lower than that in lymphoblastoid cells (Fig. 2A).

**Effect of EGF on mRNA Expression**—Since EGF caused a marked decrease in the amount of ATM protein, it was possible that this was due to transcriptional down-regulation. To monitor changes to ATM mRNA in lymphoblastoid cells, we employed quantitative reverse transcriptase-PCR as described.
2-fold dilutions. The amount of competitor DNA added to each sample lacking exon 38 was employed as a competitor for ATM cDNA amplification in lymphoblastoid cells as described previously (31). In these experiments a DNA fragment (402 bp) corresponding to an A-T mutant (G5319A) lacking exon 38 was employed as a competitor for ATM cDNA amplification from C3ABR cells (nucleotides 5122–5665, 544 bp) in a series of 2-fold dilutions. The amount of competitor DNA added to each sample was as follows: lane 1, 0.078 attomol/μl; lane 2, 0.156; lane 3, 0.313; lane 4, 0.625; lane 5, 1.25; lane 6, 2.5; lane 7, 5.0; and lane 8, 10 attomol/μl. Size markers appear on the left-hand side of the figure. Cells were incubated for 8 and 16 h with EGF (50 ng/ml) as indicated. These experiments were repeated three times. B, Northern blot analysis of ATM mRNA form normal human skin fibroblasts (HSF7) after treatment with EGF (50 ng/ml) for various times up to 24 h. Each lane contained 20 μg of total RNA, and glyceraldehyde-3-phosphate dehydrogenase was employed as a loading control. The ATM/glyceraldehyde-3-phosphate dehydrogenase ratio was determined by densitometry and represents the relative amounts of ATM mRNA present with time after EGF treatment.

![Figure 3](image)

**FIG. 3.** Determination of ATM mRNA transcript in lymphoblastoid cells after incubation with EGF. A, quantitative reverse transcriptase-PCR was carried out to determine ATM mRNA in C3ABR lymphoblastoid cells as described previously (31). In these experiments a DNA fragment (402 bp) corresponding to an A-T mutant (G5319A) lacking exon 38 was employed as a competitor for ATM cDNA amplification from C3ABR cells (nucleotides 5122–5665, 544 bp) in a series of 2-fold dilutions. The amount of competitor DNA added to each sample was as follows: lane 1, 0.078 attomol/μl; lane 2, 0.156; lane 3, 0.313; lane 4, 0.625; lane 5, 1.25; lane 6, 2.5; lane 7, 5.0; and lane 8, 10 attomol/μl. Size markers appear on the left-hand side of the figure. Cells were incubated for 8 and 16 h with EGF (50 ng/ml) as indicated. These experiments were repeated three times. B, Northern blot analysis of ATM mRNA form normal human skin fibroblasts (HSF7) after treatment with EGF (50 ng/ml) for various times up to 24 h. Each lane contained 20 μg of total RNA, and glyceraldehyde-3-phosphate dehydrogenase was employed as a loading control. The ATM/glyceraldehyde-3-phosphate dehydrogenase ratio was determined by densitometry and represents the relative amounts of ATM mRNA present with time after EGF treatment.

Previously (31). A DNA fragment (402 bp) corresponding to an ATM mutation (G5319A), which lacks exon 38, was used as a competitor for ATM cDNA amplification (nucleotides 5122–5665, 544 bp in size) in a series of 2-fold dilutions from C3ABR cells. After 8 h of treatment with EGF, an equivalent point was reached at 0.625 attomol/μl of competitor compared with 1.25 attomol/μl for untreated cells representing an ~2-fold reduction in ATM mRNA in response to EGF (Fig. 3A). After 16 h of treatment with EGF the equivalent point was reached at ~0.4 attomol representing a 3-fold reduction in ATM mRNA. Northern blot analysis revealed that ATM mRNA was reduced to 60% after incubation of normal fibroblasts (HSF7) with EGF for 8 h and reached a plateau at ~30% of the untreated value at 12–24 h post-treatment, which parallels the changes shown by reverse transcriptase-PCR in lymphoblastoid cells (Fig. 3B).

**Reduced Sp1 Binding Activity after EGF Treatment**—A similar genomic organization at the ATM locus exists for the human and mouse genes where ATM and nuclear protein at the A-T locus (NPAT) are arranged ~0.5 kb apart in a head-to-head configuration (Fig. 5A) (21, 22). These two genes are transcribed from a central bidirectional promoter that contains several binding sites for the transcriptional factor, Sp1 (33). Since ATM mRNA and protein were reduced after EGF treatment, we predicted that this might be due to interference with or reduced Sp1 DNA binding activity. Use of gel-shift analysis with an oligonucleotide-binding consensus sequence for Sp1 revealed the presence of a single, well defined, retarded band (Fig. 4A). The amount of Sp1 binding in extracts from C3ABR cells decreased with time after EGF treatment (Fig. 4A). A decrease of ~50% by 12 h post-treatment, leveling off at later times, paralleled the decrease in the amount of ATM mRNA and protein seen previously. To establish the specificity of this binding, we added anti-Sp1 antibody to the incubation mixture and observed a supershift of the retarded band (Fig. 4A, lower panel). A control antibody against p53 did not alter migration of the band. In addition excess cold Sp1 binding consensus oligonucleotide successfully competed for binding, whereas cold AP-1 binding oligonucleotide failed to do so (results not shown). Under these conditions the amount of Sp1 protein did not change (Fig. 4B). When Sp1 binding was determined in four additional cell lines a similar decrease was observed in all cases (Fig. 4C).

**Effect of EGF on ATM Promoter**—To confirm the effect of EGF on the transcriptional regulation of ATM, we cloned the common promoter region between ATM and NPAT into a luciferase reporter construct (Fig. 5) and carried out transient transfections in control lymphoblastoid cells. Exposure of transfected C3ABR cells to EGF for 16 h led to a significant down-regulation of luciferase activity in cells transfected with the ATM promoter construct in agreement with loss of Sp1 binding activity (Fig. 5).

**Causal Relationship between Sp1 and ATM Down-regulation**—The parallel decrease of ATM and Sp1 binding activity together with the down-regulation of ATM promoter activity after EGF treatment suggested that the decrease in Sp1 binding led to reduced ATM. To test this we employed GM-CSF that has been shown to increase markedly the DNA binding activity of Sp1 (34). As observed above, treatment of cells with EGF for 16 h down-regulated the amount of Sp1 binding activity (Fig. 6A, 3rd lane) and subsequent addition of GM-CSF to these cells caused a rapid (within 1 h) restoration of Sp1 binding activity (Fig. 6A, 5th lane). Incubation of untreated cells with GM-CSF also showed evidence of increased Sp1 binding activity (Fig. 6A, 4th lane). EGF caused a down-regulation of ATM protein (Fig. 6B, 2nd lane), and compatible with the increase in Sp1 binding activity there was also a rapid return of ATM to normal levels when cells were subsequently treated with GM-CSF (Fig. 6B, 4th lane). Under these conditions Sp1 protein levels did not change (Fig. 6B). To test whether the reduction in Sp1 binding activity and ATM down-regulation were responsible for the EGF-induced radiosensitivity, cells were exposed to radiation after pretreatment with EGF followed by GM-CSF incubation for 1 h. A normal pattern of radiosensitivity was observed compatible with restored levels of ATM protein under these conditions (Fig. 6C).

**Radiation Induces ATM Protein**—We investigated whether radiation might also attenuate the response, since it has been shown that Sp1 activity is up-regulated by radiation (32, 35), which in turn should increase the amount of ATM protein. When C3ABR cells were exposed to radiation (5 Gy) there was no change in the amount of Sp1 binding activity, but when these cells were pretreated with EGF initially for 16 h, Sp1 binding activity decreased and subsequent exposure to radiation restored the amount of Sp1 binding activity in 3 h (Fig. 7A). Under these conditions incubation of cells with EGF followed by radiation exposure led to an increase in the amount of ATM protein to untreated levels by 3 h post-irradiation (Fig. 7B). ATM kinase activity increased by 30 min post-irradiation, and the extent of this increase was somewhat lower in cells pretreated with EGF (Fig. 7B). By 3 h post-irradiation ATM kinase induction was the same in cells either untreated or
pretreated with EGF, at which time ATM protein levels were the same (Fig. 7B). These data were further supported by radiation-induced stabilization of p53 and serine 15 phosphorylation of p53 with or without prior incubation with EGF (Fig. 7B). The latter provides an in vivo measure of ATM kinase activation.

DISCUSSION

Activation of ATM kinase by ionizing radiation and other DNA-damaging agents is critical for modulating its activity in several DNA damage signaling pathways (36, 37). We have demonstrated here that ATM can also be altered at the transcriptional level by continuous incubation with EGF over a 16-h period. Under these conditions EGF-treated cells were more sensitive to subsequent ionizing radiation exposure than untreated, irradiated cells. Evidence exists for both radiosensitizing and radioprotective effects of EGF, which is dependent upon both the cell type and position in the cell cycle (25–29, 38–40). In human squamous carcinoma cells, radiosensitization by EGF appears to be in part due to increasing the magnitude of radiation-induced G2/M arrest (39). In CaSkI cells the enhancement of radiosensitivity was evident from a reduction in the shoulder region of the survival curves (27). The degree of radiosensitization in these cells by EGF was of the same order as that observed in the present study. None of the previous studies have provided a mechanistic explanation for radiosensitization, but the down-regulation of ATM in this study provides a potential explanation. Failure to observe a sensitizing effect of radiation in A-T cells provides further support for an

Anti-p53 antibody (Oncogene Science) was used as a control. Quantitation of binding activity was determined by densitometry. B, Sp1 levels were determined by immunoblotting 20 μg of nuclear extract from each sample in A. Actin was used as a loading control. C, several different lymphoblastoid cells (C3ABR, C28ABR, C35ABR, and C31ABR) were treated with EGF for 16 h prior to preparation of extracts for binding to the Sp1 consensus sequence as described above.
The catalytic component (DNA-PKcs) of a second protein involved in DNA damage recognition, DNA-dependent protein kinase (42), was also shown to be down-regulated by EGF, and this would also be expected to increase the radiosensitivity of cells. Down-regulation of both ATM and DNA-PKcs suggests that they may have common regulatory elements responsive to EGF. Indeed this appears to be the case since both genes are organized in head-to-head configuration with other genes, and their putative promoters contain CCAAT boxes and Sp1 consensus sequences. Approximately 0.5 kb separates the 5′ end of the ATM transcript from the 5′ end of another NPAT/E14/CAND3 gene, indicating that they share a bidirectional promoter (21, 22, 34). NPAT plays a role in facilitating the entry of cells into S phase (43), and ATM activates the p53 pathway to delay the passage of cells from G1 to S phase after ionizing radiation exposure (44, 45). DNA-PKcs and Cdc21 (coding for a protein involved in the initiation of DNA replication (46)) also share a bidirectional promoter that contains several CCAAT boxes and Sp1 consensus sites similar to the ATM/NPAT promoter (34, 47). Clearly Sp1 is a candidate for common regulation of these four genes, and its down-regulation or inactivation might account for the effect of EGF in reducing the amounts of ATM and DNA-PKcs. Evidence to support this was provided in a recent report which demonstrated that nitric oxide causes a 4–5-fold increase in expression of DNA-PKcs and protects cells from the toxic effects of a number of agents including ionizing radiation (48). The increased expression of DNA-PKcs was shown to be due to increased binding of Sp1 to the DNA-PKcs promoter. The mechanism by which nitric oxide enhanced Sp1 binding was not elucidated. We have shown here that a decrease in Sp1 DNA binding activity paralleled the EGF-induced decrease in ATM protein, and when the ATM promoter was linked to a reporter gene, transcriptional activation was reduced by EGF. We have established here that GM-CSF which enhances the amount of Sp1 DNA-binding activity rapidly reverses the EGF-induced down-regulation of ATM. Thus there appears to be a causal relationship between the reduction in Sp1 DNA binding activity in EGF-treated cells and the decrease in ATM protein. Evidence for a role for EGF in reducing the amount of Sp1 protein in a rat pituitary cell line (GH4) has been reported (49). This reduction was accompanied by a 50% reduction in Sp1 binding to a GC-rich element in the gastrin promoter, a 50–60% decrease in Sp1-mediated transactivation of reporter genes and a decrease in cell proliferation. The results obtained here with several normal human fibroblasts and lymphoblastoid cells differ from the data with GH4 cells, in that Sp1 protein remains unchanged as the DNA binding activity decreases with time after EGF incubation, and in this case also there was no decrease in cell proliferation (results not shown). In our case, it is possible that modification of Sp1 might account for decreased DNA binding activity (35). Several protein kinases alter the DNA binding activity of Sp1 (50), and thus alteration in specific sites of phosphorylation of Sp1 could account for the changes in DNA binding activity reported here.

The human glioblastoma cell line MO59J is characterized by lack of expression of DNA-PKcs and low levels of ATM protein and is extremely sensitive to ionizing radiation (47). Thus it was somewhat surprising in the present study that EGF did

prior to addition of GM-CSF (10 ng/ml) for 2 h. A, Sp1 DNA binding activity was determined by electrophoretic mobility shift assay. B, changes in amount of ATM protein detected with anti-ATM antibody. Sp1 protein levels were measured with Sp1 antibody, and actin was used as a loading control. C, radiosensitivity was determined as described in the legend to Fig. 1. GM-CSF (10 ng/ml) was added to cells for 1 h after 16 h of EGF treatment.
not increase markedly the radiosensitivity of cells in which both ATM and DNA-PKcs were down-regulated. This can be explained by the ability of ionizing radiation to both transcriptionally increase the amount of ATM protein and activate its kinase activity. Compared with activation of pre-existing ATM protein which reaches optimal levels by 20 min post-irradiation (14, 15), EGF-pretreated cells respond more slowly to radiation showing some kinase activity by 30 min but only reaching maximum levels by 3 h post-irradiation. This delay in restoring ATM protein to constitutive levels and in activating ATM kinase appears to account for the reduction in survival in EGF-pretreated cells, post-irradiation, since up-regulation of ATM by GM-CSF restored survival to normal levels. Failure of A-T cells to show any additional radiosensitization also supports this. Although it is likely that EGF operates via Sp1 in reducing the amount of ATM protein, it is apparent that radiation counteracts this. We have shown here that the amount of Sp1 DNA binding activity is rapidly restored when EGF-pretreated cells are exposed to radiation, accounting for the increase in ATM. Previous results have revealed that Sp1 DNA binding activity is increased rapidly in human melanoma cells exposed to ionizing radiation (32, 35).

Clarke et al. (19) were the first to report alterations of ATM protein in a specific tissue when they failed to detect ATM in normal breast myoepithelial cells, but a significant level of expression was present in the proliferative myoepithelium of sclerosing adenosis. Immunoblotting extracts from freshly isolated PBMCs established that ATM was either absent or present at low levels, but increased 10-fold in response to mitogenic stimuli over 3–4 days (20). To add to this we have demonstrated here that ATM protein can be down-regulated by EGF to radiosensitize cells, but radiation restores ATM to normal levels and activates its kinase activity, and this is mediated at least in part by the Sp1 transcription factor that has several consensus binding sites in the ATM bidirectional promoter. These observations provide greater insight into the
regulation of ATM that involves not only activation of pre-existing protein but also alterations in the amount of ATM protein in response to different stimuli. Determination of ATM expression levels in different tissues/cell types may provide further insight into the response of different tissues to radiation and the factors involved in transcriptional control of the ATM/TPAT promoter. Finally, the results described here raise a fundamental issue in relation to radiosensitization by attenuating ATM protein. Reduction of ATM, in this case by EGF, is countered by radiation exposure that rapidly restores ATM to constitutive levels and would be expected to lessen any therapeutic benefit of radiation. A combination of attenuating ATM, together with inhibitors of its activity, may be a more effective solution.

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