Exposure to ionizing radiation leads to induction of the immediate-early gene, early growth response-1 (Egr-1). Previous studies have suggested distinct cell type- and inducer-specific roles for EGR-1 protein in cellular growth inhibition. The present study was undertaken to determine the functional role of EGR-1 in growth inhibition caused by exposure of tumor cells to ionizing radiation. Exposure to ionizing radiation caused induction of EGR-1 protein in human melanoma cells A375-C6. Inhibition of either the function of EGR-1 protein by stable transfection with a dominant-negative mutant or the expression of EGR-1 by transient transfection with an antisense oligomer resulted in a diminished growth-inhibitory response to ionizing radiation. Because previous studies have suggested that mutations in the tumor-suppressor gene p53 confer radio-resistance, we examined the p53 status of A375-C6 cells. Interestingly, both the parental and the transfected A375-C6 cells showed trisomy for wild-type p53 alleles. Exposure to ionizing radiation resulted in induction of p53 protein that localized to the nucleus in A375-C6 cells. These data suggest that inhibition of EGR-1 function confers radio resistance despite the induction of wild-type nuclear p53. Thus, EGR-1 is required for the growth-inhibitory response to ionizing radiation in A375-C6 cells.

Exposure to ionizing radiation leads to inhibition of tumor cell growth that often shows characteristic features of apoptosis (1). The process of growth inhibition requires the coordinate expression of specific genes. Gene expression studies have documented the association of the transcription factor p53 (2) with growth inhibition and apoptosis caused by ionizing radiation. On the basis of circumstantial evidence, it has been suggested that the immediate-early gene, early growth response-1 (Egr) family of transcription factors EGR-1 (reviewed in Ref. 3) may cause down-regulation of the expression of specific genes. Gene expression studies have documented the association of the transcription factor p53 (2) with growth inhibition and apoptosis caused by ionizing radiation. On the basis of circumstantial evidence, it has been suggested that the immediate-early gene, early growth response-1 (Egr) family of transcription factors EGR-1 (reviewed in Ref. 3) may cause down-regulation of the expression of specific genes. Gene expression studies have documented the association of the transcription factor p53 (2) with growth inhibition and apoptosis caused by ionizing radiation. On the basis of circumstantial evidence, it has been suggested that the immediate-early gene, early growth response-1 (Egr) family of transcription factors EGR-1 (reviewed in Ref. 3) may cause down-regulation of the expression of specific genes.

**Materials and Methods**

Cell Culture and Plasmid Constructs—Plasmid pCMV-WT1-EGR1, which encodes a dominant-negative mutant of EGR-1, contains a WT1-EGR-1 chimera downstream of the cytomegalovirus (CMV) promoter in vector pCB6 (12). The WT1 domain of the construct provides the transcription repression function to the chimera, whereas the EGR-1 domain that contains the three zinc fingers provides the DNA binding function. Plasmid pCMV-M-EGR1, which encodes full-length mouse EGR-1 protein, contains mouse EGR-1 cDNA downstream of the CMV promoter in vector pCB6 (12). Human melanoma cells A375-C6 and transfected cell lines A375-C6/WT1-EGR1.L13 and A375-C6/WT1-EGR1.L4 expressing the chimera, A375-C6/M-EGR1.L1 expressing mouse EGR-1, or A375-C6/vector.L1 and A375-C6/vector.L2 expressing the pCB6 vector were cultured as described previously (10, 11, 13). Each of these cell lines was obtained by pooling about 200 G418-resistant transfected clones.

Irradiation—A 100-kV industrial x-ray machine (Phillips, Nether-
lands) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm aluminum plus 1-mm beryllium filter was 1.85 Gy at a focus-surface distance of 30 cm.

Assay for \[^3H\]Thymidine Incorporation—These experiments were performed as described previously (13). Cells were plated at a density of 2,000/200 \( \mu \)l in 96-well plates, and 24 h later they were left unexposed or exposed to different doses of radiation (5 or 20 Gy). The cells were cultured for 48 h and then pulsed for 8 h with \[^3H\]thymidine (2 Ci/mmol; 0.5 \( \mu \)Ci/well). Radioactivity incorporated into the cells was determined, and percent growth inhibition was calculated as described (13).

Immunocytochemistry—EGR-1 or p53 expression was determined by immunocytochemical analysis, as we described previously (10, 11). Cells were left unexposed or exposed to a 5-Gy dose of radiation, incubated for the indicated period of time at 37 °C, and then subjected to immunocytochemistry with the anti-EGR-1 antibody sc-110 (Santa Cruz Biotechnology, Inc.) or the anti-p53 antibody DO-1 (Oncogene Science, MA) and the Elite ABC kit (Vector Laboratories, Burlingame, CA).

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EGR-1 Induction Is Required for Maximal Radiosensitivity in A375-C6 Melanoma Cells*

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Quantitation of Apoptosis—To quantify apoptosis, the ApopTag in situ end labeling kit (Oncor, Gaithersburg, MD), which detects DNA strand breaks by terminal transferase-mediated dUTP-digoxigenin end labeling (TUNEL) was used as described (11). To determine the percentage of cells showing apoptosis, four experiments in total were performed, and approximately 1,000 cells were counted in each experiment.

Western Blot Analysis—A375-C6 cells were left untreated or exposed to a 5-Gy dose of radiation and then incubated for various time intervals. Thereafter, total cell extracts were prepared, and 30 μg of each extract protein was electrophoresed on 7.5% polyacrylamide-SDS gels and subjected to Western blot analysis using the anti-EGR-1 antibody sc-110, the anti-p53 antibody D0–1, or for loading control the β-actin antibody (Sigma) and β-Actin protein A, as described (10, 11).

Antisense Oligomer-mediated Inhibition of EGR-1 and Effect on Response to Ionizing Radiation—Phosphorothioate-capped human Egr-1 antisense (AS) and non-sense (NS) oligodeoxynucleotides, as described previously (10, 11), were used in the experiments aimed at blocking endogenous EGR-1 expression in human A375-C6 cells or in A375-C6/M-EGR1.L13 cells. The oligodeoxynucleotides were added to the cells 4 h before irradiation at a concentration of 25 nM, which has been shown to be nontoxic to the cells while still blocking EGR-1 expression (10, 11). The cells were then exposed to the indicated dose of radiation, and after 48 h growth inhibition was determined by [3H]thymidine incorporation assays.

Polymerase Chain Reaction-Single-stranded Conformation Polymorphism (PCR-SSCP) and Fluorescence in Situ Hybridization (FISH) Analysis of p53 Gene—To identify mutations in the p53 gene, PCR-SSCP was performed. Total DNA extracted from A375-C6 or A375-C6/M-EGR1.L13 cells was used to amplify exons 2 through 11 by PCR, SSCP was performed. Total DNA extracted from A375-C6 or A375-C6/M-EGR1.L13 cells was used to amplify exons 2 through 11 by PCR, which employed 32P end-labeled primers that were specific for the human p53 gene. Mutations were detected by electrophoresis in mutation detection enhancement gel (Hydrolink, AT Biochem). To evaluate human p53 gene. Mutations were detected by electrophoresis in mutation detection enhancement gel (Hydrolink, AT Biochem). To evaluate human p53 gene. Mutations were detected by electrophoresis in mutation detection enhancement gel (Hydrolink, AT Biochem). To evaluate human p53 gene. Mutations were detected by electrophoresis in mutation detection enhancement gel (Hydrolink, AT Biochem). To evaluate human p53 gene. Mutations were detected by electrophoresis in mutation detection enhancement gel (Hydrolink, AT Biochem). To evaluate human p53 gene. Mutations were detected by electrophoresis in mutation detection enhancement gel (Hydrolink, AT Biochem). To evaluate human p53 gene. Mutations were detected by electrophoresis in mutation detection enhancement gel (Hydrolink, AT Biochem).

RESULTS

Exposure to Ionizing Radiation Causes Growth Inhibition in A375-C6 Cells.—To study the consequences of exposure to ionizing radiation, a dose-response study was performed by exposing A375-C6 cells to various doses of ionizing radiation and determining growth inhibition by [3H]thymidine incorporation assays. As seen in Fig. 1, irradiation caused growth inhibition in A375-C6 cells, exposure to a 5-Gy dose caused growth inhibition in about 80% of the cells, and exposure to a 20-Gy dose caused growth inhibition in about 90% of the cells. These data suggest that ionizing radiation causes dose-dependent growth inhibition in A375-C6 cells.

Ionomizing Radiation Induces Egr-1 Expression in A375-C6 Cells—To determine whether radiation causes induction of EGR-1 in A375-C6 cells, these cells were exposed to a 5-Gy dose of ionizing radiation and subjected to immunostaining 1 h after the exposure. In addition, to quantify EGR-1 expression levels, whole cell protein extracts were prepared from the cells at different time intervals after exposure to ionizing radiation and subjected to Western blot analysis. As shown in Fig. 2a, fewer than 5% of the cells expressed EGR-1 when left unexposed. However, exposure to a 5-Gy dose of ionizing radiation resulted in a marked increase in the number of cells expressing EGR-1; more than 98% of the cells expressed EGR-1 protein in the nucleus (Fig. 2a). Consistent with these data, Western blot analysis indicated that when exposed to a 5-Gy dose of radiation, EGR-1 protein was unaltered as compared with unexposed cells in the first 15 min but was induced within 30 min after the exposure (Fig. 2b). Peak levels of EGR-1 were attained at 45 min after the exposure; thereafter, EGR-1 levels diminished at 60 min and returned to basal levels at 120 min after the exposure (Fig. 2b). Thus, EGR-1 protein was transiently induced in response to the 5-Gy dose of radiation.

The Dominant-Negative Mutant of Egr-1 Confers Resistance to Ionizing Radiation-induced Growth Inhibition.—We have shown previously (10) that in A375-C6 cells, the WT1-EGR1 chimera competes effectively with EGR-1 transactivation through the EGR-1 target binding site and thus acts as a dominant-negative mutant of Egr-1. To study the effect of the chimera on the ability of ionizing radiation to induce growth inhibition, A375-C6 cells were transfected with the WT1-EGR1 expression construct or with the pCB6' empty vector, and stable transfectants were selected with G418 as described previously (10, 11). The results from 13 different transfection experiments revealed that about the same number (mean ± S.D.) of G418-resistant transfected clones were obtained in
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Ionizing radiation causes growth inhibition and apoptosis in diverse cell types (1, 2). To determine whether ionizing radiation causes apoptosis in A375-C6 cells and whether EGR-1 function is required for apoptosis in these cells, A375-C6/vector,L1, A375-C6/vector,L2, A375-C6/WT1-EGR1.L4, and A375-C6/WT1-EGR1.L13 transfected cell lines were irradiated at a 5-Gy dose and subjected to TUNEL after 48 h. A375-C6/vector,L1 or A375-C6/vector,L2 cells exposed to a 5-Gy dose showed 1.5 or 2% TUNEL-positive cells, respectively, whereas A375-C6/WT1-EGR1.L4 or A375-C6/WT1-EGR1.L13 cells exposed to this dose showed 0.4 or 0.6% TUNEL-positive cells, respectively (Fig. 3c). These differences in the apoptotic response of cells transfected with vector alone or the chimera were highly reproducible in four different experiments and were statistically significant (p < 0.001) by the Student’s t test. These results suggest that A375-C6 cells show an apoptotic response to ionizing radiation and that the dominant-negative mutant of Egr-1 provides these cells protection from ionizing radiation-inducible apoptosis.

The Antisense Egr-1 Oligomer Protects A375-C6 Cells from Ionizing Radiation-inducible Growth Inhibition—Since the WT1-EGR-1 chimera is expected to act as a dominant-negative mutant for all the gene members of the Egr family that share the EGR-1 binding sequence, the effects of the chimera on growth inhibition or apoptosis could be due to competitive inhibition of function of a relative of EGR-1 or of EGR-1 itself. To directly determine the specific function of EGR-1 in ionizing radiation-inducible growth inhibition, we used an AS oligomer (17) described previously (10, 11) to block radiation-inducible expression of Egr-1. A375-C6 cells were left untreated or treated with the AS oligomer or an NS oligomer for control, and 4 h later the cells were exposed to a 5-Gy dose of radiation. The consequences of exposure to radiation were examined by [3H]thymidine incorporation assays. As shown in Fig. 4a, the AS oligomer protected the A375-C6 cells from ionizing radiation-inducible growth inhibition at a 5-Gy dose; cells that were pretreated with any oligomer showed about 75% growth inhibition, whereas cells pretreated with the AS oligomer showed about 50% growth inhibition. Cells that were treated with the NS oligomer, for a control, showed about 75% growth inhibition after exposure to a 5-Gy dose of radiation (Fig. 4a). This difference in response to ionizing radiation between the AS pretreated and untreated or NS-treated A375-C6 cultures was statistically significant (p < 0.001) by the Student’s t test.

Our previous studies in A375-C6 cells (11) have suggested that the AS oligomer protects A375-C6 cells expressing the AS oligomer but not the NS oligomer. We reasoned that if Egr-1 inhibition caused by the AS oligomer is owing to inhibition of expression of endogenous Egr-1, transfection of the cells with mouse Egr-1 whose expression was not expected to be inhibited by the oligomer would result in rescue of the cells from this effect. By contrast, if the effect of the AS oligomer was owing to inhibition of expression of genes other than Egr-1, transfection of the cells with mouse Egr-1 would...
To address this question, we first transfected A375-C6 cells with the mouse-EGR-1 expression construct, pCMV-M-EGR1, or the empty vector pCB6 and selected transfected clones with G418-sulfate. 12 different transfection experiments were performed, and a similar number (mean ± S.D.) of transfected clones were obtained with pCMV-M-EGR1 (220 ± 35) or vector alone (255 ± 29). From these different transfection experiments, we obtained 12 different transfected cell lines (A375-C6/M-EGR1.L1-L12) for the mouse EGR-1 expression construct; each cell line was a pool of about 200 G418-resistant clones. The growth rate of A375-C6/M-EGR1.L1-L12 cell lines was similar to that of A375-C6/vector.L1 or A375-C6/vector.L2 cell line (data not shown).

Next, we performed Western blot analysis to ascertain that A375-C6/M-EGR1 cell lines showed increased expression of EGR-1 protein and to study the effect of the AS-Egr-1 oligomer on the expression of ectopic mouse Egr-1. A375-C6/M-EGR1.L1 cells were treated with the AS-Egr-1 or NS oligomer for 4 h or left untreated, and whole cell protein extracts were prepared and subjected to Western blot analysis with the EGR-1 antibody sc-110, which is expected to detect both endogenous human EGR-1 and ectopically expressed mouse EGR-1 proteins. As a control for endogenous human EGR-1 protein levels in these experiments, we used whole cell protein extract from A375-C6/vector.L1 cell line. As seen in Fig. 4b, A375-C6/M-EGR1.L1 cells showed a 4-fold increase in expression of EGR-1 protein over that seen in A375-C6/vector.L1 cells. Treatment of A375-C6/M-EGR1.L1 cells with the NS oligomer did not inhibit the expression of EGR-1 protein (Fig. 4b). A375-C6/M-EGR1.L1 cells treated with the AS oligomer showed a 3-fold increase in EGR-1 expression over that seen in A375-C6/vector.L1 cells (Fig. 4b), suggesting that total EGR-1 protein levels were minimally affected in these M-EGR-1 over-expressors by the AS oligomer. Thus, A375-C6/M-EGR1.L1 cells treated with the AS oligomer, which is directed against human Egr-1, continue to show expression of ectopic mouse Egr-1. When two other pCMV/M-EGR1 transfected cell lines, A375-C6/M-EGR1.L2 and A375-C6/M-EGR1.L3, were examined by Western blot analysis, they showed a 4-fold increase in expression of EGR-1 relative to that in parental or A375-C6/vector.L2 cells, and treatment with the AS oligomer resulted in a 0.8-fold decrease in EGR-1 expression (data not shown). Thus, the AS oligomer does not affect total EGR-1 protein much in the murine EGR-1 over-expressors (Fig. 4b), whereas it does diminish the expression of endogenous human EGR-1 in A375-C6 cells (11). We then determined whether the AS oligomer inhibited the ability of ionizing radiation to cause growth inhibition in A375-C6/M-EGR1.L1 cells. The cells were treated with AS or NS oligomers or left untreated with the oligomers for 4 h and were further left unexposed or exposed to a 5-Gy dose of ionizing radiation. The consequences of exposure to radiation were examined by [3H]thymidine incorporation assays. As seen in Fig. 4c, the A375-C6/M-EGR1.L1 cells showed about 85% growth inhibition when exposed to a 5-Gy dose of radiation. When the exposed to a 5- or 20-Gy dose of radiation. 48 h later, [3H]thymidine incorporation assays were performed, and percent growth inhibition was calculated. Each data point represents a mean of 48 observations from three separate experiments; error bars indicate ± standard deviations. c, the dominant-negative mutant of Egr-1 protects A375-C6 cells from radiation-inducible apoptosis. Cultures of A375-C6/vector.L1, A375-C6/vector.L2, A375-C6/WT1-EGR1.L13 or A375-C6/WT1-EGR1.L13 cells were left untreated (UT) or treated with a 5-Gy dose of radiation and subjected to TUNEL after 48 h. Four separate experiments were performed, and approximately 1,000 cells in total were scored for TUNEL-positive nuclei in each experiment. Data show percent TUNEL-positive cells as a function of irradiation; error bars indicate ± standard deviations.

Fig. 3. Effect of ionizing radiation on the growth of transfected cell lines. a and b, the dominant-negative mutant of Egr-1 protects A375-C6 cells from radiation-inducible growth inhibition, A375-C6/WT1-EGR1.L13 and A375-C6/vector.L1 cells (a) or A375-C6/WT1-EGR1.L4 and A375-C6/vector.L2 cells (b) were left unexposed or not rescue the cells from this effect. To address this question, we first transfected A375-C6 cells with the mouse-EGR-1 expression construct, pCMV-M-EGR1, or the empty vector pCB6 and selected transfected clones with G418-sulfate. 12 different transfection experiments were performed, and a similar number (mean ± S.D.) of transfected clones were obtained with pCMV-M-EGR1 (220 ± 35) or vector alone (255 ± 29). From these different transfection experiments, we obtained 12 different transfected cell lines (A375-C6/M-EGR1.L1-L12) for the mouse EGR-1 expression construct; each cell line was a pool of about 200 G418-resistant clones. The growth rate of A375-C6/M-EGR1.L1-L12 cell lines was similar to that of A375-C6/vector.L1 or A375-C6/vector.L2 cell line (data not shown).
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FIG. 4. Egr-1 AS oligomer protects A375-C6 cells but not A375-C6/M-EGR1.L1 cells from ionizing radiation-inducible growth inhibition. a and c, A375-C6 cells or A375-C6/M-EGR1.L1 cells were left unexposed or exposed to the Egr-1 AS or NS oligomer for 4 h and then left further unexposed (0 Gy) or exposed to a 5-Gy dose of radiation. After 48 h, the cells were pulsed with [3H]thymidine, and incorporation was determined. b, A375-C6 or A375-C6/M-EGR1.L1 cells were left unexposed or exposed to the AS or NS oligomer for 4 h and then left further unexposed (0 Gy) or exposed to a 5-Gy dose of radiation. After 48 h, the cells were pulsed with [3H]thymidine, and incorporation was determined. Relative counts per minute (indicative of growth) as a function of the radiation dose are shown. Each data point represents a mean of 48 observations from three separate experiments; error bars indicate ± standard deviations. b, to determine the effect of the AS or NS oligomer on EGR-1 expression in A375-C6/M-EGR1.L1 cells, the cells were exposed to AS or NS oligomers for 4 h or left unexposed. Then, whole cell protein extracts were prepared, and 30 μg of each extract was subjected to Western blot analysis by using the anti-EGR-1 antibody sc-110. Whole cell protein extracts prepared from untreated A375-C6/vector.L1 cells were used as controls for endogenous human EGR-1 protein levels. The blot was subsequently probed with the anti-actin antibody (data for actin not shown), to determine the effect of the AS or NS oligomer on EGR-1 expression in A375-C6/M-EGR1.L1 cells. The numbers (i.e., 1.0, 3.3, 4.8, 4.1) showing relative expression of EGR-1 are ratios of the normalized levels of EGR-1 in the corresponding lane to the normalized level of EGR-1 in the A375-C6/vector.L1 cells.

Figure 4 shows the relative expression of EGR-1 in A375-C6 cells treated with or without Egr-1 AS or NS oligomers. The data indicate that the Egr-1 AS oligomer protected A375-C6 cells from growth inhibition caused by ionizing radiation, whereas the NS oligomer did not. The relative expression of EGR-1 was compared between untreated and treated cells, with the untreated cells serving as a control.

The Radio-resistant Phenotype of Transfected A375-C6 Cells Expressing the Dominant-Negative Mutant of EGR-1 Is Independent of p53 Expression—Wild-type p53 plays a functional role in DNA repair by arresting cells in the late G1 phase of the cell cycle after irradiation (5, 6). It is also known that the cellular levels of p53 protein increase after exposure to ionizing radiation (2). On the other hand, the expression of mutant forms of p53 may confer cellular resistance to growth inhibition caused by ionizing radiation (20). We therefore examined the status of p53 in parental and transfected A375-C6 cells by using PCR-SSCP analysis. Total genomic DNA from the placenta was used as a negative control for p53 gene mutations, and the pancreatic tumor cell line CFPAC-1 was used as a positive control for mutation in exon 7 of the p53 gene. Each exon was amplified by PCR using specific radiolabeled primers, and the products were denatured and then subjected to mutation detection enhancement gel electrophoresis. Variations in single strand conformation were analyzed by autoradiography. These studies indicated that the A375-C6 parental cells, the A375-C6/WT1-EGR1.L13 transfected cells, and the placenta showed similar single strand conformations for the p53 gene exons 2 through 11 (Fig. 5a), suggesting that the p53 gene in the A375-C6 cells is wild type. As expected, the CFPAC-1 DNA showed mutation in exon 7 (Fig. 5a, lane 2). These studies showed that neither the parental nor the A375-C6/WT1-EGR1.L13 transfected cells show mutation in the p53 gene.

To further confirm the results of PCR-SSCP, nucleotide sequence analysis was performed on two overlapping DNA fragments that were generated by using p53-specific primers and PCR, as described under “Materials and Methods.” These DNA fragments encompassed the region from nucleotide 119 that is located upstream of the p53 translation initiation ATG sequence (nucleotides 215–217) to nucleotide 1428, which is located downstream of the p53 stop codon (TGA, nucleotides 1394–1396). The nucleotide sequence data (not shown) indicated that the two overlapping DNA fragments showed sequence that was identical to that of human wild-type p53 cDNA (16). Thus, consistent with the results of PCR-SSCP, there were no alterations in the coding region of the p53 gene in A375-C6 cells.

FISH analysis, performed by using a digoxigenin-labeled p53 cosmid probe, showed that the proportion of nuclei with two signals for the p53 gene was 20–25% and that the proportion of nuclei with three signals was 75–80% (data not shown). These data imply that A375-C6 cells contain 3 alleles of the p53 gene. Western blot analysis was then performed to examine whether exposure to ionizing radiation caused induction of p53 protein. A375-C6/vector.L1 and the A375-C6/WT1-EGR1.L13 transfected cells were either left untreated or exposed to a 5-Gy dose of ionizing radiation. At 5 or 8 h after the exposure, the cells were harvested, and whole cell protein extracts were subjected to Western blot analysis for p53 protein. As seen in Fig. 5c, A375-C6/vector.L1 and A375-C6/WT1-EGR1.L13 cells that were left untreated showed a modest basal expression of p53.
FIG. 5. **p53 gene status and protein expression in A375-C6 cells.**

**a**. Wild-type status of the p53 gene in A375-C6 parental and transfected cells. Total genomic DNA from the placenta (*lane 1*); a pancreatic tumor cell line, CFPAC-1, as a positive control for exon 7 mutation (*lane 2*); A375-C6 cells (*lane 3*); or A375-C6/WT1-EGR1.L13 cells (*lane 4*) were subjected to the PCR-SSCP assays to detect mutations in p53 exons 2 through 11. Note that CFPAC-1 DNA shows mutation in exon 7 (*lane 2*), as expected.

**b**. Nuclear expression of p53 protein in A375-C6 cells. A375-C6/vector.L1 or A375-C6/WT1-EGR1.L13 cells were left untreated or irradiated with a 5-Gy dose. 5 h after the exposure, the cells were subjected to immunocytochemistry studies for p53 protein. Brown diaminobenzidine stain is indicative of p53-positive cells, whereas methyl green counterstain is indicative of p53-negative cells.

**c**. p53 protein is induced by ionizing radiation. A375-C6/vector.L1 or A375-C6/WT1-EGR1.L13 cells were left untreated or treated with a 5-Gy dose of ionizing radiation. Protein extracts were prepared at 5 or 8 h after irradiation (as indicated), and 40 μg of each extract was subjected to Western blot analysis for p53 protein. The blot was subsequently probed with an antibody for β-actin to verify equal loading of proteins in each lane. Autoradiography was performed by exposing the blot probed with anti-p53 antibody to x-ray film for 24 h or that probed with the anti-actin antibody to x-ray film for 15 min.
protein. Cells that were exposed to radiation showed a strong induction of p53 protein at 5 or 8 h after the exposure (Fig. 5c).

Because p53 protein should be present in the nucleus if it is to be functional (21), we used immunocytochemistry to determine whether p53 protein that was induced by ionizing radiation in A375-C6/vector.L1 or A375-C6/WT1-EGR1.L13 cells was actually present in the nucleus. The cells were left unexposed or exposed to a 5-Gy dose of radiation. 5 h after the exposure, the cells were subjected to immunocytochemistry for p53 protein. As seen in Fig. 5b, unexposed cultures of A375-C6/vector.L1 or A375-C6/WT1-EGR1.L13 cells showed about 40–50% p53-positive cells. When these cultures were exposed to a 5-Gy dose of radiation and incubated for 5 h after the exposure, more than 95% of the cells were identified as p53 positive (Fig. 5b). Interestingly, p53 expression was primarily localized to the nucleus in unexposed or irradiated cultures of A375-C6/vector.L1 and A375-C6/WT1-EGR1.L13 cells (Fig. 5b). Together, these data indicate that exposure to ionizing radiation results in increased expression of nuclear p53 protein in cells that are transfected with the vector alone or with the dominant-negative mutant of EGR-1. Thus, despite the induction of wild-type p53 protein, blocking EGR-1 function in A375-C6/vector.L1 and A375-C6/WT1-EGR1.L13 cells conferred resistance to ionizing radiation.

DISCUSSION

The present study suggests that ionizing radiation causes growth inhibition and apoptosis in human melanoma cells A375-C6. Western blot analysis and immunocytochemistry studies indicated that Egr-1 is induced in the melanoma cells by ionizing radiation in a dose-dependent manner. Functional studies using A375-C6 cells expressing the Egr-1 dominant-negative mutant, whose product competes functionally with EGR-1 (10), or an antisense Egr-1 oligomer, which blocks the expression of Egr-1 (10, 11), suggested that EGR-1 function is required for the radiation-inducible growth inhibition. A previous study has indicated that EGR-1 may confer resistance to growth inhibition caused by ultraviolet radiation in the mouse fibroblast NIH 3T3 cells (22). This apparent difference in the role of EGR-1 in A375-C6 and NIH 3T3 cells indicates that EGR-1 function is influenced by the host cell background. Recent studies (14, 23) have identified corepressor proteins that bind to EGR-1 and modulate its activity, and it is necessary to determine whether differences in the expression of specific EGR-1 binding proteins are responsible for the distinct EGR-1 functions.

The inhibition of EGR-1 expression with the AS oligomer or function with the dominant-negative mutant leads to partial, but not complete, radioresistance. Because in A375-C6 cells the dominant-negative mutant abrogates EGR-1-dependent transactivation substantially, but not completely (10), and because treatment with the AS oligomer does not result in complete ablation of endogenous EGR-1 levels (11), it is possible that incomplete radioresistance may, in part, be due to incomplete interference with endogenous EGR-1. In addition, however, there exists the possibility that the induction of other redundant growth inhibitory pathways by ionizing radiation may prevent complete radioresistance despite ablation of EGR-1. Thus, EGR-1 may control only a specific component of the radioresistant pathways.

Because p53 protein has been linked to cell cycle arrest (2, 4–6) and apoptosis (7, 8), and because p53 mutations have been reported to increase resistance to growth inhibition by ionizing radiation (20) in diverse tumor cell types, we sought to determine the status and expression of p53 in A375-C6 cells. These studies indicated that the melanoma cells A375-C6 have an extra copy of the p53 gene with trisomy for chromosome 17 and that none of the three alleles of p53 contains mutations in exons 2 through 11. Exposure to ionizing radiation led to an induction of p53 protein in the nucleus of A375-C6/vector.L1 and A375-C6/WT1-EGR1.L13 transfected cells. It is possible that induction of p53 protein may have rendered the parental cells radiosensitive, leading to growth inhibition and apoptosis. However, despite the induction of wild-type nuclear p53 protein, the transfected cells expressing the dominant-negative mutant of Egr-1 were resistant to ionizing radiation. These data suggest that Egr-1 plays a key functional role in the growth-inhibitory response to ionizing radiation. Further studies will identify the downstream events influenced by EGR-1 that lead to growth inhibition.

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