Demethylation of the Estrogen Receptor Gene in Estrogen Receptor-negative Breast Cancer Cells Can Reactivate Estrogen Receptor Gene Expression

Anne T. Ferguson, Rena G. Lapidus, Stephen B. Baylin, and Nancy E. Davidson

The Johns Hopkins Oncology Center, Baltimore, Maryland 21231

Abstract

Approximately one third of breast cancers grow independently of estrogen, lack detectable estrogen receptor (ER) protein, and rarely respond to hormonal treatment. Previous studies correlated the lack of ER gene expression in ER-negative breast tumor cells with hypermethylation of a CpG island in the 5' regulatory region of the ER gene. In order to determine whether demethylation of the ER gene in the ER-negative human breast cancer cell line MDA-MB-231 could affect ER transcription, cells were treated with two inhibitors of DNA methylation, 5-aza-2'-deoxycytidine or 5-aza-2'-deoxyctydine. DNA from cells treated with either drug became partially demethylated at several methylation-sensitive restriction enzyme sites, including HhaI, NotI, and SacII, within the ER CpG island. This demethylation correlated with reexpression of the ER gene as detected by reverse transcriptase-PCR and production of ER protein as detected by Western blot analysis. ER produced in drug-treated cells was functionally active as demonstrated by its ability to activate transcription of estrogen-responsive genes. These results suggest that DNA methylation of the ER CpG island may play a role in suppression of ER gene expression in ER-negative breast cancer cells.

Introduction

Numerous experimental and clinical studies have established that estrogen plays a major role in the initiation and progression of breast cancer (1). Approximately two thirds of breast cancers express the ER3 gene (ER+), and their growth is stimulated by estrogen. For these tumors, therapeutic strategies include estrogen ablation or antiestrogens. However, the remaining fraction of primary breast cancers lack detectable ER protein (ER−) and are rarely responsive to hormonal treatment (2).

Frequently, these ER− tumors lack ER gene expression (3, 4), yet this is not due to mutations within the ER gene (5). Therefore, acquired loss of ER transcription is a potential mechanism for hormone resistance. ER gene expression in ER− breast cancers may be silenced by methylation of a cytosine-rich area, termed a CpG island (6), in the 5' regulatory region and first exon of the gene (4). In normal adult tissue, CpG islands are unmethylated with the exception of transcriptionally silent genes on the inactive X chromosome and some imprinted genes (7, 8). Methylation of these islands has been shown to directly inhibit transcription or stabilize structural changes in chromatin that prevent transcription (9, 10).

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Materials and Methods

Cell Culture and Reagents. MDA-MB-231 cells were obtained from Dr. Marc Lippmann (Lombardi Cancer Center) and maintained in DMEM with 5% FCS. Azac or deoxyC (Sigma) were freshly prepared in distilled water, and cells were treated with the indicated doses of drug every 4 days. At each time point, genomic DNA, total cellular RNA, and protein were extracted from cells using standard protocols.

Southern Blot Analysis. For the analysis of the ER gene methylation status, 10 μg genomic DNA were digested first with 10 units/μg BsmI, followed by either 15 units/μg HhaI, 10 units/μg HpaII, 25 units/μg NotI, 25 units/μg SacII, or 5 units/μg SmaI, and subjected to electrophoresis in a 1.5% agarose gel. DNA was transferred to zetaprobe filters as described previously. Filters were hybridized to a 1.3-kb BsmI fragment from the plasmid pGHER1 (generously provided by Dr. P. Chambon, Institut de Chimie Biologique, Strasbourg, France; Ref. 12), which was oligolabeled using a multiprime labeling kit (Amersham).

RT-PCR. RT-PCR was performed as described previously (13). Approximately 3 μg total cellular RNA were used for each reverse transcription reaction, and one tenth of this reaction was used for PCR. Primers were designed that amplify between exons 7 and 8 of ER (5'-GCACCCCTGAAGTTCTCTGGAA-3', 5'-TGCGTAAAGGTGTCGTTGAT-3'), exons 3 and 4 of PR (5'-TGTCAGGCTGGCATGGTCCTTG-3', 5'-GACGGGTGACTGCA-GAAAATCCC-3'), and exons 1 and 3 of actin (5'-ACATGGATGATGAGCACCCCTGAAGTTCTCTGGAA-3', 5'-CATGGATGATGAGCACCCCTGAAGTTCTCTGGAA-3'). The annealing temperatures used for the ER, PR, and actin primers were 55°C, 65°C, and 60°C, respectively. The PCR sample was subjected to electrophoresis in a 1.0% agarose gel.

Western Blot Analysis. Total cellular proteins extracted from 106 cells were resolved by electrophoresis in an 8% denaturing polyacrylamide gel, and proteins were electroblotted to nitrocellulose membranes. Immunoblot analysis with the ER or PR mAbs, ER1D1 or PRIOA9 (Immunotech, Inc.), and horseradish peroxidase-conjugated rabbit anti-mouse antisera was performed using standard protocols.

Construction of the EREtk-Luciferase/5V-neo Plasmid. A 274-bp KpnI-BglII DNA fragment containing the EREtk transcriptional unit from the EREtk-Luciferase plasmid was amplified in TAE Tris acetate EDTA buffer using the primers ERE-A (5'-CTGTTGCCCATCTGAAACATCC-3') and ERE-B (5'-GAAACATCCAAAGTTCTCTGGAAAAGGAGGCT-3'). The amplified product was cut with KpnI and BglII, and ligated into the KpnI-BglII sites of the pSV-neo plasmid (14).
2-ERE-tk-CAT (a generous gift from Dr. Benita Katzenellenbogen; Ref. 14) was ligated to KpnI-BglII digested pGL2-Basic (Promega). The resulting plasmid is named ERE2-tk-luciferase. Next, a 1.5-kb Accl-XmaI DNA fragment containing the SV40 promoter-neomycin gene-polyadenylation site from pCEA3 (Invitrogen Corp.) was blunt ended using the Klenow fragment of DNA polymerase I and ligated to Smal-linearized ERE2-tk-luciferase to yield the plasmid named ERE2-tk-luciferase/SV-neo. This plasmid contains two EREs that drive expression of the luciferase reporter gene and can monitor ER-E2-induced transcription.

Creation of the Stable Cell Lines MDA-MB-231ELuc.1-10. Two 100-mm-diameter plates of 50% confluent MDA-MB-231 cells were transfected with 10 μg ERE2-tk-luciferase/SV-neo (15). At 48 h posttransfection, the cells were split into ten 100-mm-diameter plates, and G418-resistant cells were selected in medium that contained 1 mg/ml Geneticin (G418 sulfate; Gibco-BRL). After 2 weeks of selection, 10 clonal populations of G418-resistant cells were isolated and named MDA-MB-231ELuc.1-10.

Luciferase Assays. Cell lysates were made, and luciferase assays were performed as described previously (15).

Results

Demethylation of the ER CpG Island Region after Treatment with Demethylating Agents. Since previous studies revealed a correlation between the absence of ER gene expression and extensive methylation of the ER gene CpG island in ER- human breast cancer cells, ER- MDA-MB-231 cells were treated with deoxyC in an attempt to demethylate the CpG island. The ER CpG island is contained within a 1.3-kb fragment of DNA that is flanked by two BsmI restriction enzyme recognition sites. Within this CpG island are many recognition sites for methylation-sensitive restriction enzymes, including Hhal, HpaII, NotI, SacII, and Smal (Fig. 1A). Genomic DNA from drug-treated and untreated cells was digested with the methylation-insensitive enzyme BsmI and one of the five aforementioned methylation-sensitive enzymes, and examined by Southern blot analysis using the 1.3-kb BsmI ER DNA fragment as a probe. DNA that is completely methylated at the restriction enzyme sites yields a 1.3-kb flanking DNA fragment. If the DNA is partially or fully demethylated, smaller sized DNA fragments are observed (Fig. 1A).

As expected, DNA from untreated MDA-MB-231 cells was methylated at the Hhal sites as indicated by the presence of the 1.3-kb band in Fig. 1B, Lane 1. By comparison, BsmI-Hhal digestion of DNA from cells treated with 0.5 or 0.75 μM deoxyC for 14 days yielded smaller DNA fragments ranging in size from 0.2–1.0 kb (Fig. 1B, Lanes 2 and 3). This BsmI-Hhal digestion pattern indicates that partial demethylation occurred at several of the Hhal sites. Similar results were obtained at the NotI and SacII recognition sites. DNA from untreated cells showed a fully methylated pattern when digested with BsmI-NotI or BsmI-SacII (Fig. 1B, Lanes 4 and 7). Again, DNA from drug-treated cells was digested by NotI into 1.0- and 0.3-kb DNA fragments, which indicates that the NotI site became partially demethylated (Fig. 1B, Lanes 5 and 6: 0.3-kb band undetectable), while SacII digestion produced 1.0-kb, 0.8-kb, and 0.3-kb DNA fragments. Analogous DNA digestion patterns were observed using the same restriction enzymes and DNA from MDA-MB-231 cells treated for 5 days with deoxyC or 2 weeks with 1.0, 2.5, or 5.0 μM azaC (data not shown). In the absence of any treatment, two other methylation-sensitive DNA sites within the ER CpG island, HpaII and Smal, are partially methylated (data not shown). In summary, these results clearly demonstrate that deoxyC and azaC cause partial demethylation at several sites within the ER CpG island.

Expression of the ER Gene after Treatment with Demethylating Agents. It was important to demonstrate that the partial demethylation of the ER CpG island in deoxyC-treated MDA-MB-231 cells is associated with reexpression of the ER gene and production of ER protein. ER gene expression was undetectable by RT-PCR using RNA from untreated MDA-MB-231 cells (Fig. 2A, Lane 3). However, within 5 days of treatment with 0.5 or 0.75 μM deoxyC, cells began to express the gene at levels detectable by RT-PCR (Fig. 2A). Similar results were obtained using 2.5 or 5.0 μM azaC treatment of MDA-MB-231 cells as well as another ER- cell line, Hs578t, known to be methylated at the ER CpG island (Ref. 4; data not shown). More notably, ER protein was detected by Western blot analysis using lysates from MDA-MB-231 cells exposed to 0.5 or 0.75 μM deoxyC (Fig. 2B) and 2.5 or 5.0 μM azaC (data not shown). Taken together, these results suggest that demethylation plays a significant role in reactivating ER gene expression.

Functional Analysis of ER Induced by Demethylating Agents. In order to show that ER produced in deoxyC-treated MDA-MB-231 cells was functional, the ability of the cells to activate estrogen-responsive gene expression was analyzed in two ways. Ligand-bound ER can activate expression of the PR gene as well as reporter genes linked to EREs with the basal thymidine kinase promoter (14, 16). Therefore, it was first tested whether the induced ER could activate PR transcription. RT-PCR using PR-specific PCR primers was performed on RNA isolated from deoxyC-treated cells. Fig. 3A demonstrates that untreated ER-' MDA-MB-231 cells that are grown in the presence of estrogen (5% FCS with phenol red) lack PR transcript (Fig. 3A, Lane 3). However, within 5 days of deoxyC treatment, the PR gene is expressed (Fig. 3A, Lane 8). This time course parallels that seen for ER. Similar results were obtained with RNA from MDA-MB-231 and Hs578t cells treated with 1 or 2.5 μM azaC for 4 days.
Fig. 2. ER gene expression and protein production in deoxyC-treated MDA-MB-231 cells. A, RT-PCR was used to detect ER mRNA (470 bp) and actin mRNA (400 bp). +/- indicate whether reverse transcriptase was added to (+) or omitted from (-) the reaction. Lane 1: negative control for PCR reaction, water only; Lane 2: positive control, ER* MCF-7 cells; Lane 3: untreated ER~ MDA-MB-231 cells; Lanes 4-12: MDA-MB-231 cells; Lane 4: 0.5 µM deoxyC for 1 day; Lane 5: 0.75 µM deoxyC for 1 day; Lane 6: 0.5 µM deoxyC for 3 days; Lane 7: 0.75 µM deoxyC for 3 days; Lane 8: 0.5 µM deoxyC for 5 days; Lane 9: 0.5 µM deoxyC for 7 days; Lane 10: 0.75 µM deoxyC for 7 days; Lane 11: 0.5 µM deoxyC for 14 days; and Lane 12: 0.75 µM deoxyC for 14 days. B, Western blot analysis detects the 67-kd ER protein. Lane 1: positive control, ER* MCF-7 cells; Lane 2: negative control, untreated ER~ MDA-MB-231 cells; Lanes 3-10: MDA-MB-231 cells; Lane 3: 0.5 µM deoxyC for 5 days; Lane 4: 0.75 µM deoxyC for 5 days; Lane 5: 0.5 µM deoxyC for 7 days; Lane 6: 0.75 µM deoxyC for 7 days; Lane 7: 0.5 µM deoxyC for 10 days; Lane 8: 0.75 µM deoxyC for 10 days; Lane 9: 0.5 µM deoxyC for 14 days; and Lane 10: 0.75 µM deoxyC for 14 days.

Fig. 3. PR gene expression and protein production in deoxyC-treated MDA-MB-231 cells. A, RT-PCR was used to detect PR mRNA (460 bp) and actin mRNA (400 bp). Lanes are as described in the legend for Fig. 2. B, Western blot analysis detects the 109-kd isoform of PR protein. Lane 1: positive control, PR* T47D cells; Lane 2: negative control, untreated ER~ MDA-MB-231 cells; Lane 3: 0.75 µM deoxyC for 5 days; and Lane 4: 0.75 µM deoxyC for 7 days.

(data not shown). In addition, after 5-day exposure of MDA-MB-231 cells to 0.75 µM deoxyC, PR was detected by Western blot analysis (Fig. 3B).

An additional test for ER function utilized the stable cell lines containing ERE~tk-luciferase, MDA-MB-231ELuc.1-10. Cells grown in the presence of E2 (5% FCS with phenol red) were treated with 0.75 µM deoxyC for the indicated number of days, and lysates from these cells were analyzed for luciferase activity. DeoxyC treatment of the cell clones increased luciferase activity by 1.5-5.6-fold above the background level of the untreated cells. The effect of deoxyC treatment on one clone,
231ELuc.6 cells were treated with 0.75 J·m deoxyC for up to 7 days. At each time point, 10^6 cells were harvested and analyzed for luciferase activity. Fold induction, ratio of the luciferase activity achieved with deoxyC treatment to that achieved without deoxyC treatment. The results reflect the average of two sets of independent experiments.

MDA-MB-231ELuc.6, is shown in Fig. 4. Taken together, these findings demonstrate that the ER protein made in response to a demethylating agent is functional as a transcriptional activator.

Discussion

Abnormal DNA methylation of CpG islands is an early event in the progression of some human cancers (17). This may be a result of an increased capacity of the cancer cells to methylate DNA as indicated by increased levels of intracellular DMT RNA and enzyme activity (18). Hypermethylation of the CpG island of two tumor suppressor genes, Von Hippel Lindau and p16, is associated with loss of gene expression in human cancers (19). With respect to breast cancer, ER~ (18). Hypermethylation of the CpG island of two tumor suppressor genes is associated with loss of gene expression in human cancers (4). This may be a result of hypermethylation of the ER gene and hormone resistance in some human breast cancer cells. Treatment of ER~ human breast cancer cells with demethylating agents led to partial demethylation of the ER CpG island, which may reflect that either a fraction of DNA was demethylated in each cell or that DNA was demethylated in only a percentage of the cells. Interestingly, the DNA sites that became demethylated with drug treatment are in a 300-bp region of the CpG island that has the highest CpG density. The ER gene may be similar to the human γ-globin gene in that its reexpression requires a minimum length of methylation-free DNA rather than demethylation of specific CpG dinucleotides (23). Drug treatment of the ER~ cells also led to expression of ER mRNA and subsequent production of a functional protein. Future studies will be directed at understanding the biological effects of reactivating ER in this setting. In this regard, it was demonstrated that expression of exogenous ER in MDA-MB-231 cells leads to an increase in cell doubling time, arrest at G0-G1, and cell death (24). Therefore, it is conceivable that treatment of ER~ cells with demethylating agents may itself suppress cell growth or secondarily render these cells responsive to hormonal therapies like tamoxifen.

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References


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