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

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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' 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 and 5-aza-2'-deoxycytidine. DNA from cells treated with either drug became partially demethylated at several methylation-sensitive restriction enzyme sites, including HhaI, NorI, 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 demethylation 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- and guanine-rich area, termed 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 (7, 8). Methylation of these islands has been shown to directly inhibit transcription or stabilize structural changes in chromatin that prevent transcription (9, 10).

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 NorI, 25 units/μg SacI, 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 pHGER1 (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'-GCACCTGGAAGTCTCTGGAA-3', 5'-TGGCTAAAGTGGTGCATGAT-3'), exons 3 and 4 of PR (5'-TGTCAGGCTGGATCTTCGG3'-5'-GACGGGTTGACTGCGAACAATCC-3'), and exons 1 and 3 of actin (5'-ACCATGGGATTGATGAAGAC-3', 5'-ATCAGCTGGGGGTTGGAAG-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 PR10A9 (Immunotech, Inc.), and horseradish peroxidase-conjugated rabbit anti-mouse antiserum (Southern Biotechnology Associates, Inc.) were performed using standard protocols. Western blot reactions were detected by a chemiluminescence-based photoblot system (ECL; Amersham).

Construction of the ERE-tk-Luciferase/SV-Neo Plasmid. A 274-bp KpnI-BglII DNA fragment containing the ERE-tk transcriptional unit from

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3 The abbreviations used are: ER, estrogen receptor; azaC, 5-aza-2'-deoxycytidine; deoxyC, 5-aza-2'-deoxycytidine; DMT, DNA methyltransferase; E½, 17β-estradiol; ER*, estrogen receptor positive; ERE, estrogen-responsive element; FR, progesterone receptor; PR*, progesterone receptor positive; PR', progesterone receptor negative; RT, reverse transcriptase.

Work in cultured human breast cancer cells has shown that the absence of ER gene expression in ER~ human breast cancer cells is associated with an increased capacity to methylate DNA as well as extensive methylation of the CpG island of the ER gene (4). The current study was designed to determine, by functional analysis, whether this CpG island methylation plays a direct role in suppressing ER gene expression in ER~ breast cancer cells. The nucleoside and nucleotide analogues, azaC and deoxyC, were used to inhibit DNA methylation in the ER~ tumor cell line MDA-MB-231 (11). Our results show that treatment of MDA-MB-231 cells with either drug is associated with partial demethylation of the ER CpG island, reexpression of ER mRNA, and ER protein synthesis. Furthermore, the ER protein is functional as demonstrated by reactivation of ER-E2-induced gene expression. These results suggest that it may be possible to use azaC or deoxyC to improve the response of ER~ human breast cancers to hormonal therapies.
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-231 ELuc.1-10.

**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 HhaI, Hpall, 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 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 HhaI sites as indicated by the presence of the 1.3-kb band in Fig. 1B, Lane 1. By comparison, BsmI-HhaI 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-HhaI digestion pattern indicates that partial demethylation occurred at several of the HhaI 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−/PR− MDA-MB-231 cells that are grown in estrogen-free conditions do not express PR (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 ERE2-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,
inactivity of this 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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