Regulation of DNA Methylation in Human Breast Cancer

EFFECT ON THE UROKINASE-TYPE PLASMINOGEN ACTIVATOR GENE PRODUCTION AND TUMOR INVASION

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Urokinase-type plasminogen activator (uPA) is a member of the serine protease family and can break down various components of the extracellular matrix to promote growth, invasion, and metastasis of several malignancies including breast cancer. In the current study we examined the role that the DNA methylation machinery might be playing in regulating differential uPA gene expression in breast cancer cell lines. uPA mRNA is expressed in the highly invasive, hormone-insensitive human breast cancer cell line MDA-MB-231 but not in hormone-responsive cell line MCF-7. Using methylation-sensitive PCR, we show that 90% of CpG dinucleotides in the uPA promoter are methylated in MCF-7 cells, whereas fully demethylated CpGs were detected in MDA-MB-231 cells. uPA promoter activity, which is directly regulated by the Ets-1 transcription factor, is inhibited by methylation as determined by uPA promoter-luciferase reporter assays. We then tested whether the state of expression and methylation of the uPA promoter correlates with the global level of DNA methyltransferase and demethylase activities in these cell lines. We show that maintenance DNA methyltransferase activity is significantly higher in MCF-7 cells than in MDA-MB-231 cells, whereas demethylase activity is higher in MDA-MB-231 cells. We suggest that the combination of increased DNA methyltransferase activity with reduced demethylase activity contributes to the methylation and silencing of uPA expression in MCF-7 cells. The converse is true in MDA-MB-231 cells, which expresses a late stage highly invasive breast cancer. The histone deacetylase inhibitor, Trichostatin A, induces the expression of the uPA gene in MDA-MB-231 cells but not in MCF-7 cells. This supports the hypothesis that DNA methylation is the dominant mechanism involved in the silencing of uPA gene expression. Taken together, these results provide insight into the mechanism regulating the transcription of the uPA gene in the complex multistep process of breast cancer progression.

The breakdown of the extracellular matrix involves a variety of growth factors and proteases and is an important step in the process of tumor invasion and metastasis (1, 2). Urokinase-type plasminogen activator (uPA) and its cell surface glycoprophosphotidyl inositol-linked receptor (uPAR) play important roles in several malignancies (1). uPA produced by tumor cells and the surrounding stroma is intimately involved in tumor cell invasion, migration, and proliferation (1). The uPAR localizes the proteolytic effects of uPA within the tumor cell environment. Additionally, uPA enhances neovascularization of tumors thus further contributing to the process of tumor progression (1). Numerous data demonstrate the causal role of uPA in tumor growth and metastasis (1). First, expression of uPA or uPAR was shown to enhance tumor growth and metastasis (3, 4). Second, increased uPA gene expression in various malignancies including breast cancer is closely related with disease stage (1, 5). Third, inoculation of human breast cancer cells in mice lacking the uPA gene results in tumors of significantly smaller volume than tumors implanted in wild type mice (3). Fourth, we have demonstrated previously that a peptide derived from the non-receptor binding domain of uPA (A6) decreases breast cancer invasion, growth, and metastases because of its pro-apoptotic and anti-angiogenic effects (6). Fifth, the active site inhibitor of uPA (B-428), alone or in combination with the anti-estrogen tamoxifen, blocks the growth and metastasis of prostate and breast cancers (7, 8). Sixth, antibodies directed against uPA or uPAR are able to decrease tumor growth (9–11).

DNA methylation marks inactive genes and can suppress gene expression directly by interfering with the binding of transcription factors or indirectly by attracting methylated DNA binding factors that recruit histone deacetylases and precipitate an inactive chromatin structure (12, 13). Aberrant DNA methylation patterns are commonly observed in cancer (13). Neoplastic cells have the ability to simultaneously harbor widespread hypomethylation and regional hypermethylation that contribute to tumor progression (13, 14). Whereas hypermethylation and silencing of tumor suppressor genes has attracted much attention recently (13, 15, 16), the molecular mechanisms underlying hypomethylation of tumor progression factors such as ras, myc, hox11, and xmrk that are up-regulated during cancer development are poorly described. This suggests that hypomethylation may also play an important role in regulating gene expression during tumorigenesis similar to hypermethylation (17–20).

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Methylation of uPA Gene in Breast Cancer

In the current study, we tested the hypothesis that the expression of uPA, a well defined marker of highly invasive tumor cells and activated at the late stages of breast cancer, can be regulated via changes in the methylation status of its promoter region. As a follow-up to our previous studies where we demonstrated that the uPA gene is transcriptionally suppressed by DNA methylation (21), we have now focused on the examination of the role of DNA demethylation and DNA methylating enzymes such as DNA methyltransferase (DNMT) and demethylase (DMase) in regulating uPA expression during breast cancer progression.

MATERIALS AND METHODS

Cells and Cell Culture—All human breast cancer cell lines obtained from the American Type Culture Collection (ATCC; Manassas, VA). MDA-MB-231 cells were maintained in t-15 medium (Invitrogen) with 2 mM glutamine, 1% fetal bovine serum (FBS), and 100 units/ml of penicillin-streptomycin sul fate (Invitrogen). MCF-7 cells was maintained in minimum Eagle’s medium (Earle’s salts; Invitrogen) with 2 mM glutamine, 10% FBS, 10 μg/ml insulin, and 100 units/ml of penicillin-streptomycin sul fate. T47D and BT474 cells were maintained in RPMI 1640 (Invitrogen) with 2 mM glutamine, 10% FBS, and 100 units/ml of penicillin-streptomycin sul fate. T47D cells were supplemented with 1% charcoal-stripped insulin. HS578T and BT549 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 2 mM glutamine, 5% FBS, and 100 units/ml of penicillin-streptomycin sul fate. All cells were incubated at 37°C in 5% CO2 with the exception of MDA-MB-231 cells that are grown in the absence of CO2.

Northern Blot Analysis—MCF-7 cells were treated with or without 5-aza-2′-deoxycytidine (5-azaCdR) (Sigma) at a concentration of 20 μM for 3 days and were cultured in the absence of the drug for an additional 24 h. MDA-MB-231 and MCF-7 cells were treated with Trichostatin A (TSA) (Sigma) at a concentration of 100 ng/ml for 2 days. RNA from these cells was isolated using the Trizol method following the manufacturer’s instructions (Invitrogen), fractionated on a 0.8% agarose gel (22, 23). The filters were hybridized with a 32P-labeled human uPA cDNA at 65°C for 24 h, which was then stripped of the probe and re-hybridized with an 18 S RNA probe to normalize for the amount of RNA loaded in each lane. Autoradiography of the filters was carried out for 18 h at 37°C using XAR film (Eastman Kodak Co., Rochester, NY). The intensity of hybridization to uPA mRNA and 18 S RNA was quantified by densitometric scanning. The normalized uPA mRNA expression was determined by dividing the intensity of the uPA signal per each lane.

Boyden Chamber Matrigel Invasion Assay—Cell invasive capacity was determined using two compartment Boyden chamber Matrigel invasion chambers (8-μm pore membrane filter) with the manufacturer’s instructions (Corning Corporation, Burlington, MA) (6, 7). The 8-μm pore polycarbonate filters were coated with basement membrane Matrigel (50 μg/filter). 5 × 104 cells treated with or without 5-azaCdR in 0.1 ml of medium were added to the upper chamber and placed on top of a lower chamber pre-filled with 0.8 ml of serum-free medium supplemented with 25 μg/ml fibronectin (Sigma) and then incubated at 37°C for 24 h. After the incubation, medium was removed, and the polycarbonate filters with invaded cells were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde (Sigma) in 0.1 M phosphate buffer, pH 7.4, at room temperature for 30 min. They were then stained with 1.5% toluidine blue, washed, and mounted onto glass slides. Number of cells invaded was examined under a light microscope. Ten fields of vision were randomly selected, and the mean cell number was calculated.

 Luciferase Reporter Assay—The uPA promoter region (−745 to +30) was cut from uPA-chloramphenicol acetyltransferase reporter vector (gift from Dr. F Blasi, Milan, Italy) at AvrII and Smal sites and then inserted into a luciferase reporter vector pGL-3 basic (Promega, Madison, WI) digested with NheI and Smal to generate uPA-luc plasmid. Unmethylated uPA promoter construct was obtained by treating uPA- luc plasmid with S-adenosylmethionine (AdoMet) in the absence of the bacterial CpG methylases. The uPA-luc plasmid was methylated at different Cpg sites in vitro using methylases (mSssI, mHpaII, and mHhaI) and the methyl donor as recommended by the manufacturer (New England Biolabs, Mississauga, ON, Canada). Complete methylation was confirmed by resistance to HpaII and HhaI restriction enzymes. PGL-3 basic was used as a negative control. The different treated plasmids were transiently transfected into MDA-MB-231 cells using LipofectAMINE as a carrier according to the manufacturer’s protocol (Invitrogen). pEVR0 or pEVR-F-Ets-1 were gifts from B. J. Graves at University of Utah School of Medicine, Salt Lake City, UT. FSV-β-gal (Promega) containing the β-galactosidase gene under transcriptional control of SV40 promoter was maintained in 293T cells stably transfected at a concentration of 0.5 μg/sample to normalize for transfection efficiency. 48 h after transfection, cells were scrapped in 1× reporter lysis buffer (Promega) followed by centrifugation at 14,000 rpm. Luciferase activity in the supernatants was then analyzed by mixing 45 μl of cell lysate, 6 μl of luciferase assay reagent (30 mAT ATP; Promega), 300 μl of 50 mM MgCl2, and 100 μl of 1× reporter lysis buffer (25 μl) for 20 s in a luminometer (Monolight 2010). For β-galactosidase activity, 50-μl lysates were mixed with 200 μl of β-galactosidase assay buffer (24 μl NaHPO4, 16 μl NaH2PO4, 4 μl KCl, 400 mM MgCl2, 20 μM β-mercaptoethanol, and 0.3 mg O-nitrophenyl-β-galactopyranoside per sample) in a 96-well plate and incubated for 4 h at 37°C. The reaction was stopped by adding 50 μl of 1 M sodium carbonate solution. Absorbance was measured at 420 nm in a V-max plate reader ( Molecular Devices). Activity was determined by comparison to a standard curve. Luciferase reporter activity in relative luminescence units was normalized to β-galactosidase activity as described (24).
Methylation of uPA Gene in Breast Cancer

Nuclear Extracts—Total nuclear extracts were obtained by lysing cells in 500 μl of Buffer A (10 mM Tris, pH 8.0, 1.5 mM MgCl₂, 5 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) followed by centrifugation at 2000 rpm for 10 min. The precipitated nuclear pellets were resuspended gently in 30 μl of Buffer B (20 mM Tris, pH 8.0, 1.5 mM MgCl₂, 25% glycerol, 0.5 mM dithiothreitol, 0.4 mM NaN₃, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 13,000 rpm for 30 min. Protein content was determined and aliquots stored at −80 °C.

Electrophoretic Mobility Shift Assay—PEVRF-Eta-1 was transiently transfected into MDA-MB-231 cells using LipofectAMINE according to the manufacturer’s protocol (Invitrogen). Nuclear extracts were isolated as described and used for electrophoretic mobility shift assay. Double-stranded synthetic oligonucleotides (ODN) corresponding to methylated or unmethylated Ets binding sites were generated using the following oligonucleotides: methylated sense, TGTTGCAGCCCTGAGCTGC; methylated antisense, ACACCTGGAAACCCTGG; unmethylated sense, GTGTTGCAGCCCTTGAGCTGC; unmethylated antisense, ACACCTGGAAACCCTGG.

DNA Methylation Transferase Activity Assay—5 μg of the nuclear extract obtained were incubated with the methyl donor [³H]-AdoMet (5-adenosyl-l-methyl-[³H]-methionine; Amersham Biosciences) and hemimethylated double stranded oligos (poly(dI-dC); Amersham). Total radioactivity retained by GF/C filters from these cells were digested with non-methylation sensitive enzyme HpaII (lanes a, b, and c) or with methylation sensitive enzymes HpaI (lanes a1, b1, and c1) or with methylation sensitive enzymes HpaI (lanes a1, b1, and c2) and HhaI (lanes a3, b3, and c3). DNA digests were resolved on 0.8% agarose gel and blotted to nylon membrane. All blots were probed with a [³P]-labeled human uPA and 18 S cDNA, as described under “Materials and Methods”. The invasive capacity of MDA-MB-231, MCF-7, and MCF-7 cells treated with 5-aza-CdR was assessed by Boyden chamber invasion assay as described under “Materials and Methods”. Southern blot analysis of MDA-MB-231, MCF-7, and MCF-7 cells treated with 5-aza-CdR is shown in C. 10 μg of genomic DNA isolated from these cells were digested with non-methylation sensitive enzyme PstI (lanes a1, b1, and c1) or with methylation sensitive enzymes HpaII (lanes a2, b2, and c2) and HhaI (lanes a3, b3, and c3). DNA digests were resolved on 0.8% agarose gel, and all blots were probed with a 788-bp human uPA promoter fragment (C). Results are representative of at least three different experiments. Data of invasion assay (B) are expressed as mean ± S.E. of values from three independent experiments. Significant difference in the number of invading from control is represented by an asterisk (p < 0.05).

RESULTS

Evaluation of uPA mRNA Expression and Its Effect on Tumor Cell Invasion—To test the hypothesis that uPA might play a role in breast cancer cell invasion, we first examined the expression of uPA mRNA in the estrogen receptor (ER)-positive, hormone-sensitive human breast cancer cell line MCF-7 and in highly invasive ER-negative, hormone-insensitive MDA-MB-231 cells using Northern blot analysis. Additionally, mRNA expression was also evaluated in MCF-7 cells following treatment with the DNA methyltransferase inhibitor 5-aza-CdR. As seen in Fig. 1A, uPA mRNA is detected in MDA-MB-231 and MCF-7 cells and in 5-aza-CdR treated MCF-7 cells indicating that this potent demethylating agent is capable of inducing the re-activation of uPA gene expression in MCF-7 cells. To examine the effect of uPA on the invasive capacity of these cells, we carried out a modified Boyden chamber Matrigel invasion assay. These studies show that MDA-MB-231 cells expressing high levels of uPA are able to invade through the Matrigel, whereas MCF-7 cells that do not express uPA are unable to invade. Furthermore, the non-invasive nature of MCF-7 cells was reversed, and a significant number of tumor cells invaded through the Matrigel, following 5-aza-CdR treatment of MCF-7 cells (Fig. 1B). This increase in tumor cell invasive capacity following demethylation correlated with induction of uPA.
mRNA. These results are consistent with previous studies by us and others showing that uPA expression is directly related to the invasive capacity of the tumor cell (30).

Analysis of uPA Gene Methylation in Breast Cancer—We then determined whether this switch in expression of uPA is controlled epigenetically. The change in uPA gene methylation status was examined as a potential molecular mechanism regulating the differential expression of uPA in tumor cells representing early (MCF-7) and late (MDA-MB-231) stage human breast cancer. Genomic DNA was isolated from these cell lines and digested first with the methylation-insensitive endonuclease PstI (Fig. 1C, lane a) and then with the methylation-sensitive (HpaII, HhaI) endonucleases (Fig. 1C, lanes b and c). These samples were subjected to a Southern blot analysis and hybridized with a probe recognizing the uPA promoter region. Because this region of the gene bears multiple CCGG and CGCG sites, it can be cleaved extensively by HpaII and HhaI restriction enzymes when it is unmethylated, as is the case with DNA prepared from MDA-MB-231 cells expressing abundant amounts of uPA (Fig. 1C, lanes b1 and c1). In contrast, in MCF-7 cells, both HpaII and HhaI failed to cleave this region resulting in an identical pattern to the one observed after PstI digestion alone (Fig. 1C, lanes b2 and c2). To further confirm that this differential digestion with HpaII and HhaI is because of DNA methylation, MCF-7 cells were treated with 5-azaCdr and subjected to a similar Southern blot analysis. The pattern of cleavage of the uPA promoter was identical to the one observed in MDA-MB-231 cells (Fig. 1C, lanes b3 and c3).

Characterization of Human Breast Cancer Cell Lines for uPA Gene Expression, Cell Invasive Capacity, and Methylation Status within the uPA Promoter Region—To determine whether the correlation among uPA expression, cell invasiveness, and uPA promoter methylation is representative of all breast cancer cell lines, we characterized several additional cell lines representing different stages of breast cancer. Using reverse transcriptase-PCR, we found that, like MCF-7 cells, T47D and BT474 cells that are known to be ER-positive do not express uPA. (Fig. 2A). On the other hand, HS578T and BT549, which are ER-negative, were found to express uPA, as does MDA-MB-231 (Fig. 2A). To identify whether a correlation between uPA expression and cell invasive capacity exists in all of these cell lines, we carried out a modified Boyden chamber Matrigel invasion assay. These studies show that HS578T and BT549 cells, which express uPA, act in a similar fashion to MDA-MB-231 and are able to invade through the Matrigel. T47D and BT474, in addition to MCF-7, are found to have low invasive capacity (Fig. 2B).

Complete characterization of the methylation status of the uPA promoter region in human breast cancer cell lines was carried out by MSP. For these studies, we selected PCR primers that could amplify a 475-bp DNA fragment that included part of the promoter and of exon 1 within the uPA gene. This area is rich in CpG dinucleotides (Fig. 2C), which can serve as sites for methylation modification. The analysis of this region was also carried out by a TESS (transcription element search system) DNA analysis program showing the presence of several important DNA transcription factor binding sites in this region, which may play a significant role in regulating uPA expression (Fig. 2C). MSP analysis revealed that greater than 80% of cytosines of CpG dinucleotides are methylated in MCF-7 cells that do not express uPA. Similarly, non-uPA expressing and low invasive T47D and BT474 cells also have a high degree of methylation in the uPA promoter at 21.2 and 18.2%, respectively. By contrast, in uPA expressing MDA-MB-231, HS578T, and BT549 cells, the uPA promoter was completely unmethylated (Fig. 2C). In Fig. 3 we have provided the complete DNA sequence from the results of MSP analysis of two of the most commonly described highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cell lines. Overall, these results are in agreement with methylation of the uPA gene in MCF-7 cells resulting in silencing of uPA gene expression and provide a clear correlation among uPA expression, cell invasive capacity, and uPA promoter methylation.

Analysis of the Impact of Methylation on uPA Gene Promoter Activity in Human Breast Cancer Cells—After we established a correlation between promoter methylation and uPA gene transcription, we examined the effect of changes in promoter methylation on gene transcription. For these studies, the uPA promoter reporter construct (-745 to +30 bp) uPA-luc was methylated by mHpaII, mHhaI, or mSssI methylases or mock-
treated in vitro and was transiently transfected into the human breast cancer cells MDA-MB-231. Luciferase reporter activity was determined 48 h after transfection. As a control we used a pGL-3 basic luciferase construct lacking the promoter. The mock-treated unmethylated uPA-luc plasmid exhibited at least 4-fold higher luciferase activity as compared with cells transfected with the promoterless control plasmid as expected. Methylation of the different plasmids with different methylases reduced the promoter activity significantly (Fig. 4A).

Transcription factor Ets-1 activates uPA promoter and induces its gene expression (31, 32). As shown in Fig. 2C, Ets-1 binding site coincides with methylation sites and is therefore a candidate to be affected by DNA methylation. We then determined whether the inducible effect of Ets-1 could be blocked by methylation. Methylated or unmethylated uPA-luc was co-transfected with either pEVRF0 (empty vector for pEVRF-Ets-1; lane 2) or pEVRF-Ets-1 (lane 3) encoding Ets-1 as shown in Fig. 4B. Although pEVRF-Ets-1 activates unmethylated uPA-luc more than 5-fold, it fails to stimulate the transcription activity of all methylated uPA-luc plasmids (Fig. 4B). This marked suppression is consistent with methylation regulating the uPA promoter. Although there are several methylated cytosines in regions surrounding Sp1 binding sequences, transfection of Sp1 plasmid failed to cause any significant change in the uPA-mediated luciferase activity, as was observed following Ets-1 plasmid transfection (data not shown).

Electrophoretic Mobility Shift Assay of Methylated Versus Unmethylated ETS Binding Site—To further evaluate the involvement of the Ets-1 transcription site in the regulation of the uPA promoter, we next examined the ability of the Ets-1 protein to bind to both the methylated and unmethylated Ets-1 binding sites by electrophoretic mobility shift assay. Methylated and unmethylated oligonucleotides corresponding to the Ets-1 binding site within the uPA promoter were synthesized, labeled, and incubated with nuclear extracts from MDA-MB-231 cells where the Ets-1-binding protein was overexpressed. The resulting DNA-protein complex indicates that there is a greater amount of binding with the unmethylated ODN than with the methylated ODN (Fig. 5). Addition of 10–50-fold excess of unlabeled methylated oligonucleotide did not effect the Ets-1 binding activity. By contrast, addition of a 10–50-fold excess of unlabeled unmethylated ODN resulted in a dose-dependent displacement in binding of Ets-1-binding protein to the unmethylated oligonucleotide (Fig. 5). These results confirm that the Ets-1-binding protein selectively binds to the unmethylated, and not the methylated, Ets-1 transcription factor site within the uPA promoter.

Maintenance and de NocDNMT and DMase Enzyme Activities in Human Breast Cancer Cells—We then examined whether differences in the state of methylation of the uPA promoter between MDA-MB-231 and MCF-7 cells reflect a global change in the DNA methylation machinery. We first examined the levels of de novo and maintenance DNA DNMT activities in the two cell lines by quantitation of the total amount of [3H]-methyl group that has been catalyzed by nuclear extracts prepared from these cells onto either hemimethylated (maintenance DNMT activity) or unmethylated DNA substrates (de novo DNMT activity). As shown in Fig. 6A, the overall maintenance DNA DNMT activity observed was 3-fold higher in MCF-7 cells (lane 5), which have a methylated uPA promoter as compared with MDA-MB-231 cells, where the uPA promoter is unmethylated (lane 3). In contrast, as shown in Fig. 6B, endogenous de novo DNMT activity was found to be significantly higher in MDA-MB-231 cells (lane 3) as compared
with MCF-7 cells (lane 5). Thus, the maintenance but not the de novo DNMT activity correlates with the state of methylation of the uPA gene.

We then determined whether there are differences in global DNA DNase activity in MCF-7 cells in comparison with MDA-MB-231 cells. It has been suggested previously that tumor cells have high levels of DNase activity that may be responsible for the hypomethylation observed in these cells. Active removal of the [3H]-CH₃ moiety from methylated cytosines in vitro methylated DNA has been shown previously to result in a release of a volatile residue that was identified as methanol (28). Using a volatile assay we quantitated DNase activity in these breast cancer cells (26–28). DNase activity was 3–4-fold higher in MDA-MB-231 cells where the uPA promoter is hypomethylated, as compared with MCF-7 cells, where the promoter is methylated (Fig. 6C). Thus, global DNase activity correlates with the state of methylation of the uPA promoter in breast cancer cells.
ulation of uPA during tumor progression. After fully characterizing several human breast cancer cell lines representing various stages of breast cancer, we focused on 2 cell lines, MCF-7, a hormone-sensitive and low invasive cell line that represents early stage human breast cancer, and MDA-MB-231 cells, which are hormone-insensitive and represent late stage breast cancer. Animals inoculated with MDA-MB-231 develop large tumors that can metastasize to several sites in vivo (6). In these cells, levels of uPA expression correlate with tumor cell invasive capacity and provide a link between uPA expression and tumor stage (5, 37). Using this model we examined whether the methylation status of the uPA gene plays a role in regulating the differential expression of this gene through the multistep process of tumor progression. Our data show that the methylation status of the uPA promoter correlates with its state of expression as demonstrated by methylation-sensitive endonuclease Southern blot analysis and sodium bisulfate mapping. DNA methylation plays a causal role in controlling the uPA expression, because the DNMT inhibitor 5-azaCdr induces the expression of uPA in MCF-7 cells and increases their invasive capacity. Further support for the causal role of DNA methylation in regulating uPA gene expression is drawn

A 50 columns. Following elution with a continuously increasing gradient salt buffer, eluted fractions (500 μl × 10) from the total nuclear extract were collected. A 20-μl sample of each eluted fraction was incubated with [3H]-methyl-DNA substrate overnight at 37°C. Amount of generated volatized [3H]-CH3OH was counted for each cell line (C). Results are expressed as the mean disintegration per min per sample ± S.E. of cell extracts, each of which was assayed in triplicate.

Fig. 6. Maintenance and de novo DNMT and DMase enzyme activities in human breast cancer cells. Total nuclear extracts (5 μg) obtained from MDA-MB-231 and MCF-7 breast cancer cells were incubated with the methyl donor [3H]-AdoMet and substrate (hemimethylated poly(mdC-dG-dC-dG) oligo or unmethylated poly(dI-dC-dI-dC)). After incubating for 3 h at 37°C, the mixtures were passed through GF/C filters and precipitated with trichloroacetic acid. Total counts of mean disintegration/min of [3H]-CH3 incorporation retained from the GF/C filters represent the level of maintenance (A) or de novo (B) DNMT activity in these breast cancer cell lines, as marked by + signs, respectively (lanes 3 and 5 in A and B). CTL represents the reaction mixture containing no substrate oligo or nuclear extract. Total radioactivity retained by GF/C filters from reaction mixtures of respective cell lines in the absence of substrate oligo are shown by − signs (lanes 2 and 4 in A and B). Results are expressed as the mean disintegration/min of [3H]-CH3 incorporated into substrate oligonucleotide per μg of nuclear protein ± S.E. of triplicate cell extracts, each of which was assayed in triplicate. Total nuclear extracts (6 μg) obtained from MDA-MB-231 and MCF-7 cells were loaded onto DEAE-Sephadex

Fig. 7. Expression of uPA gene with TSA treatment in human breast cancer cells. Total cellular RNA was extracted from MDA-MB-231 and MCF-7 cells treated with or without 100 ng/ml TSA for 2 days. 15 μg of total cellular RNA from each cell line was analyzed by Northern blot analysis to monitor the level of UPA mRNA expression. All blots were probed with [32P]-labeled uPA or 18 S cDNA, which were then scanned by laser densitometric scanning. Changes in UPA mRNA expression as determined by plotting the ratio of UPA/18 S mRNA is shown. Results represent ± S.E. of four different experiments. Significant difference from control is represented by asterisks (p < 0.05).
from experiments that show that in vitro methylation inhibits uPA promoter activity and its transactivation by the transcription factor Ets-1. Ets-1 is required for the expression of uPA in a number of tissues (31, 32, 38) and interacts with sequences that are close to CpG dinucleotides as shown in Fig. 2. Methylation might inhibit uPA promoter activity by either inhibiting the interaction of Ets-1 with methylated CpGs in its recognition sequence or by recruiting methylated DNA-binding proteins that results in an inactive chromatin structure (12, 39, 40). These results demonstrate that the uPA promoter region involved in these effects contains an Ets-1 binding site; however, further experiments are required to determine the exact role of the Ets-1 site.

Aberrations in the DNA methylation machinery resulting in global hypomethylation and regional hypermethylation are well documented in cancer. It has been suggested that induction of both DNMT and DMase activities might play a role in the complex changes in DNA methylation observed in cancer cells (15). However, most of the attention in the field has been directed to the hypermethylation of tumor suppressor genes in cancer cells and its potential role in tumorigenesis (41). A large number of studies have demonstrated that inhibition of DNMT reverses tumor growth, and in addition, antisense DNMT1 inhibitors are currently in clinical trials (42–44). Our data, however, suggest that the involvement of methylation in tumorigenesis is more complex and that hypomethylation of certain genes might play a critical role in tumor progression. Therefore, activities that are responsible for demethylating genes required for tumor invasion might be important anticancer targets.

Our data are consistent with a model in which DNMT and DMase activities are expressed differentially and play distinct roles at different stages of tumor progression (Fig. 8). Early stage cancer cells such as MCF-7 show higher maintenance DNMT activity that may be required for maintaining the transformed state and involved in the silencing of the tumor suppressor genes. However, in the later stages of tumor progression, increased DMase activity is vital to induce the expression of genes that are silenced by DNA methylation but are critical for tumor invasion such as uPA. We show that in later stage MDA-MB-231 cells, maintenance DNMT activity is reduced whereas DMase activity is increased. It has been shown previously that ectopic expression of the ras oncogene can lead to increased DMase activity, and an active DMase was purified recently from human lung carcinoma cells (29, 45). It is not yet clear which of the DMases is specifically induced in MCF-7 cells, and further experiments are required to characterize this DMase. Nevertheless our results demonstrate that DNA methylation activities do undergo distinct changes during tumor progression and might play different roles at specific stages.

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REFERENCES

1. Rabbani, S. A., and Mazar A. P. (2001) Surg. Oncol. Clin. N. Am. 10, 383–415
2. Mueller, B. M. (1996) Curr. Top. Microbiol. Immunol. 213, 65–80
3. Frandsen, T. L., Holst-Hansen, C., Nielsen, B. S., Christensen, I. J., Nyengaard, J. R., Carmeliet, P., and Brunner, N. (2001) Cancer Res. 61, 522–527
4. Xing, R. H., and Rabbani, S. A. (1996) Int. J. Cancer 67, 423–429
5. Schnallfeldt, B., Prechelt, D., Harting, K., Spathe, K., Rutke, S., Konik, E., Frödin, R., Berger, U., Schmitt, M., Kuhn, W., and Lengyel, E. (2001) Clin. Cancer Res. 7, 2396–2404
6. Guo, Y., Higazi, A. A., Arakelian, A., Sachs, B. S., Cines, D., Goldfarb, R. H., Jones, T. R., Kwaan, H., Mazar, A. P., and Rabbani, S. A. (2000) FASEB J. 14, 1440–1450
7. Xing, R. H., Mazar, A., Henkin, J., and Rabbani, S. A. (1997) Cancer Res. 57, 3585–3593
8. Rabbani, S. A., Harakidas, P., Davidson, D. J., Henkin, J., and Mazar, A. P. (1995) Int. J. Cancer 63, 840–845
9. Ignar, D. M., Andrews, J. L., Witherspoon, S. M., Leray, J. D., Clay, W. C., Gehribe, C. W., and Ehrlich, M. (1993) Nucleic Acids Res. 11, 6883–6894
10. Szyf, M. (2001) Front. Biosci. 6, D599–D609
11. Van Zee, K. J., Calvano, J. E., and Bisogna, M. (1998) Oncogene 16, 2723–2727
12. Watt, P. M., Kumar, R., and Kees, U. R. (2000) Genes Chromosomes Cancer 29, 371–377
13. Mompour, R. L., and Bovenzi, V. (2000) J. Cell. Physiol. 183, 145–154
14. Gama-Sosa, M. A., Slager, A. V., Trewn, R. W., Oxenhandedler, R., Kuo, C., Gehribe, C. W., and Ehrlich, M. (1993) Nucleic Acids Res. 11, 6883–6894
15. Szyf, M. (2001) Front. Biosci. 6, D599–D609
16. Van Zee, K. J., Calvano, J. E., and Bisogna, M. (1998) Oncogene 16, 2723–2727
17. Kost, T. A., Ignar, D. M., Clay, W. C., Andrews, J., Leray, J. D., Overton, L., Hoffman, C. R., Kilpatrick, K. E., Ellis, B., and Emerson, D. L. (1997) Gene 190, 139–144
18. Rabbani, S. A., and Gladu, J. (2002) Cancer Res. 62, 2390–2397
19. Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000) Nat Genet. 25, 269–277
20. Momparler, R. L., and Bovenzi, V. (2000) J. Cell. Physiol. 183, 145–154
21. Gama-Sosa, M. A., Slager, A. V., Trewn, R. W., Oxenhandedler, R., Kuo, C., Gehribe, C. W., and Ehrlich, M. (1993) Nucleic Acids Res. 11, 6883–6894
22. Szyf, M. (2001) Front. Biosci. 6, D599–D609
23. Xing, R. H., and Rabbani, S. A. (1999) Int. J. Cancer 83, 443–450
24. Ricci, A., Grimaldi, G., Verde, P., Sebastiani, G., Roast, S., and Blasi, F. (1985) Nucleic Acids Res. 13, 2759–2771
25. Herman J. G., Graff J. R., Mysharnik S., Nelkin B. D., and Baylin S. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8821–8826
26. Rosenthal, N. (1987) Methods Enzymol. 152, 704–720
27. Issa, J. P., Verito, P. M., Wu, J., Szaezal, S., Celano, P., Nelkin, B. D., Hamilton, S. R., and Baylin, S. B. (1998) J. Natl. Cancer Inst. 88, 1235–1240
28. Han, B., Liu, N., Yang, X., Sun, H. B., and Yang, Y. C. (2001) J. Biol. Chem. 276, 7937–7942
27. Szyf, M., and Bhattacharya, S. (2002) Methods in Molecular Biology: Measuring DNA Demethylase Activity In Vitro, Humana Press, Totowa, NJ
28. Szyf, M., and Bhattacharya, S. (2002) Methods in Molecular Biology: Extracting DNA Demethylase Activity from Mammalian Cells, Humana Press, Totowa, NJ
29. Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6107–6112
30. Liu, D. F., and Rabbani, S. A. (1995) Prostate 27, 269–276
31. Nakada, M., Yamashita, J., Okada, Y., and Sato, H. (1999) J. Neuropathol. Exp. Neurol. 58, 329–334
32. Watabe, T., Yoshida, K., Shindoh, M., Kaya, M., Fujikawa, K., Sato, H., Seiki, M., Ishii, S., and Fujinaga, K. (1998) Int. J. Cancer 77, 128–137
33. Dobosy, J. R., and Selker, E. U. (2001) Cell Mol. Life Sci. 58, 721–727
34. Ng, H. H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999) Nat. Genet. 23, 58–61
35. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998) Nature 393, 386–389
36. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. (1997) Int. J. Cancer 72, 1–22
37. Rha, S. Y., Yang, W. I., Gong, S. J., Kim, J. J., Yoo, N. C., Roh, J. K., Min, J. S., Lee, K. S., Kim, B. S., and Chung, H. C. (2000) Cancer Lett. 150, 137–145
38. Naito, S., Shimizu, K., Nakashima, M., Nakayama, T., Ito, T., Ito, M., Yamashita, S., and Sekine, I. (2000) Pathol. Res. Pract. 196, 103–109
39. Fujita, N., Shimotake, N., Okai, I., Chiba, T., Saya, H., Shirakawa, M., and Nakan, M. (2000) Mol. Cell. Biol. 20, 5107–5118
40. Hermann, H., and Doerfler, W. (1991) FEBS Lett. 281, 191–195
41. Yang, X., Yan, L., and Davidson, N. E. (2001) Endocr. Relat. Cancer 8, 115–127
42. Lubbert, M. (2000) Curr. Top. Microbiol. Immunol. 249, 135–164
43. MacLeod, A. R., and Szyf, M. (1995) J. Biol. Chem. 270, 8037–8043
44. Juttermann, R., Li, E., and Jaenisch, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11797–11801
45. Szyf, M., Theberge, J., and Bozovic, V. (1995) J. Biol. Chem. 270, 12690–12696