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A Potential Role for Epigenetic Modulatory Drugs in the Enhancement of Cancer/Germ-Line Antigen Vaccine Efficacy

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Abstract

The discovery of epigenetic silencing as a key mechanism of tumor suppressor gene inactivation in human cancer has led to great interest in utilizing epigenetic modulatory drugs as cancer therapeutics. It is less appreciated that medically important tumor-associated antigens, particularly the Cancer Testis or Cancer/Germ-line family of antigens (CG antigens), which are being actively tested as cancer vaccine targets, are epigenetically activated in many human cancers. However, a major limitation to the therapeutic value of CG antigen-directed vaccines is the limited and heterogeneous expression of CG antigens in tumors. Recent work has begun to dissect the specific epigenetic mechanisms controlling differential expression of CG antigen genes in human cancers. From a clinical perspective, convincing data indicate that epigenetic modulatory agents, including DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors, robustly promote the expression of CG antigens, as well as class I major histocompatibility complex (MHC I) and other immune costimulatory molecules, in tumors. Importantly, the effects of these agents on CG antigen gene expression often show marked specificity for tumor cells as compared to normal cells. Taken together, these data encourage clinical evaluation of combination therapies involving epigenetic modulatory drugs and CG antigen-directed tumor vaccines for the treatment of human malignancies.

Keywords

cancer-testis antigens; cancer/germ-line antigens; epigenetics; DNA methylation; 5-aza-2′-deoxycytidine; immune response; MAGE-A1; NY-ESO-1; HDAC inhibitors; DNMTs

Epigenetic Regulation of CG Antigens

CG antigens are a large family of genes of mostly unknown function that show highly restricted expression, limited to germ cells of the testis and ovary, trophoblast, and a variety of human cancers.[1] CG antigen genes have been grouped based on their chromosomal localization, with approximately half of these genes encoded on the X chromosome (CG-X genes), and half on autosomes (non-X CG antigens).[1] The founding member of the gene family, *MAGE-1* (later renamed *MAGE-A1*)[2] was discovered in 1991 by Boon and coworkers, who identified it as a gene encoding an MHC I-restricted antigen recognized specifically by cytotoxic T lymphocytes (CTL) from a melanoma patient.[3] Since that time, over 80 additional members of the gene family have been identified, by either cytotoxic T-lymphocyte (CTL) or seroreactivity studies, and/or by virtue of their expression pattern in somatic, gametogeneic, and cancer tissues.[1,4]

The first evidence that CG antigen genes are epigenetically regulated was provided by the observation that treatment with 5-aza-2′-deoxycytidine (DAC), a classical DNA methyltransferase inhibitor,[5] activates *MAGE-A1* expression in human melanoma cell lines.[6] Additionally, hypomethylation of specific CpG sites in the *MAGE-A1* gene was found to

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correlate with gene expression.[7,8] A number of other CG antigen genes, including *MAGE-A2*, *-A3*, *-A4*, *NY-ESO-1*, *LAGE-1* and *XAGE-1* were also found to be regulated by promoter DNA methylation,[9-11] suggesting that DNA methylation may be a primary regulator of expression of this gene class.[10] Compared with tumor suppressor genes (TSGs), CG antigen genes show the opposite methylation pattern in normal somatic tissues and tumors. TSGs are typically unmethylated and expressed in normal tissues but can become silenced in association with DNA methylation in cancer.[12] In contrast, CG antigen genes are methylated and silent in normal tissues but become hypomethylated and activated in certain human cancers. Notably, CG antigen promoters, which often contain CpG islands,[13] show a similar change in methylation (hypomethylation) as does global genomic DNA in many human cancers.[14,15] An early study has investigated the relationship between CG antigen gene expression and global hypomethylation in cancer.[8] Using a panel of cancer cell lines showing variable levels of *MAGE-A1* expression, De Smet et al. found a general correlation between *MAGE-A1* expression and genomic DNA hypomethylation.[8] Additional investigations utilizing clinical tumor sample isolates and multiple CG antigen genes are required to firmly establish this relationship.

A number of recent studies have provided additional information about the epigenetic mechanisms controlling CG antigen gene expression (Fig. 1). There are three enzymatically active DNMT enzymes in mammalian cells, DNMT1, DNMT3a, and DNMT3b[16] and any combination of these enzymes could theoretically be involved in CG antigen gene regulation. Kozlowski et al. reported that in HCT116 colon cancer cells, which normally do not express CG antigen genes, genetic knockout of both DNMT1 and DNMT3b, but not of either enzyme alone, induces CG antigen gene expression.[17] In addition, two recent studies have found that DNMT1 may play a more prominent independent role in directing CG antigen gene repression in other cancer cell types.[18,19] There may also be important distinctions with regard to DNMTs involvement in CG antigen gene methylation, based on the specific CG antigen gene under study. For example, we have recently shown that methylation of the MAGE-A1 and NY-ESO-1 promoters is cooperatively maintained by DNMT1 and DNMT3b in HCT116 cells (functional redundancy), while methylation at the XAGE-1 promoter requires the activity of both enzymes.[19]

Histone modifications also appear to play a critical role in epigenetic regulation of CG antigen gene expression. An intriguing observation in this context was provided by the work of Shinkai and colleagues, who performed an oligonucleotide microarray screen for genes upregulated in murine ES cells sustaining a genetic knockout of the euchromatic histone methyltransferase enzyme G9a.[20] These authors reported that *MAGE-A2* expression was activated in G9a$^{-/-}$ ES cells, and that this correlated with changes in the histone modification pattern at the MAGE-A2 promoter, including loss of dimethylated H3K9 (diMe-H3K9), and gains in dimethylated H3K4 (diMe-H3K4) and acetylated H3K9/K14.[20] Additionally, genetic knockout of the highly related histone methyltransferase GLP, also in mouse ES cells, showed a similar phenotype with respect to *MAGE-A2* expression and histone code modifications.[21] Whether other CG antigen genes besides *MAGE-A2* are affected by G9a/GLP loss in this manner, and whether G9a/GLP are involved in CG antigen gene regulation in humans is unknown. With regard to histone modifications, our recent work, using HCT116 cells, has shown that loss of diMe-H3K9 is not sufficient for activation of CG antigen genes, including *MAGE-A1*, *NY-ESO-1* and *XAGE-1*, because the levels of this modification are reduced in DNMT1 or DNMT3b knockout cells that do not express significant levels of these genes.[19] However, we observe a strong association between increased levels of diMe-H3K4 and Ac-H3K9 and CG antigen gene activation, which is only seen in DNMT1/3b double knockout HCT116 cells.[19]

Recently, Lobanenkov and coworkers have provided an important conceptual advance in our understanding of the mechanism of regulation of CG antigen genes.[22,23] These studies

investigated the role of the *Brother of Regulator of Imprinted Sites* (BORIS), a germ-cell specific CCCTC-binding factor (CTCF) paralog,[24,25] in the regulation of various CG antigen genes, including *MAGE-A1* and *NY-ESO-1*.[22,23] CTCF is a epigenetic modulatory protein with a well-established role in reading gene-imprinting marks in soma.[26] CTCF and BORIS share a conserved central 11 Zinc Finger DNA binding domain (11ZF DBB) but have divergent N and C termini.[25] Conditional expression of BORIS was shown to activate expression of CG antigen genes, and kinetic studies showed that DAC treatment activates *BORIS* expression prior to inducing the expression of other CG antigen genes.[22] Furthermore, downregulation of BORIS by RNA interference prior to DAC treatment reduced the capacity of DAC to activate *MAGE-A1* expression.[22] BORIS was also shown to bind directly to the MAGE-A1 and NY-ESO-1 promoters and to displace CTCF at these loci.[22,23] These data provide intriguing evidence that aberrant expression of BORIS in human cancers could play a role in the initiation of CG antigen gene expression. Interestingly, BORIS is coexpressed in testicular germ cells with CG antigen genes, suggesting that it may regulate the expression of CG antigen genes during normal development.[24,25] The relationship between BORIS, CTCF, and other epigenetic control mechanisms in the regulation of CG antigen gene expression in human cancer is an important area for continued investigation.

Activation of CG Antigen Expression and Immune Recognition Using Epigenetic Modulatory Drugs

As described above, the expression of CG antigens is activated by treatment with the classical DNA methyltransferase inhibitor DAC. We have recently shown, using a microarray approach, that DAC treatment simultaneously activates many CG antigens, and the level of induction is more robust than other types of genes induced by this agent.[27] Other epigenetic modulatory drugs have also been shown to activate CG antigen gene expression. Treatment of cancer cell lines with zebularine, a second generation DNMT inhibitor and cytidine analog, also leads to robust CG antigen gene activation.[28] Additionally, combination treatment of DAC and depsipeptide, an HDAC inhibitor, leads to enhanced expression of the CG antigen gene *NY-ESO-1* in thoracic cancer cells.[29] It was also recently shown that combination treatments utilizing DAC and the classical HDAC inhibitor Trichostatin A synergistically activate the expression of *MAGE-A* class genes in human cancer cell lines.[30] A summary of the published data reporting CG antigen gene activation by epigenetic modulators is shown in Table 1.

Perhaps most importantly, both DAC and zebularine appear to activate CG antigen gene expression specifically in tumor-derived cells.[27,28] In our studies, we performed cDNA microarray analyses of three carcinoma and one normal epithelial cell line treated with DAC, and observed robust induction of >16 distinct CG antigen genes in all three carcinoma cell lines but not in normal epithelial cells.[27] Differential inhibition of DNMT enzymes did not appear to account for this phenomenon as covalent DNMT1 adduct formation and global genomic DNA methylation occurred in both the tumor and normal cell lines.[27] Similarly, a microarray study by Peter Jones and colleagues found that zebularine treatment induced eight different CG antigens in three different tumor cell lines, with no induction in four different normal fibroblast cell lines.[28]

Immune recognition of CG antigens is dependent on proper display of CG antigen-derived peptides in the context of MHC I molecules on the surface of tumor cells.[31] Thus, it is critical to note that DAC treatment also induces MHC I expression in cancer cells[27,32-34] (Table 1). MHC I downregulation is a common mechanism of immune evasion in human cancer,[35] and *MHC I* and *MHC II* gene expression are repressed by DNA methylation in various malignancies and possibly also during development.[36] In addition to MHC, other immune costimulatory molecules, including ICAM-1 and LFA-3, are induced in tumor cells by DAC treatment.[34] CG antigen peptide/MHC cell surface complexes are recognized by cytotoxic T-lymphocytes

(CTLs). Thus, it would be predicted that treatment with epigenetic modulators such as DAC would enhance CTL-directed killing of tumor cells following induction of CG antigen genes. In fact, this has been observed in numerous cell-based studies (Table 1). These data provide a critical validation that CG antigen gene activation by treatment with epigenetic modulators not only activates CG antigen gene expression, but that it also leads to functional CG antigen presentation and recognition by immune cells. Remarkably, Guo et al recently reported a novel strategy in which tumor cell lines were treated with DAC to induce the expression of a specific CG antigen (P1A) de novo.[37] One of these cell lines, the mammary tumor line 4T1, was used to challenge mice which received adoptive transfer of P1A-specific CTL cells.[37] Sequential DAC treatment and adoptive transfer in syngeneic BALB/c mice resulted in pronounced regression of 4T1 lung tumor metastases, as compared to control animals receiving adoptive transfer but not DAC treatment.[37] This "chemoimmunotherapy" study suggests that it is possible to activate immune responses to previously CG antigen-negative tumors by treatment with epigenetic modulators prior to, or concurrent with, vaccination. This strategy could make possible the clinical use of CG antigen-directed vaccines for classes of tumors not previously known to express CG antigens.

We have recently shown, in a Phase I clinical trial for the treatment of solid tumors, that low dose infusion of DAC (Decitabine) elicits hypomethylation of the MAGE-A1 promoter in vivo, in peripheral blood mononuclear cells (PBMCs).[38] Because we did not obtain tumor tissue in this study, it remains unclear whether similarly favorable pharmacodynamics would also be observed in solid tumors, although one would anticipate similar results in liquid tumor tissues. We did not measure MAGE-A1 expression in this clinical study; however, in vitro experiments indicate that normal cells have a reduced capacity to express MAGE-A1, even if the promoter is hypomethylated.[27] Thus, while specificity remains a paramount issue, DNA methyltransferase inhibitors may yield high therapeutic indices, with regard to activation of CG antigen expression in vivo. In this context, Sigalotti et al reported that peripheral blood and bone marrow cells from DAC-treated myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients showed increased expression of multiple CG antigen genes at the mRNA level.[39] This important finding further highlights the potential clinical utility of epigenetic modulators for activating CG antigen-directed immune responses.

Concluding Remarks

CG antigen vaccines are under clinical development, and formulations based on specific peptides, full-length proteins, and DNA vaccination strategies are under active investigation.[1] A recent placebo-controlled clinical trial using recombinant NY-ESO-1 protein and a novel adjuvant generated antibody, $CD4^+$, and $CD8^+$ T-cell immune responses, as well as superior clinical outcomes in melanoma patients.[40] Thus, CG antigen vaccines are both safe and have the capacity to be highly immunogenic. However, heterogeneous expression of CG antigens, both within individual tumors and throughout the patient population, presents a significant barrier to the clinical utility of the single agent vaccine approach. Recent clinical experience with DAC, and the related compound 5-azacytidine, have demonstrated that low dose treatments with these agents show low toxicity, along with high response rates in hematopoetic malignancies.[41,42] Taken together, data showing robust CG antigen and MHC I gene activation by epigenetic modulators in vitro and in vivo, along with the generally low in vivo toxicity of both CG antigen vaccines and epigenetic modulators, suggests that prior or concurrent administration of epigenetic modulatory drugs could significantly increase immunological and clinical responses to CG antigen-directed vaccines.

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Abbreviations

CG antigen
: cancer/germ-line antigen

DAC
: 5-aza-2′-deoxycytidine or decitabine

DNMT

DNA methyltransferase

HDAC
histone deacetylase

MHC
major histocompatability complex

CTL
cytotoxic T lymphocytes

TSG
tumor suppressor genes

BORIS
brother of the the regulator of imprinted sites

CTCF
CCCTC-binding factor

diMe-H3K9
dimethylated histone H3 lysine 9

diMe-H3K4
dimethylated histone H3 lysine 4

Ac-H3K9
acetylated histone H3K9

MDS
myelodysplastic syndrome

AML
acute myeloid leukemia

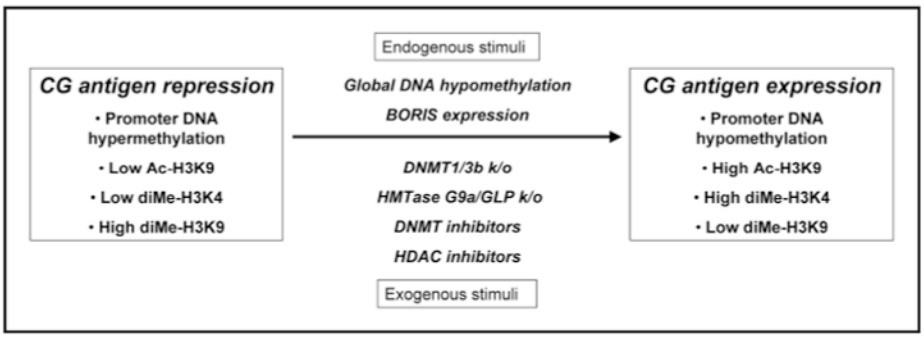

Figure 1.

Epigenetic regulation of CG antigen gene expression. Repression of CG antigen genes in somatic tissues and human cancer cells is associated with DNA hypermethylation, low levels of acetylated lysine 9 and dimethylated lysine 4 of histone H3 (Ac-H3K9 and diMeH3K4), and high levels of diMe-H3K9. In contrast, activation of CG antigen genes, seen in germ cells of the testis and ovary and in some human cancers, is associated with promoter hypomethylation and the opposite pattern of histone code modifications. Endogenous stimuli proposed to lead to CG antigen gene expression include global genomic DNA hypomethylation and expression of the CTCF paralog BORIS. Exogenous stimuli that promote CG antigen gene expression include siRNA knockdown of DNMT1, or genetic knockout of both DNMT1 and DNMT3b in human cancer cells. In murine ES cells, genetic knockout of the euchromatic histone methyltransferase enzymes G9a or GLP causes CG antigen gene activation. Finally, treatment of human cancer cells with epigenetic modulatory drugs, including DNMT and HDAC inhibitors, activates CG antigen gene expression.

Table 1

CG antigen gene induction using epigenetic modulatory agents

Epigenetic Modulator	Induced CG Antigen and/or Costimulatory Molecule	Tumor Cell Type	Enhanced Tumor Cell Lysis by CTL	Ref.
DAC[a]	MAGE-A1	Melanoma	Yes	6
DAC	MAGE-A1, MHC I, ICAM-1, LFA-3	Melanoma	N/d	34
DAC	MHC I	Colon	N/d	32
DAC/Depsipeptide	NY-ESO-1	Thoracic	Yes	29
DAC	Numerous CG antigens	Renal Cell	Yes	43
DAC	Numerous CG antigens	Bladder	N/d	44
DAC	Numerous CG antigens	Mesothelioma	N/d	45
DAC[b]	MAGE-A1, NY-ESO-1, SSX	MDS, AML	N/d	39
Zebularine	Numerous CG antigens	Bladder, Pancreas, Colon	N/d	28
DAC	Numerous CG antigens, MHC I, β-2-microglobulin	Colon	N/d	27
DAC	MAGE-A3	Melanoma	Yes	46
DAC	XAGE-1	Gastric, Colon	N/d	11
DAC	P1A	Multiple types[c]	Yes	37
DAC/Trichostatin A	MAGE-A1, -A2, -A3, -A12	Multiple types	N/d	30

[a] 5-aza-2′-deoxycytidine.

[b] in vivo experiment involving treated patients.

[c] includes in vivo activation of P1A in treated mice.

N/d, not determined.