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Epigenetic Agents Induce MHC class I Surface Expression on Tumor Cells and Reexpress an Aberrantly Silent Marker of Myeloid Neoplasms

Ph.D. Thesis

Supervisor

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Abbreviations

Ac acetylated

AKT Agammaglobulinemia tyrosine kinase

AML Acute myeloid leukemia

AML-TLD/AML-MDS AML with trilineage dysplasia

APC Antigen presenting cell

ATP Adenosine triphosphate

5-azaC 5-azacytidine

CIITA MHC class II transactivator

CML chronic myelogenous leukemia

DAC 5-aza-2'-deoxycytidine, decitabine

DNA Deoxyribonucleic acid

Dnmt1 DNA methyltransferase 1

Dnmt2 DNA methyltransferase 2

Dnmt3a DNA methyltransferase 3a

Dnmt3b DNA methyltransferase 3b

Dnmt3L DNA methyltransferase 3L

DNMTi DNA methyltransferase inhibitor

CBP CREB binding protein

ChIP Chromatin immunoprecipitation

CpG CG dinucleotide

CR Complete response

CTL Cytotoxic T Lymphocytes

ER endoplasmic reticulum

ES embryonic stem cells

HAT Histone acetyltransferases

HDACi Histone deacetylase inhibitors

HMT Histone methyltransferases

HLA Human Leukocyte Antigen

HPV Human Papilloma Virus

IFN-γ interferon gamma

ISRE interferon-sensitive response element

logFC log fold change

LOH Loss of heterozygosity

MAPK mitogen activated protein kinases

MBP Methyl binding protein

MDS Myelodysplastic Syndrome

MHC Major histocompatibility complex

NK cells Natural killer cells

PR Partial response

PRMT Protein arginine methyltransferases

RB Retinoblastoma

TNF-α Tumor necrosis factor alpha

TRA Tumor rejection antigens

TSA Trichostatin A

VLP Virus-like particle

Introduction

Epigenetic modifications of the human genome, namely histone deacetylation or aberrant DNA methylation, represent tumorigenic events that are functionally equivalent to genetic changes (Rountree et al., 2001). In tumor cells, reexpression of genes downregulated by promoter DNA methylation can be achieved by the administration of inhibitors of DNA methyltransferase (DNMTi) and the effect can be increased with inhibitors of histone deacetylases (HDACi), such as trichostatin A (TSA) (Yoshida et al., 1990) or sodium butyrate (Chiurazzi et al, 1999). HDACi in some instances have been shown to be effective agents in the treatment of some tumor cells. The anti-tumor therapeutic potential of these compounds has been tested in clinical studies and the targets of choice in the experimental setting are usually epigenetically silenced tumor suppressor genes (Egger et al., 2004). It has been hypothesized that epigenetic changes may also represent one of the basal mechanisms underlying tumor cell escape from immune responses.

Tumor cells have the ability to evade specific immune responses mediated by cytotoxic T cells (CTL) by downmodulating major histocompatibility complex (MHC) class I molecules on their surface (Garrido et al., 1997). There are several phenotypes of MHC class I-deficient tumors described by Garrido et al (1976) that might be classified either as irreversible or reversible. The mechanisms associated with irreversible changes including β-microglobulin mutations, haplotype-specific loss and HLA loss have been described (Garrido et al., 1997). The inhibition of transcription of the genes involved in antigen processing commonly known as antigen processing machinery (APM) (Hicklin et al., 1999; Seliger et al., 2000), namely proteasome subunits *LMP2* and *LMP7* and transporters associated with antigen processing *TAP1* and *TAP2* often leads to reversible, downmodulated MHC class I molecules on tumor cells. Reexpression of APM genes can be frequently induced by cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) resulting in the

induction of surface MHC class I molecules which are subsequently recognized and lysed by cytotoxic T lymphocytes (CTL) (Lu et al., 2001; Mikyskova et al., 2005).

The mechanism linking antigen processing machinery to the induction of MHC class I molecules in relation to the administration of IFN-γ is not completely understood. The possibility that epigenetic mechanisms may also be involved in the silencing of APM genes have been raised (Bubenik, 2004). The expression of immunological important molecules such as MHC class I or MHC class II, co-stimulatory and adhesive molecules on tumor cells, cytokine production as well as the expression of tumor rejection antigens can also be directly or indirectly modulated by epigenetic mechanisms (Maeda et al., 2000; Nie et al., 2002; Chou et al., 2005; Sanchez-Abarca et al., 2009). Hence, the reversal of the immune escape phenotype of tumor cells represents one of the possible modalities by which epigenetic agents can inhibit growth of the tumor (Tomasi et al., 2006).

Hypermethylated promoter regions lead to aberrantly silent genes whose status can be reversed by DNMTi such as 5-azacytidine (5-azaC) and 5-aza-2'-deoxycytidine (DAC). These inhibitors carry a modified cytidine nucleoside ring which permits them to inhibit DNA methyltransferases (Dnmt). As these agents enter the cell, they are converted to nucleotides by kinases and incorporated as deoxynucleotides or ribonucleosides during S phase of the cell cycle (Momparler, 2005). Normally, cytosine analogues that are incorporated into DNA act as substrates for DNA methyltransferase in S phase but, the modified cytosine ring effectively binds the enzyme inhibiting the activity of the Dnmt. This leads to a hypomethylated, transcribed promoter. The HDACi trichostatin A (TSA) and sodium butyrate work in combination with DNMTi or alone remodeling the chromatin found around the promoter regions of genes. These drugs target many genes that are epigenetically modified in cancer including those involved in cell cycle control which are consequently demethylated or

reacetylated, nonfunctioning apoptotic effector genes, and DNA repair genes that contribute to the instability of neoplasia. Additionally, these epigenetic agents reverse downregulated immune recognition molecules, inhibit angiogenesis and tumor cell invasion as well as metastasis. In recent years, both DNMTi and HDACi have successfully been used in phase I and II human trials (Gore et al., 2006; Issa et al., 2004) exhibiting outstanding results including patient tolerance, decrease of tumor size, promoter demethylation and reexpression of silenced genes. These agents are seen as promising anti-cancer drugs or as adjuvants to be used in combination with other therapies due to their ability to affect many different epigenetic abnormalities in neoplastic cells. This thesis focuses on the effect of DNA methyltransferase and histone deacetylase inhibitors on MHC class I negative human papilloma virus tumor cells, the genes responsible for the induction of these molecules on the surface of these cells and the tolerance/response of patients treated with a combination of these drugs.

Mechanisms of antigen presentation in the context of MHC molecules

The major histocompatibility complex describes two types of molecules that present processed peptide antigen to T cells. MHC class I molecules bind and present peptides that have been processed in the cytosol of an infected cell. Therefore, are able to present viral proteins on the surface of the cell. Proteins brought into the cell via phagocytosis or endocytosis are degraded within intracellular vesicles of macrophages, B cells and bind MHC class II molecules. The expression of both MHC classes is enhanced by cytokines released during the immune response, specifically interferon gamma (IFN-γ). Additionally, IFN-γ favorably affects the antigen presenting ability of MHC class I molecules by increasing the expression of internal components that are responsible for the degradation and transport of processed peptides.

In normal cells, viral proteins are processed into peptides in the proteasome,

transported to the lumen of the endoplasmic reticulum (ER), adhere to the preassembled MHC class I molecule and eventually presented on the surface of the cell (Jensen, 2007). The antigenic peptide is degraded to smaller sequences of 8-10 amino acids in the proteasome by the IFN-γ inducible proteins LMP2 and LMP7 and LMP10 (Niedermann et al, 1999) and escorted to the lumen of the ER where it encounters the transporter associated proteins TAP1 and TAP2, which bring the antigen inside the ER and attach the peptide to the assembled MHC class I molecule (Lankat-Buttgereit and Tampe, 1999).

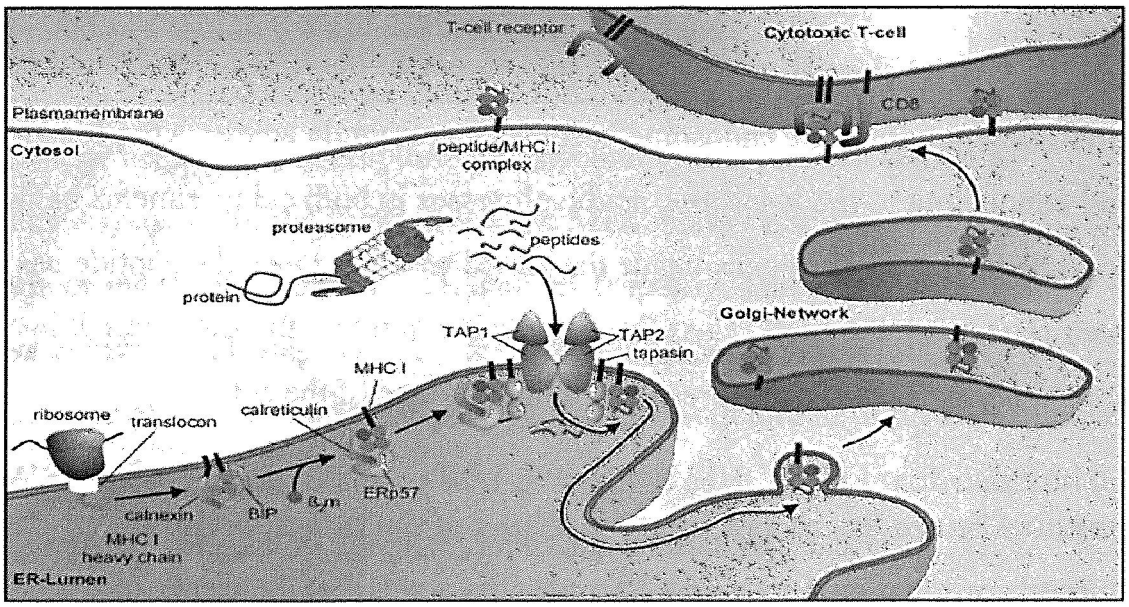

Fig.1. Antigen processing pathway. MHC class I heavy chain is translocated to the ER and joins the immunoglobulin binding protein β_2-microglobulin and chaperone calnexin which assists in the proper folding of the heavy chain. Additional molecules that comprise the peptide loading complex (ERp57, calreticulin, tapasin, TAP1 and TAP2) attach to the MHC molecule. Degraded peptides arrive at the opening of the TAP gates and adhere to the waiting MHC class I molecule. Loaded MHC class I molecules leave the ER travel through the Golgi complex and appear on the surface of the cell and are targeted by a $CD8^+$ T cell which subsequently destroys the cell (adapted from Schmitt and Tampe 2005).

The MHC class I molecule and its attached peptide travels through the Golgi and presented on the cell surface and recognized by $CD8^+$ cytotoxic T

lymphocytes.

Whereas protein processing of MHC class I pathway begins in the proteasome and continues in the ER, MHC class II pathway process begins with endocytosis of the pathogen. Cross presentation by professional antigen presenting cells enables cells to internalize antigen and also present in the context of the MHC class I molecule. Macrophages, immature dendritic cells, B cells and other antigen presenting cells engulf and place pathogens within acidified vesicles called endosomes. This internal structure does not present the pathogen protein to the proteasome but contains proteases that breakdown the proteins to peptides that bind MHC class II. As the endosome moves further inside of the cell, the environment within the endosome becomes increasingly acidic. After locating and connecting to the lysosome, acidic proteases in both compartments begin to degrade the protein antigens inside the joined vesicles. Once the peptide has been processed, the MHC class II molecule is taken into the ER where it is processed further and then presented on the surface of the cell.

Immune Surveillance and Immunoediting in the tumor environment

Burnet's original immune surveillance theory states that thousands of tumor cells produced every day, antigenic in the host, are cornered by immune responses and eradicated lends itself to the concept of tumor rejection antigens (TRA) (Thomas, 1959; Burnet, 1957). Tumor rejection antigens in the mouse and some human tumors have been verified and are recognized on the surface of tumor cells by CTL. Under certain conditions, these antigens play a role in the suppression of the growth of early tumors and inhibit the progression of these malignant cells i.e. T cell-mediated surveillance. The lack of MHC class I molecules on tumor cells in turn activate a cascade of other immune cells such as Natural killer (NK) cells, which recognize and lyse MHC class I negative tumor cells. Therefore, these cells play a very important role in immunological control in tumor growth.

γ:δ T cells are important players in immune surveillance. They are a source of IFN-γ which induces the expression of MHC class I molecules on tumor cells. If MHC class I expression is detected, CTL functions as the anti-tumor effector. However, should the number of MHC class I molecules drop below a certain threshold, NK cells become more active in surveillance. Hence, if downregulated MHC class I is detected (below a threshold) in the tumor environment, CTL response will be replaced by NK response but, tumor that loses all of its MHC class I molecules becomes invisible to CTL. NK cells express inhibitory receptors on the surface that bind MHC class I molecules of the tumor and should the number of MHC class I molecules decrease, a signal is sent to NK cells to destroy the tumor. Moreover, the tumor cell can express ligands for negative NK receptors that enable it to escape from NK cell-mediated lysis. Also, if a tumor loses one MHC class I molecule from its surface, the tumor may elude both the CTL and NK response. There may be a link between innate and adaptive anti-tumor immunity beginning with the appearance of NK cells that release IFN-γ in an environment of downregulated MHC class I tumor cells. This in turn upregulates MHC class I molecules on the surface stimulating MHC adaptive immunity followed by a strong CTL-mediated tumor response (Bubenik, 2003).

Immunoediting, the recognition and eradication of cancer cells, has been described in three steps: elimination, equilibrium and escape (Dunn et al., 2002). In the process of elimination, there are several stages that lead to cessation and removal of the tumor. Early in elimination, a tumor grows to a certain size and becomes invasive. Inflammation activates tumor-infiltrating NK, Natural killer T cells (NKT), γ:δ T cells, macrophages and dendritic cells (Girardi et al., 2001; Smyth et al., 2001; Matzinger, 2000) which then produce IFN-γ. This action results in some tumor death via apoptosis (Kumar et al, 1997) or impedes cell proliferation (Bromberg et al., 1996) followed by chemokines CXCL10 (interferon-inducible protein-10), CXCL9 (monokine induced by IFN-γ or MIG)

and CXCL11 (interferon-inducible T cell chemoattractant, ITAC) produced from the tumor cell itself, as well as, from surrounding tissue (Luster and Leder, 1993; Liao et al., 1995; Cole et al., 1998). These chemokines promote angiogenesis leading to further tumor death (Coughlin, 1998; Qin and Blankenstein, 2000). Dendritic cells quickly remove tumor cell debris that formed as a result (directly or indirectly) of IFN-γ release and relocate to draining lymph nodes. The release of these chemokines also recruits additional NK cells and macrophages to assist in the removal of the remaining tumors. IL-12 is then produced by the interaction of tumor-infiltrating NK cells and macrophages killing more tumors. Mechanistically, TNF-related apoptosis-inducing ligand, perforin and reactive oxygen/nitrogen intermediates mediate further eradication of tumors (Smyth et al., 2000; Takeda et al., 2002). In the draining lymph node, dendritic cells signal $CD4^+$ IFN-γ cells (Th1) which then promote the development of $CD8^+$ cells (Pardoll, 2002; Gerosa et al., 2002; Ferlazzo et al, 2002). Toward the end of the elimination stage, $CD4^+$ and $CD8^+$ T cells proceed to the site of inflammation, recognizing antigen bearing tumor cells.

Any tumor cells that have survived the elimination process are captured in the second step, equilibrium. During this phase of surveillance, selective pressure is applied by lymphocytes and IFN-γ which attempts to remove genetically unstable and rapidly mutating tumor cells. While some of the variants that escaped the elimination stage are removed, other new variants arise with new mutations and are not detected by CTL. This stage of the process can last many years meanwhile, newly formed tumor beds appear. Eventually, these tumor variants completely evade the immune response.

The tumors variants that survive detection to this point have masked themselves in order to avoid immunological recognition and elimination either by genetic or epigenetic changes. This leads to the escape of the tumor resulting in malignant disease within the host. Some of these escape variants can be targeted, reversing

genes that are downregulated thus supporting their proliferation. In the next section, the manner that the antigen processing machinery can be targeted to reverse downregulated MHC class I molecules will be discussed.

Mechanisms of irreversible and reversible MHC class I downregulation

Cell surface expression of MHC class I molecules depends on the proper function of the assembly of the MHC molecule, protein processing within the proteasome and transport of the peptide to the endoplasmic reticulum through the TAP portal. In the case of improper function which leads to downregulation of MHC class I molecule, the state of downregulation can be reversed or remain in a non-expressed form. There are four different phenotypes that represent downregulated surface expression of MHC class I molecules on tumor cells. These phenotypes were first described in mice lymphomas (Garrido et al., 1976) and later in human tumors (Garrido et al., 1997). The authors assigned a phenotype to describe the loss of antigen presentation and cataloged them as reversible or irreversible. Phenotype I with the most notable genetic alterations being mutations or deletions in β_2-microglobulin (D'Urso, 1991), TAP-associated (Cromme et al., 1994) or structural MHC class I genes (Bonal et al., 1986). These irreversible abnormalities have been associated with HLA class I loss, downregulation or effects on HLA allo-specificities (Caberra, 2007; Mendez et al., 2008) but are varied in their frequency in certain types of tumors. For example, *β_2-microglobulin* mutations in the colon have been observed at 21 percent of colon carcinomas and 15 percent of melanomas (Seliger et al., 2006). Although MHC class I downregulation has been observed in renal cell carcinomas, bladder and laryngeal tumors, no mutations in *β_2-microglobulin* have been detected (Atkins et al., 2004; Fernandez et al., 2000; Romero et al, 2005). Haplotype-specific loss leading to loss of heterozygosity (LOH) on chromosome 6p21 has been noted also in different tumors including renal cell carcinoma (12 percent) and head and neck squamous cell carcinoma (36

percent) (Maleno et al, 2004). Loss of an HLA haplotype which has been categorized as phenotype II, has been recorded in only a few tumor cell lines. has been speculated that this loss was due to chromosomal non-disjunction or mitotic recombination (Torres et al., 1996). In the case of phenotype III, the downregulation of HLA locus loss is thought to be transcriptional since HLA class I locus promoter sequences and mRNA levels for some class I alleles in tumor cells differ from those in normal cells. For example, increased *c-myc* transcription which interferes with *HLA-B* at the promoter level leads to downregulation in melanoma (Versteeg et al, 1989; Schrier et al, 1991). The l expression of these HLA genes can be restored by cytokines. Phenotype IV which is not reversible represents allelic HLA loss that possibly occurs as a result of a point mutation(s), partial deletion(s) (Browning et al., 1993), chromosomal breakage or somatic recombination (Browning et al., 1996) of HLA genes. Some tumors do not fall into any of these categories, likely due tc multiple events that produce a combination of phenotypes. A tumor may expr a single HLA class I allelic product because of events that occur over time during the progression of the cancer via immunoselection. Therefore treatmen strategies would be even more difficult to develop.

Immunotherapy as a means to induce the expression of MHC class I molecule on tumor cells has been one of the focuses of immunologists. Although upmodulating MHC class I molecules with IFN-γ has been successful in some instances, cytokines other than IFN-γ and TNF-α that have produced positive results. IL-12 was shown to have anti-angiogenic properties stimulating IFN-γ and TNF-α production by NK cells (Nanni et al, 1998) after peritumoral injection. IL-2 also seemed to enhance IFN- γ production by both T and NK cells upregulating MHC class I and MHC class II molecules (Bubenik, 1999). Cytokine therapy of MHC class I negative tumors has encountered some succe in mice, however, attempts to reverse MHC class I negative tumors completel or even partially have not been observed in immunotherapy trials. In order to

administer an effective therapy, a clear understanding of the MHC class I status of the tumor must be known beforehand and if cells do not present MHC class I molecules, can they be stimulated to produce IFN-γ. Perhaps to learn more about the MHC class I status of the tumors, a closer examination of the genes responsible for the induction of the molecules on the surface tumor cells may be studied. If the genes are not transcriptionally active, either due to genetic or epigenetic error, specific drug therapies could be developed to target the genes responsible for inducing MHC class I molecules. Recognizing the ability of DNA methyltransferase inhibitors to reverse aberrantly silent genes in tumors, Bubenik (2003) proposed therapeutic, epigenetic protocols used in other tumor models to upregulate MHC class I expression. For example, Primeau et al (2003) utilized the combination of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor, depsipeptide, and observed the reactivation of aberrantly silent genes maspin and gelsolin in human breast carcinoma cell lines. Soon thereafter, the first experiments analyzing the effect of these agents on MHC class I negative tumors resulted in the induction of MHC class I surface molecules after treatment with the combination of 5-azacytidine and trichostatin A (Bubenik, 2003). In addition to these promising results, histone deacetylase inhibitors in their own right have been shown to induce the expression of these surface molecules (Magner et al., 2000; Khan et al., 2004; Magner and Tomasi, 2005).

Epigenetics: mechanisms that influence gene silencing

The first experimental work performed utilizing inhibitors of methylation was done by Frantisek Šorm and his colleagues at the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences (Šorm et al., 1964). They synthesized and demonstrated that azapyrimidines 5-azacytidine and 6-azacytidine, inhibitors of nucleic acid biosynthesis, had significant antimetabolic and cancerostatic effects. Using *Escherichia coli* as a model, the

researchers discovered that 50 percent of the cells did not survive in media supplemented with 0.25 ug 5-azacytidine.This was a fantastic finding after comparing this inhibition to 6-azacytidine which exhibited a much weaker bacteriostatic effect that also acts as an agent against cancer. Additionally, analysis of 6-azauridine had a similar inhibitory affect in *E. coli*, albeit much weaker than both of the previously tested agents. To confirm that the effects of these agents occurred at the molecular level, uracil, uridine and cytidine was added to the culture resulting in the return of normal growth indicating that 5-azacytidine interfered with the biosynthesis of pyrimidines within the nucleic acid chain or with biopolymers. Unfortunately, 5-azacytidine was shown to be toxic to mammals through research with AK mice which are relatively resistant to chemotherapy. When these mice were exposed to this azapyrimidine agent, they exhibited lower levels of bone marrow and hypoplastic shrinking of their lymphatic systems, a primary symptom of toxic infection. However, these same mice suffering from lymphoid leukemia, who received lower dosages of this agent (still exhibiting some toxic side effects), lived considerably longer. More than 20 days later, 75 percent of the mice survived after only one treatment with 5-azacytidine (Šorm and Veselý, 1964). A dosage of 10 times less, given every other day over a five day period increased the number of survivors to 100 percent. This was exciting and promising results that began the intense research required to fully understand the effect of these agents. Šorm and his colleagues continued to analyze the effect of these agents in AKR leukemic mice (Veselý et al., 1967), on ribosome synthesis (Pačes et al., 1968), and additionally on mechanistic effect of 5-aza-2'deoxycytidine on *E.coli* (Doskočil and Šorm 1969). This early data supplied information that lead to the mechanism behind the action of 5-azacytidine (Jones and Taylor, 1980) which eventually was approved as a chemotherapeutic drug by the Federal Drug Administration of the United States. It is currently used in phase I/II trials at many institutes that specialize in the treatment of terminally ill cancer patients. Unbeknownst to these gentlemen,

their work would be instrumental in the development of the field of epigenomics and it role in cancer. Epigenetics is defined as heritable changes in gene expression that does not depend on changes in DNA sequences. It is hypothesized that the silencing of genes epigenetically occurs at least as often as mutations and deletions (Jones and Baylin, 2002; Herman and Baylin, 2003). The most notable studied epigenetic changes are DNA methylation and histone deacetylation both playing a significant role in the development of cancer.

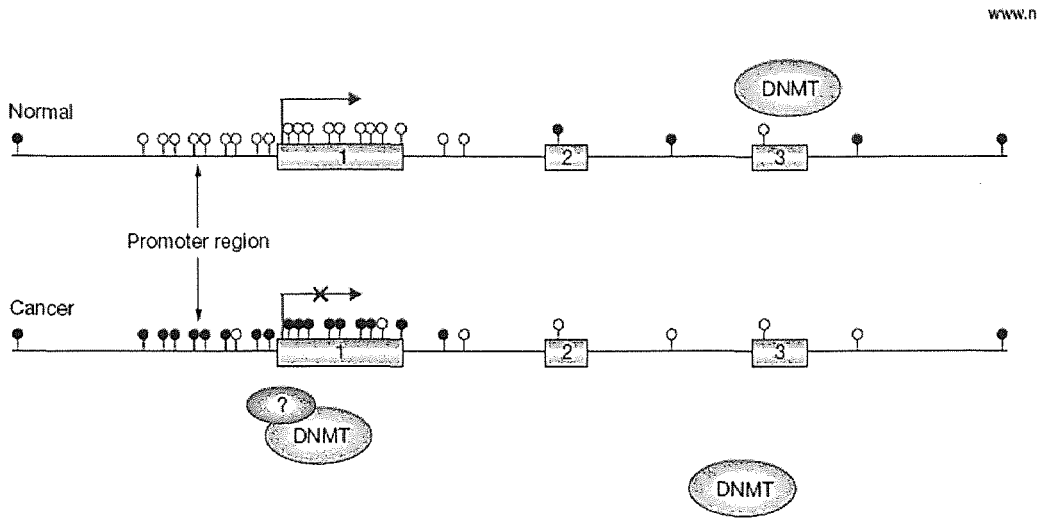

Fig. 2. DNA methylation in normal and cancer cells. The circles denote CpG dinucleotides, white-unmethylated and black-methylated. Exons within the gene are designated 1, 2 and 3. Dnmt act on hypermethylated promoter in cancerous gene. X depicts transcription repression (Adapted from Baylin, 2005).

In cancer development, initial studies of DNA methylation found that in comparing normal and neoplastic tissues, global loss of 5-methylcytosine was observed in cancerous tissue which suggested that methylation was a common feature of carcinogenesis (Lapeyre and Becker, 1979). The importance of demethylation of cytosines within specific genes provided data of mechanistic activation of gene expression (Feinberg and Vogelstein, 1983). On the other hand, it was observed that methylation was needed to maintain chromosomal integrity (Ehrlich, 2000). It has been shown that hypomethylation influences

tumor progression by destabilizing the genome via demethylation of transposons, pericentric repeats and inducing the expression of many genes including oncogenes (Nishigaki et al., 2005). It was realized as well that DNA methylation in cancer was not only affected by global hypomethylation but also gene specific methylation (Baylin, 2005). The observation of many genes that harbored an increase in DNA methylation particularly at the promoter region resulted in the silencing of gene expression and loss of protein function (Herman and Baylin, 2003). It has been hypothesized that epigenetic silencing is a mechanism that inactivates tumor suppressors such as hMLH1 mismatch repair gene (Jones and Laird, 1998). Other investigators have confirmed that DNA promoter methylation is instrumental in cancer progression and metastasis.

While mutations in oncogenes lead to a gain of function and mutations or deletions in tumor suppressors result in loss or inactivation of negative regulators, DNA methylation can be detrimental to the function of tumor suppressors. When the promoter regions of DNA are methylated, transcription factors are unable to bind sufficiently rendering the gene inactivated (Bird, 2002; Jones and Baylin 2002). This phenomenon is often found in tumor cells resulting in aberrant silencing of normal tumor suppressor function (Jones and Baylin 2002; Herman and Baylin 2003).

These promoter alterations are reversible with inhibitors of methylation 5-azacytidine and 5-aza-2'-deoxycytidine derepressing affected tumor suppressors and restoring normal promoter function. Therapeutic use of these agents has provided a new and effective option for patients with leukemias and related diseases.

DNA methylation occurs at the 5'carbon of the cytosine ring which is covalently modified by a methyl group resulting in 5-methylcytosine. The methyl group projects into the major groove of DNA inhibiting the binding of transcription factors. Approximately four percent of the genome consists of 5-methylcytosine usually paired with guanosine nucleotides commonly referred to as CpGs. CpGs

dinucleotides are rarely observed in the genome but, when found, they are located in short clusters called CpG islands within and around promoter regions of genes. In normal DNA, most CpG dinucleotides within the genome are heavily methylated, but CpG islands in germ-line tissue and promoters of somatic cells are free of methylation. DNA methylation is important in regards to maintaining the silent state of non-expressed or noncoding DNA such as condensed, transcriptionally inactive pericentric heterochromatin. This ensures that DNA of this region is late-replicating, suppressing the expression of viral sequences or transposons that may have integrated into these sites (Bestor, 2000; Bird, 2002; Jones and Baylin 2002). However, these some of these sites are unmethylated in euchromatin, genomic DNA that is accessible to transcription factors. In addition, in mammalian cells some sequences of DNA are methylated to maintain an inactive state such as CpG islands in the promoters of genes that are found on the inactivated X chromosome of females. Also, specific imprinted genes are methylated, silencing the expression of either the maternal of paternal allele. Methyl groups are added to the 5' carbon of the cytosine ring by the catalyst DNA methyltransferase.

In mammalian cells, three enzymes are involved in the additon of a methyl group to cytosine including *Dnmt1*, *Dnmt3a* and *Dnmt3b* (Okano et al., 1998 Okano et al., 1999; Bestor, 2000). Each has a unique role; Dnmt1, maintenance of established methylation patterns, *Dnmt3a* and *Dnmt3b* mediate the establishment of new or de novo DNA methylation patterns (Okano et al, 1998; Okano et al., 1999; Bestor, 2000). In cancer cells, the function of these enzymes may differ in the sense that *Dnmt1* interacts with *Dnmt3b* to maintain abnormal gene hypermethylation (Rhee et al., 2000; Rhee et al., 2002). The loss of *Dnmt1* is lethal to cells although embryonic stem cells (ES cells) continue to proliferate (Li et al., 1992). If the function of this gene is disturbed, the genome becomes hypomethylated, followed by the upregulation of genes that are normally unexpressed as was the case of several mesodermal markers in *Xenopus laevis*

embryos (Stancheva and Meehan, 2000). This resulted in embryonic death due to p53-induced apoptosis (Stancheva et al, 2001). Cultured fibroblast from conditional *Dnmt1* knockout mice also succumbed to the same fate (Jackson-Grusby et al, 2001). Additionally, reactivated placental and germ line markers were found in fibroblasts signifying that *Dnmt1* exhibited tissue-specific gene expression. *Dnmt1* has been shown to interact with the methyl binding protein (MBP) MeCP2 (Fuks et al, 2003) as well as with MBD2 and MBD3 to recruit histone methyltransferases to hemi-methylated DNA (Tatematsu et al., 2000) to repress gene expression. The role of MBP in DNA methylation and histone deacetylation will be discussed in greater detail later in this chapter.
Dnmt3a and *Dnmt3b* targeting has demonstrated that these methyltransferases are vital for early development in mice (Okano et al., 1999). Inactivation of these methyltransferases permitted ES cells to become methylated but did not affect the maintenance methylation of imprinting loci. Both genes seem to overlap in expression. However, differences in their functions have been noted. Phenotypically, *Dnmt3a* knockout mice show no physical abnormalities and survive up to 4 weeks after birth. In sharp contrast, *Dnmt3b* knockouts die before birth. *Dnmt3a*, *Dnmt3b* double knockout mice exhibit developmental defects at and die just before gastrulation. Investigators examined the CpG islands of the X-linked homeobox genes *Rhox6* and *Rhox9* which are expressed in the trophectoderm of embryos and discovered that both methyltransferases were important however, the established methylation pattern of *Dnmt3b* was more dense than *Dnmt3a* (Oda et al, 2006). Therefore, the data suggests that some of the functions of the genes are shared. Data produced by Xu et al (2006) provided further data of the role of *Dnmt3b* by demonstrating that the mutation of *Dnmt3b* but not *Dnmt3a* lead to a recessive autosomal disorder called immunodeficiency, centomeric instability, and facial abnormalities (ICF) syndromes. The disease is characterized by decreased serum immunoglobin levels that result a number of infectious diseases in children before they reach

adulthood. In ICF, DNA hypomethylation via a mutation in *Dnmt3B* is found chromosome one, nine and sixteen leads to the elongation of juxtacentromeric heterochromatin and the formation of complex multiradiate chromosomes (Xu et al., 1999) is the observable genotype of this disease.

Recent research has examined the role of a fourth DNA methyltransferase, Dnmt3L. Dnmt3L has been found to work in unison with both Dnmt3a and Dnmt3b (Hata et al, 2002), establishes areas of maternal imprinting, has a zinc ginger-like PHD domain that recruits histone deacetylases and may play a role in the repression of newly configured imprinted regions (Aapola et al., 2002). In heterochromatin, DNA methyltransferases may recruit histone deacetylases (HDAC) and other chromatin binding proteins to the promoter to regulate gene silencing (Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000; Fuks et al., 2001; Bachman et al., 2001). Additionally, MBP that attach to the methylated CpG island are required to render the gene transcriptionally silent. Currently five MBP (MBD1, MBD 2, MBD3, MBD4 and MeCP2) all which have similar methyl binding domains, combine with different co-repressor complexes to regulate specific sets of genes according to various developmental and environment conditions. MBP have been extensively studied and their binding domains have been shown to interact with other proteins allowing them to perform various functions. One example is the protein Sin3-HDAC, a co-repressor to DNA, is recruited and bound by MeCp2, the first MBP discovered (Jones et al., 1998). Mutations in *MeCP2* have been linked to Rett syndrome, an X-linked neurodevelopment disorder that primarily affects girls who present severe autistic-like features i.e. small head, hands, feet, nonvocal and nonambulatory (Amir et al., 1999). *MBD1* is a transcriptional repressor that is able to bind methylated as well as unmethylated DNA (Fujita et al., 2000). It functions in unison with the histone methyltransferases Suvh391 (Fujita et al., 2003) and SetDB1 (Sarraf and Stancheva, 2004) which methylate Lysine 9 of histone H3 intensifying transcriptional repression of methylated DNA. *MBD1*

can be negatively regulated by the small ubiquitin-related modifier (SUMO) ligases (attach to proteins, large protein complexes and subcellular structures at lysine residues that lead to the negative regulation of transcription) PIAS1 and PIAS3 rendering MBD1protein incapable of binding SetDB1. This appears to be a mechanism that determines the extent of methylation required at a MDB1 bound sequence. MBD2 has been shown to associate with HDAC1 and HDAC2 in transcriptional repression and can be deterred by histone deacetylase inhibitors in the Mi-NuRD chromatin remodeling complex (Zhang et al., 1999). MBD2 also affects helper T cell differentiation (Hutchins et al., 2002) rendering *Mbd2*(-/-) mice that normally require the activator, GATA3 for IL-4 induction, to express IL-4 in differentiated helper T cells without the assistance of GATA3. Sequence homology of MBD3, a component of the Mi-2NuRD complex is similar to that of MBD2 but, is unable to bind methylated DNA (Saito and Ishikawa, 2002; Zhang et al., 1999). Although MBD2 and MBD3 associate within the chromatin remodeling complex, they exhibit very different phenotypes in mice during early development. *MBD2* null mice appeared to be healthy with the exception of the inability or incapability to nurse their young. *MBD3* knockout embryos possess severe defects and die soon after birth (Kaji et al., 2006; Kaji et al., 2007). In addition, it was observed by Kaji and associates (2006, 2007) that ES cells were unable to differentiate in MBD3 embryos and markers of undifferentiated ES cells including *Oct4*, *Rex1* and *Nanog* were highly expressed. MBD4 is a thymine glycosylase that targets and repairs cytosine deamination i.e. C to T nucleotide change (Hendrich et al., 1999). There are relatively few CpG dinucleotides in methylated genomes because of the spontaneous hydrolytic deamination of methylated cytosines. 5-methylcytosines are responsible for the CpG to TpG transition (Bird, 1980). MBD4 also acts on nonmethylated genomes changing the CpG dinucleotide to UpG (Hendrich et al., 1999). As a transcriptional repressor, MBD4 has been shown to act with the same intensity as MeCP2 and MBD2 (Kondo et al., 2005)

in combination with Sin3 and HDAC1. It is important to remember that while methylation influences the expression of genes, it does not act alone to repress transcription. Chromatin structure also plays a role in the accessibility of transcription factors to the gene promoters.

The state of chromatin plays an important role in epigenetic gene regulation. There are numerous covalent modifications of histone tail residues and within the histone itself that are linked to changes in higher-order chromatin. These modifications include phosphorylation, ubiquitination, sumoylation, acetylation and methylation. The histones possess marks that are recognized by non-histone proteins that affect chromatin structure and gene expression. In eukaryotes, the genome is linked together by nucleosomes which are comprised of 146 base

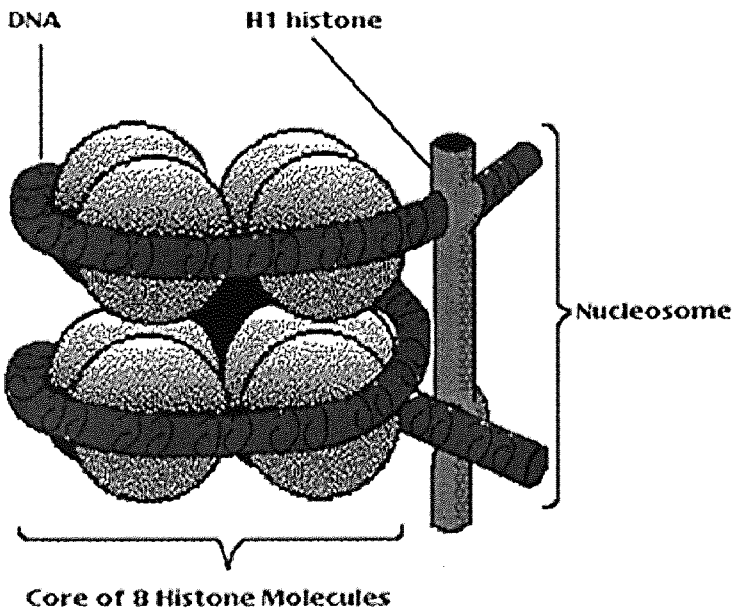

Fig. 3. A representative eukaryote nucleosome comprised of a histone octamer; two H3, H4 tetramers and two H2A, H2B histone dimers, In red, 146 base pairs of DNA wrapped around the octet. Approximately 200 bp of linker H1 histone (green) DNA separates each nucleosome is located externally.

pairs of DNA wrapped around a histone octamer (two H3, H4 tetramers and two H2A, H2B dimers). There are approximately 200 bp of linker DNA (H1) that separates each nucleosome facilitates the packing of the genome into the nucleus as well as inhibits access of certain

regulatory proteins to DNA. When genes are active, the amount of nucleosomes have been shown to be inversely proportional to transcription initiation rate at the promoter (Lee et al., 2004) which agrees with earlier studies of increased nuclease hypersensitivity and loss of histone-DNA contacts during activation of genes within the promoter.

Chromatin is constantly active as opposed to a static state. Jenuwin and Allis (2001) proposed the idea of the histone code from accumulated histone methylation data that suggested that various post-transcriptional modifications influence the binding of proteins at certain marks within chromatin. Regulation of the accessibility of transcription factors, co-factors and enzymes involved in transcription influence or changes chromatin structure, as well as, gene expression. However, some data has shown that an increasing number of potential residue targets are being discovered signifying that a histone code may be more complex. Data has been presented demonstrating that the overall charge on histone tails, which does not depend on the position of a covalent modification, may be a key element in gene expression (Zheng and Hayes, 2003). Therefore, it appears that both global effects and a position-dependent histone code at specific residues are involved in gene expression. Researchers are now attempting to determine what are the exact positions and combinations that are important in gene expression.

All of the epigenetic marks currently known on histones are reversible as are the enzymes that remove these marks. The enzymes counteract each other maintaining a balanced state between heterochromatin and euchromatin. The enzymes include seven histone acetyltransferases (HAT) that acetylate histones and those involved in the removal of acetyl groups, histone deacetylases (HDAC). The HAT enzyme, CREB binding protein p300 (CBP/p300), has broad specificity affecting the acetylation of histones (Strahl and Allis, 2000) and non-histone proteins (Gu and Roeder, 1997). HDAC consists of three classes: class I HDAC (HDAC1, 2, 3 and 8) (Bjerling et al, 2002) which are

expressed in most cell types; class II (HDAC 5, 6, 7, 9 and 10) (Fischle et al, 2002) exhibit a more restricted expression and the family HDAC11 (Gao et al, 2002) which are related to class I but with low sequence homology. Currently, seventeen histone methyltransferases (HMT) and only one demethylase, LSD-1, have been discovered. Of the HMT, nine methylate histone lysine residues and the remainder methylated arginines. All HMT have a highly conserved evolutionary domain such as Su(var) 3-9, enhancer of zeste (E(z)) or trithorax (trx) all named from work with Drosophila melanogaster. These domains are targeted by SET-domain-containing enzymes (such as SETDB1) that catalyze the methylation of specific lysines on histone H3 and H4. For example, enzymes SET1 and SET2 are known to methylate Lys4 and Lys36 of histone H3 respectively with H3K4 methylation noted as the primary mark of actively transcribed genes (Shilatifard, 2006). HMT Set 9 seems to favor lysine H3 but, also methylates a lysine residue in p53, stabilizing the protein which affects non-histone proteins. Each histone methyltransferase has the ability to regulate different genes and cellular processes even though different HMT are capable of sharing the same substrate. Lysine sites can be methylated in one of three states: mono, di or trimethylation all with different activities related to their stability. The most stable of the three is trimethylation. The residues currently linked to gene repression are H3K9me, H3K27me3 and H4K20me3 and known activating marks are H3K4me3, H3K36me3 and H3K79me3. H3K9, H3K14 and H4K16 acetylation have also been shown to be associated with activation. Of note, H4K16 is a prominent residue acetylation site that relaxes condensed chromatin and facilitates the binding of transcription factors resulting in gene expression (Shogren-Knaak et al, 2006). Histone arginine modifications were believed to occur less frequently that lysine, however, it was determined that they could be epigenetically altered by protein arginine methyltransferases (PRMT) (Bedford and Richard, 2005). These arginine altering enzymes have been described in cell signaling, DNA repair, apoptosis and transcriptional regulation. PRMT are also

recruited to non-histone proteins as arginine methylation induces increased HAT activity (Chevillard-Briet et al., 2002). Little is known regarding PRMT in immune gene regulation but, *PRMT4* or *CARM1* has been shown to be connected to MHC class II gene expression that is induced by IFN-γ. Tzortzakaki et al. (2003) provided evidence that PRMT4 joins the complex of MHC class II transactivator (CIITA) and cofactor steroid co-receptor (SRC) resulting in the activation of the CIITA promoter.

Along with methylation and acetylation, histone phosphorylation of certain residues leads to increased transcription and gene activation within chromatin. Kinase pathways which are induced by environmental stimuli are linked to chromatin formation providing a link to transcription and gene regulation during cell division at histone H3. Histone H3 of mammalian cells is phosphorylated as a result of signals that activate the mitogen activated protein kinases (MAPK) and the stress activated protein kinases (SAPK). The end result is the activation of MSK1 and MSK2 kinase that in turn phosphorylate serine 10 and 28 on histone H3 subsequently inducing active chromatin (Soloaga et al., 2003). It has been demonstrated that H3S10 phosphorylation is quickly followed by an increase in acetylation of H3S10 and H3S14 suggesting a link between other histone modifications (Cheung et al., 2000). Therefore, phosphorylation may assist in gene activation by stimulating HAT enzymes to the same histone residue. Inflammation induces H3S10 and H3K14 phosphoacetylation and activates the MAPK pathway increasing the transcription of cytokines including IL-6, IL-8, IL-12 and the macrophage chemoattract protein MCP1. Since inflammation can act as an adjuvant in immunity, the aforementioned cytokines, NF-κB and histone phosphorylation together play a vital role in sequestering NF-κB to gene targets that participate in inflammation. Histones that have H3S10 phosphorylation seem to be particularly sensitive to acetylation when the histone deacetylase inhibitor, trichostatin A (Clayton and Mahadevan, 2003) is used providing further evidence of cooperation of histone modifications.

Stressed tumors and tumors with activated Ras oncogenes may carry H3S10/28 phosphorylation and would be key candidates for treatment with histone deacetylase agents. The transcription of intercellular adhesion molecule ICAM1 (CD54) which is responsible for controlling the interactions between leukocytes and endothelial cells during the inflammatory response is greatly enhanced when CBP/p300 is phosporylated (Huang and Chen, 2005). CBP/p300 is a phosphoprotein that has a conserved motif recognized by agammaglobulinemia tyrosine kinase or AKT. Specifically, p300 protein is phosphorylated by AKT kinase which signals histone acetyltransferase activity thereby increasing the transcription of *ICAM1*. Clinicians have found this data interesting because it shows the potential effect of histone deacetylase inhibitors on kinase pathways. Increased transcription through phosphorylation is a well documented event that demonstrates how various proteins interact with accessible chromatin resulting in gene activation.

The ubiquitination of specific histone residues plays an important role in transcription, initiation and elongation, gene silencing and DNA repair. This phenomenon appears to help maintain chromatin in a euchromatin configuration. Ubiquitin is a 76 amino acid protein that is attached to a substrate by three independent enzymes including ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and the ubiquitin-protein isopeptide ligase (E3). ATP is required to activate the joining of E1 and ubiquitin which is then followed by the E2, resulting in the formation of a thioesterbond to a cysteine residue. E3 transfers ubiquitin from E2 to a target lysine residue of a particular substrate protein. The substrate can be mono-ubiquitinated or polyubiquinated with mono marking the substrate protein to participate in a certain function, whereas, polyubiquitinated substrates target proteins for degradation via the 26S proteasome. Mono-ubiquitination of H2A and H2B is reversible by ubiquitin-specific proteases (Nijman et al., 2005). *Rad6*, discovered in yeast, plays a role in the mono-ubiquitination of H2B when linked to the E2 enzyme resulting in

transcription. However, ubiquitination of H2A and H2B is complex and does not always lead to transcription. Histones found within active immunoglobin gamma chain genes are not ubiquitinated and inactive micronuclei of Tetrahymena and mouse spermatid sexbody contain ubiquitinated histones (Zhang, 2003). Therefore, it depends on the location of the gene and possibly on other histone modifications. A phenomenon called histone crosstalk has been attributed to H2B because mono-ubiquitination is required to signal histone methylation by enzymes. Mono-ubiquitination of H2A/H2B is prevalent at transcriptionally active loci and appears to be involved in the regulation of transcription of a specific set of RNA polymerase II-transcribed genes.

Similar to ubiquitination, sumoylation involves the addition of a small ubiquitin-related modifier (SUMO) to various proteins that are active in many cellular processes including the formation of higher-order chromatin. All four histones have been shown to be modified by sumoylation at specific sites on H2A, H2B and H4 (Nathan et al., 2006). As previously noted, small polypeptides attach to proteins at lysine residues leading to the negative regulation of transcription. The activation of the SUMO polypeptide begins in an ATP dependent manner by the E1 activating enzyme. This step is followed by the joining of E1 and the conjugating enzyme E2 and subsequently binds to the ε-amino group of a target lysine within the protein of interest (Melchior, 2000; Mueller et al., 2001). In vivo, all though it is not absolutely clear, there appears to be a specific SUMO E3 ligase that is necessary for targeting of the protein that is to be modified. There are three possible mechanisms that can occur in the case of SUMO; 1) Sumoylation of a transcription factor interferes with DNA binding affecting attachment to or detachment from the promoter. SUMO carries a negative charge which would allow it to inhibit the binding of some transcription factors. However, there are cases where SUMO facilitates promoter binding as in the case of the stress-induced transcription factor heat shock factor 1 (HSF) that regulates genes involved in heat shock (Hong et al., 2001); 2) Positive or

negative interaction with proteins subsequently activate or repress gene expression; 3) SUMO may directly affect the function of enzymes that are involved in gene expression. Although the mechanisms are unclear regarding exactly how SUMO modifies and regulates the catalytic abilities of these enzymes, it has become important in the realm of epigenetics. For example, the DNA methyltransferase 3a can be sumoylated resulting in its inability to bind HDAC1 and HDAC2 leading to transcription (Ling et al., 2004; David et al., 2002; Kirsh et al., 2002). In addition, the histone acetyltransferase GCN-5, a positive regulator of transcription (Sterner et al., 2006) and the histone methyltransferase Clr4 (Shin et al., 2005) are all known to be modified by SUMO.

As the biochemical studies regarding DNA methylation and histone modification continue to illustrate a picture of the changes within chromatin and its relationship to gene repression, researchers in other disciplines, such as immunology, have begun to analyze the role of epigenetic mechanisms within their system.

Epigenetics and Immunity

Epigenetic mechanisms have been shown to play a vital role in cells of the immune system. Antigen processing machinery genes that are linked to the induction of MHC class I molecules have been closely examined for genetic or epigenetic abnormalities. Researchers have found substantial evidence of the role of epigenetics in B cell development, differentiation and in the synthesis of antibodies i.e. VDJ recombination and isotype switching although antibody responses after treatment with epigenetic agents have not be analyzed. Over the last fifteen years there has been a cascade of data supporting the role of epigenetic processes which lead to the silencing of key regulatory genes. Epigenetic mechanisms have been shown to influence the expression MHC class I, MHC class II, costimulatory, adhesion molecules and tumor-rejecting antigens

as well as the stimulation of cytokines on tumor cells (Reitz et al., 1984; Magner et al., 2000; Maeda et al., 2000; Nie et al., 2002; Chou et al., 2005).

In cancer immunity, studies with epigenetic agents have demonstrated a remarkable ability to reverse downregulated immune genes. Downmodulation of MHC class I expression due to hypermethylation of MHC class I genes has been documented in melanoma cells and the combination of 5-azaC and TSA have promoted the reexpression of HLA class I antigens and subsequent restoration of the antigen-specific immune response after demethylation (Serrano et al., 2001). Weber et al. (1994) observed the demethylation of MAGE-1 melanoma cell lines with the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, followed by the upregulation of MAGE-1 tumor antigen.

Tumor cell lines that do not present peptides on their surfaces via MHC class I molecules have been upmodulated with DNA methyltransferease inhibitors, histone deacetylase inhibitors or with a combination of these agents resulting in the upregulation of the expression of antigen processing machinery genes (Nie et al., 2001; Bubenik, 2003; Sigalotti et al., 2004). In addition, HDACi have been shown to upregulate MHC class I expression on several tumor cell lines including melanoma B16/BL6, (Komastu et al., 1998) human neuroblastoma SK-N-MC and mouse adenocarcinoma Colon 26-L5 (Reitz et al., 1984). Hence, the reversal of the immune escape phenotype of tumor cells represents one of the possible modalities by which epigenetic agents can inhibit growth of the tumor (Tomasi et al., 2006).

Seliger and colleagues (2008) recently set out to determine the differential regulatory mechanisms of heterogeneous antigen processing machinery component expression and found that the promoters of the APM genes transfected into tumor cell lines were active suggesting transcriptional and/or post-transcriptional regulation. The observed activity of the differential promoter demonstrated that the decreased or lack of antigen processing gene expression, RNA and/or protein expression was linked to a nonfunctional

promoter. Analysis of the genes involved in antigen processing have shown that there are few sequence errors (relatively uncommon in the TAP1 subunit) in small cell lung carcinoma, melanoma and cervical carcinoma (Chen et al., 1996; Seliger et al., 2001; Seliger et al., 2006). This suggests that these genes are regulated epigenetically, transcriptionally and/or post-transcriptionally. These results indicated that epigenetic mechanisms can substantially contribute to the loss of induction of surface MHC class I molecules (Reitz et al., 1984; Magner et al., 2000).

Recently, HDACi have been shown to a perform a similar function to IL-12, a cytokine that stimulates naïve $CD8^+$ cells recruited during the immune response to antigen or co-stimulation to differentiate, leading to the conclusion that cytokines promote the formation of euchromatin and increase histone acetylation (Agarwal et al., 2009). However, recent studies have indicated that in cytokine-inducible gene regulation, protein deacetylation is an important mechanism that leads to transcription and gene expression (Sakamoto et al., 2004; Nusinzon and Horvath, 2003; Chang et al., 2004). Therefore, protein acetylation appears to be just as important as protein deacetylation complicating the current understanding of the action of HDACi.

Human papilloma virus

Human papilloma virus (HPV) is a double stranded DNA virus that enters and replicates in the nucleus of differentiated kertinocytes, cells that form approximately 95 percent of the epidermis, inducing warts and papillomas. While some types are manifested as warts there are some that lead to various types of cancer including anal, vulvar, penile and cervical (Parkin, 2006). In order to replicate, the virus depends on host cell transcription machinery to transcribe its genome and process the resulting RNA into mRNA. Before its random integration into the host cell genome, the viral DNA is extrachromosomal in premalignant cervical dysplastic lesions (Howley, 1991)

Cervical neoplasms are mostly attributed to HPV infection mainly due to their "high risk" oncogenic types (zur Hausen, 2002). There have been more than 100 HPV genotypes identified, 35 of which have been found in the genital tract. Eleven of those have been classified as "high risk" including types 16, 18, 31 and 45. In approximately 60 percent of human squamous cervical carcinomas (CC), the most prevalent human papilloma virus is type 16 (zur Hansen, 1996). The double-stranded viral genome is spliced at the *E1/E2* region specifically at the *E2* section (Baker et al, 1987). The *E2* open reading frame encodes a transcriptional regulatory DNA binding protein that is rendered nonfunctional. Much of the understanding of the function of E2 came from studies with bovine papiloma virus which described the positive and negative transcriptional regulatory functions of *E2* (McBride et al, 1989). In HPV 16 and 18, E2 acts as a transcriptional repressor of *E6* and *E7* promoters (Baker et al, 1987, Smotkin and Wettstein, 1986). Therefore, *E6* and *E7* oncogenes exhibit unchecked expression in human cervical carcinomas due to the disrupted *E2* region of the viral genome.

Early viral antigens of the high risk HPV types, *E6* and *E7* oncoproteins, are necessary for the maintenance of the malignant phenotype and are expressed in all tumor cells. In normal cells, during DNA damage, the master regulater p53 inhibits cell proliferation by interfering with the Bax domain, found within the inner mitochondrial membrane, which signals the anti-apoptotic BCL-2 receptor. Also, *p21* which encodes for a cyclin-dependent kinase inhibitor, is upregulated by the tumor suppresor *p53* and inhibits the Cyclin D/Cdk4 complex. Essentially, the cell cycle remains at G1 blocking the initiation of the transcription factor E2F until the damage is repaired. However, the viral oncoprotein E6 binds and degrades p53. In turn, *p21* expression is not upregulated and cyclin-dependent kinase complex is not inhibited. E7 binds the tumor suppressor retinoblastoma (RB) a protein that prevents over growth of cells. Normally, RB, activated by dephosphorylation, binds and inactivates the transcription factor E2F1 which is

required for cell cycle progression. A number of experimental *E6/E7* targeting immunotherapeutic protocols designed to elicit a specific cytotoxic T lymphocyte mediated response are currently under development (Stanley, 2006). However, the efficiency of these protocols against E6/E7 expressing tumors with downmodulated MHC class I expression is questionable. Therefore, the mechanisms of MHC class I upregulation on HPV-associated tumors are of particular interest. Because promoter hypermethylation of multiple genes in carcinoma of the uterine cervix has been described (Dong et al., 2001; Sova et al., 2006), the possible modulation of MHC class I gene expression by demethylation is of relevance and has a therapeutic potential in cervical carcinoma (Bubenik 2003).

At the moment, vaccination is the best method for controlling HPV-related disease (Barr and Sings, 2008). A quadrivalent prophylactic vaccine from Merck called Gardisil (Merck & Co. Inc. Whitehouse station, N.J., USA) contains virus-like particle (VLP) viral capsid proteins L1 of HPV types 6, 11, 16 and 18 has become the gold standard for prevention. HPV VLP and chimeric VLPs are immunogens that induce strong antiviral B cell and T cell responses. These proteins bind to human and mouse immune APC cells i.e. dendritic cells, macrophages and B cells that display markers such as MHC class II, CD80 and CD86. Dendritic cells are potent inducers of the immune response with an important role in VLP-induced immunity. Investigators have shown that these cells were activated by chimeric HPV VLPs and induced epitope-specific T-cell responses in vitro (Lenz et al., 2001).This drug was first licensed in the United States in June 2006 and Europe in September 2006.The efficacy of the drug thus far has been impressive. Follow-up data has shown Gardisil to be effective up to 100 percent in preventing cervical cancer, precancerous lesions and external genital lesions. Another HPV vaccine, Ceravix (GlaxoSmithKline Biologicals, Rixensart, Belgium), consists of VLP L1 proteins from HPV 16 and 18 has also done well. Although there is a lot of praise regarding the performance of these

drugs, there are still questions related to efficacy. Primarily, it is not known the length of time the VLP-induced treatment persists, nor is known if the treatment of men with these drugs deters virus transfer. Most noteworthy, data has shown that women who already have the virus and receive these drugs are not adversely affected by the drug but, there appears to be no observable effect of the drug.

The success of these drugs has been an excellent therapeutic tool for the preventing the onset of precancerous and cancerous lesions. As the data of women and men who are treated with these drugs and other agents continues to be collected from future trials new and improved therapies can be developed. The experiments in the laboratory will be instrumental in determining the mechanisms responsible for HPV-related carcinogenesis.

Epigenetic therapy: From bench to bedside

The data supporting therapy with epigenetic drugs *in vitro* has continued to grow and has been used to establish the dosage parameters in Phase I/II trials. Since methylation of CpG islands is rarely observed in the promoter region of normal cells, methylation provides a tumor-specific therapeutic target. *In vitro*, hypermethylated promoters have been shown to be reversed by the sequential application of histone deacetylase inhibitors followed by DNA methyltransferase inhibitors resulting in reexpression of the promoter (Cameron et al, 2001). In myeloid leukemias, this synergistic cocktail could potentially increase response rate, the length of response or the percentage of complete responses. The key cell cycle regulating gene *p15INK4B* has been shown to be silenced by DNA methylation and reexpressed after treatment with epigenetic drugs (Corn et al., 2000; Herman et al., 1997; Herman et al., 1996; Quesnel et al., 1998). Additionally, patients suffering from myelodysplastic syndromes (MDS), those that affect bone marrow stem cells and characterized by peripheral cytopenias and dysplasia of hematopoietic progenitor cells (Au et al., 2002;

Kurzrock, 2002), have responded favorably to these agents. Patients have responded well to 5-azacytidine and 5-aza-2'-deoxycytidine suggesting that epigenetic silencing or cell regulatory genes may play a role in their pathophysiology. Both demethylation agents impede the progression of MDS to acute myeloid leukemia (Silverman et al., 2002; Saba and Wijerman, 2005). Hematological response rates ranging from 30 to 60 percent have been observed in phase I and II clinical trials (Wijermans et al., 2000; Wijermans et al., 1997; Issa et al., 2004). Demethylation is dose dependent when first administered to patients but, quickly peaks and levels off. If this level is surpassed, the dose actually inhibits the differentiation effect (Jones and Taylor, 1980). Pharmokinetics also play a role in that these agents have a short serum half-life but, it is unclear what the half-life is after the drug has been incorporated into DNA. Since the response to these chemodrugs is different than chemotherapy, clinicians must determine the optimum dosage after each clinical trial.

Our patient study set out to determine if treatment with a DNA methyltransferase inhibitor followed by a histone deacetylase inhibitor demethylates the promoter of *p15INK4* increases the acetylation of histones and if this combination is tolerated by patients with myeloid neoplasms. Histone deacetylase inhibitors have been instrumental in inhibiting histone deacetylation in human tumor cells leading to acetylated histone proteins (Marks et al., 2000; Mei et al., 2004; Drummond et al., 2005). Leukemic cells treated with HDACi have displayed growth arrest, differentiation or apoptosis. Hence, these drugs may be employed in cancer therapy in combination with methyltransferase inhibitors. Currently, there are several promising histone deacetylase inhibitors used in the clinic for treatment of hematopoietic malignancies including the natural inhibitor, trichostatin A, hydroxamic acid derivatives such as cyclic tetrapeptide or depsipeptide, benzamide derivatives MS-275 and CI-994 and aliphatic acids valproic acid and phenylbutyrate. It is generally accepted that methylation is dominant over histone acetylation since transcription cannot

occur while the promoter is methylated. By first treating with a DNA methyltransferase inhibitor followed by a histone deacetylase inhibitor, reexpression of transciptionally silent genes can occur. The histone deacetylase inhibitor phenylbutyrate, a short chain fatty acid which has been successfully used to induce fetal hemoglobin in patients stricken with sickle cell anemia and β-thalassemia (Dover et al., 1992; Collins et al., 1995) has shown promising results in combination with the demethylating agent 5-azacytidine (Gore et al., 2006).

Myelodysplasia is noted by a decrease in blood stem cell production in the human body. In healthy individuals, myeloid progenitor cells develop into erythrocytes, infection fighting leukocytes and blood clotting megakaryocytes (platelets). Myeloid cells play a vital role in both innate and adaptive immunity developing into macrophages, dendritic cells or granulocytes. In diseased individuals, stem cells do not fully develop and die in the bone marrow or immediately after entering the blood stream. A decrease in the number of these vital cells results in increased infections and/or anemia due to the lack of APC cells. Individuals who develop this disease are usually male between the ages of 65 and 75. Exposure to chemicals (benzene, cigarette smoking) and chemotherapy (drug and radiation therapy) treatment have been linked to myelodysplasia. Nearly a third of patients develop acute myeloid leukemia after treatment with anti-cancer agents. Patient symptoms vary from asymptomatic to anemia. Approximately 50 percent of patients succumb to infection or excessive bleeding. Treatment is difficult in individuals who develop acute myeloid leukemia since they do not respond well to conventional chemotherapy.

Being that the deficiency of surface MHC class I on tumors induced by HPVs are frequently caused by antigen processing machinery defects, (Keating et al., 1995) this work set out to determine whether epigenetic modifications can induce surface MHC class I expression through the silencing or activation of antigen processing genes. In addition, we attempted to study the epigenetic

therapy potential of a DNA methyltransferase inhibitor followed by a histone deacetylase inhibitor in individuals suffering from myleodysplasia or AML. The goal: to demethylate the *p15INK4B* promoter and increase the acetylation of histones associated with the promoter as well as monitor patient tolerance to the drugs.

General Aims

Using an immunogenic cell line expressing HPV E6/E7 antigens with MHC class I downmodulation due to defects in antigen processing machinery, we analyzed the impact of the HDACi trichostatin A, sodium butyrate and DNMT 5-azacytidine and 5-aza-2'deoxycytidine on the surface expression of MHC class I molecules and, moreover on the activation of antigen processing machinery genes.

To answer the question of the effect of these epigenetic agents in humans, we attempted to determine via a clinical phase I trial patient tolerance of a sequential treatment of a DNA methyltransferase inhibitor followed by an histone deacetylase inhibitor, monitor reversal of promoter methylation and determine if the deacetylase inhibitor was responsible for histone acetylation i patients afflicted with myelodysplasia.

Specific Aims

To determine the effect of epigenetic agents on the induction of MHC class I molecules on the surface of tumor cells.

To determine if the expression of downregulated antigen processing machiner genes is increased after treatment with epigenetic agents.

To demonstrate that tumor cells treated with epigenetic agents are recognized l cytotoxic T lymphocytes.

To demonstrate that epigenetic drugs target the cell cycle regulator *p15INK4B* often methylated in leukemia, increase histone acetylation status and that the drugs are tolerated by MDS patients.

Materials and Methods

Cell lines and Animals

TC-1 is a malignant, immunogenic cell line expressing HPV16 E6/E7 antigens of C57BL/6 mice origin. TC-1A9 cell line represents a TC-1 MHC class I-deficient derivative. Both cell lines express E6/E7 and exhibit the phenotype MHC class I⁻/MHC class II⁻. (Lin et al, 1996; Šmahel et al, 2003)

Two month old C57BL/6 mice purchased from Anlab in the Czech Republic, were used for these experiments.

Cells were cultured in fresh media for 24 hours after which the media was removed and the cells were grown in one of three RPMI/drug groups: 50 μM 5-azaC and 10 ng/ml TSA, 50 μM 5-azaC and 10 ng/ml TSA (all chemicals purchased from Sigma, St Louis). The medium was replaced with fresh medium after 48 hours and the cells were cultured for an additional 24 hours in drug-free media for a total of 72 hours and harvested.

Patient samples

Samples were obtained from patients who exhibited either MDS or AML. The French-American-British categorization was used to classify the patients. When appropriate, the WHO category of refractory cytopenias with multilineage dysplasia (RCMD) was applied. AML arising from MDS is called either AML with trilineage dysplasia (AML-TLD) or AML-MDS. Patients with MDS age 18 years were eligible if they had refractory anemia with excess blasts or chronic myelomonocytic leukemia (CMMoL). All patients signed Institutional Review Board–approved informed consent according to Department of Health and Human Services guidelines; additional specific informed consent was obtained for the laboratory research use of patient specimens.

Flow cytometry.

Cell surface MHC class I expression on the cell lines was determined by two-step cytoflourometric analysis using anti-mouse H-2Kb/H-2Db monoclonal antibody (clone 28-8-6) and FITC labeled goat anti mouse IgG secondary antibody or by one step labeling using PE anti- H-2Db (KH95) and PE anti- H-2Kb (AF-88.5) antibodies. The following antibodies were used for detection of CD86 and MHC class II molecules: PE anti-CD86 (GL1), FITC anti-I-A^b) and (AF6-120.1). All antibodies used, including the mouse IgG2a isotope-matching control, were obtained from Pharmingen (San Diego, CA, USA). The cells were initially pre-incubated with anti-CD16/CD32 to deter non-specific binding. Analysis of 10,000 cells was done with a FACSCAN ELITE cytometer (Coulter, Miami, FL, USA). Proportions of alive, dead and apoptotic cells were determined with propidium iodide and annexin V-FITC apoptosis detection kit (Sigma, St Louis, Mo, USA).

Reverse transcription PCR and real-time quantitative RT-PCR.

Total RNA was extracted with High Pure RNA isolation kit (Roche, Basel, Switzerland). 200 ng of RNA was reverse transcribed to cDNA using random hexamer primers from GeneAmp RNA PCR Core Kit (Applied biosystems Foster city CA, USA) in a 20 µl reaction volume at 42°C for 30 minutes. PCR analysis was done using Amplitaq polymerase (Applied biosystems Foster city CA, USA). cDNA was amplified under the following conditions: 95°C for 2 min; 25 cycles of denaturation at 95°C for 30 sec, annealing at 60oC for 1 min, elongation at 72°C for 1 min and incubation at 72°C for 5 minutes. Quantitation of PCR products was performed in 25 µl of SYBR Green Super mix (Bio-Rad, Hercules, CA, USA) using an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). DNA was denatured at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, elongation at 72°C for 1 min and incubation at 72°C for 5 minutes. Fold changes in transcript levels were

calculated using CT values standardized to β-actin, used as the endogenous control (reference gene), in order to normalize the quantitation of mRNA. All samples were run in triplicates. For statistical analysis the Student test was used. Samples exhibiting $P< 0.05$ were considered to be statistically significant.

^{51}Cr microcytotoxicity assay.

Mice (three per group) were immunized with irradiated (150 Gy) TC-1 or TC-1/A9 cells either untreated or treated with 10 ng/ml TSA. In all immunization protocols, the dosage was 10^7 cells per mouse, and the mice were immunized twice in three week intervals as previously described. Eight days after the second immunization, spleen cells from immunized and control mice were extracted and used as effector cells. After lysis of erythrocytes with Tris-NH_4Cl buffer, the effector cells were mixed with the ^{51}Cr-labelled tumor target cells (TC-1 or TC-1/A9 cells; either TSA-treated or untreated) and incubated in four different target-to-effector cell ratios 1:25, 1:50, 1:100 and 1:200 for 18 h in triplicate in 96-well round bottom microtiter plates. The media was also enriched with 10^{-5} M mercaptoethanol. To assess the role of CTL, $CD8^+$ were depleted with the monoclonal antibody 2.43 and complement prior to the mixing of effector cells with targets (Baby Rabbit Complement from Cederlane, Hornby, Ontario, Canada). The percentage of specific ^{51}Cr release was expressed according to the formula: [cpm experimental release - cpm control release/cpm maximum release - cpm control release] x 100. For statistical analyses, the Student's t-test was used.

Chromatin Immunoprecipitation Assay (ChIP)

The assay was performed using a chromatin immunoprecipitation assay kit (Millipore, Billerica, MA, USA) as per manufacturer's instructions with some modifications. Briefly, proteins were cross-linked to DNA by adding 1% formaldehyde directly to the media for ten minutes at 37°C. Cross-linking was

stopped by the addition of 0.125M glycine at 37°C for 15 minutes. The media was removed and the cells were washed with PBS containing protease inhibitors (1mM PMSF, 1ug/ml aprotinin, 1 ug/ml pepstatin). The cells were then resuspended in SDS and sonicated.

DNA/protein mixture was pre-cleared with Salmon sperm DNA/protein A agarose/50% slurry followed by an incubation with the antibody for acetylated Histone H3. After several washes, DNA bound to the immunocomplexes was obtained followed by incubation with Proteinase K. DNA was recovered via phenol/chloroform extraction. For promoter analysis, we designed the following PCR primers which span the *TAP1/LMP2* bidirectional promoter: TAPsh-F GGC AAA TCT GCC CAG AGA and TAPbd R CCT AGC CTG GGA CTC TCG AC.

Bisulfite modification and Methylation Specific PCR (MSP)

Treatment of DNA from TC-1/A9 cells with sodium bisulfite was accomplished using a previously established protocol (Herman et al, 1996). In order to identify CpG islands within the promoter region of the antigen processing genes, MSP analysis was performed with primers designed with the program MethPrimer. The product size were as follows: 204 base pairs *TAP1/LMP2*, 217 base pairs *TAP2* and 177 bases in the upstream region of *LMP7* (numbers represent the lengths of the native sequences).

Bisulfite sequencing

Genomic bisulfite sequencing of the *p15INK4B* promoter was done in selected patient samples as described to validate changes in promoter methylation (Cameron et al, 1999). Primers used were 5-TGAAGGAATAGAAATTTTTTGTTT-3 and 5-AAGCAAGCTTAAACCCTAAAACCCCAACTACCTAA-3 for the initial amplification, followed by a nested PCR using the internal primers

5-GGGGATTAGGAGTTGAG-3 and 5-ACCCTAAAACCCCAACTACC-3. The large size of this product precluded amplification from bisulfite-treated DNA in some cases. For these samples and time points, a smaller nested PCR was done initially using the primer pair 5-TAGGTTTTTTGGTTTAGTTGAAAA-3 and 5-CTCCTTCCTAAAAAACCTAAACTCAAC-3, followed by internal primers 5-TTTGTAGGTTGGTTTTTTATTTTGTTA-3 and 5-AAAACCTAAACTCAACTTCATTACCCT-3. This product includes the central 303 bp region of the *p15INK4B* CpG island. All pretreated patient samples were screened for promoter methylation of *p15INK4B*. Genes found to be methylated in the pretreatment specimen were monitored for changes in methylation.

Western Blot analysis

Whole cell protein extracts were prepared from cell lines TC-1 and TC-1/A9 using lysis buffer containing 20 mM HEPES (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride and 0.2mM protease inhibitor cocktail. Proteins (30 μg) were separated on a 7.5% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 0.1%/TBST/5% skim milk for 1 hr at room temperature and incubated over night at 40°C with goat polyclonal antibody against TAP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with anti-goat-HRP, which was followed by extensive washing, the bound antibodies were visualized using enhanced chemiluminescence. Levels of actin were analyzed with goat polyclonal antibody and were used as control for equal loading.

Microarray analysis

TC-1/A9 cells were cultured in fresh media for 24 hours after which the media was removed and replaced with RPMI supplemented with 1 uM 5-aza-2'deoxycytidine or interferon gamma. Cells were incubated for 72 hours at 37C. RNA was extracted from the cells with the High pure RNA isolation kit (catalog no. 11828665001). 50-100 ng of double stranded cDNA was resuspend in low TE (0.1 mM EDTA, 10 mM Tris HCL, pH 8.0). cDNA was hybridized to a Illumina's MouseWG-6 expression bead chip (19,000 probe sets) for whole genome analysis and processed according the standard manufacturer's protocol at the Genomics and Bioinformatics Core Facility at the IMG. The data was normalized per array using GeneSpring version 7.3 software that was used to normalize the data by dividing the raw data by the 50th percentile of all measurements on the array. All material and equipment utilized for the microarray analysis was available through the Institute for Molecular Genetics Microarray core facility. Agilent Technologies, Palo Alto, CA provided the core facility with the software for analysis.

Results

Epigenetic agents upregulate MHC class I expression on the surface of MHC class I-deficient TC-1/A9 cells

The effect of 5-azaC and TSA on MHC class I expression in MHC class I-deficient TC-1/A9 was studied. Treatment of this cell line with 5-azaC and TSA induced expression of MHC class I molecules on the cells (Fig.4). The effect was weaker than that observed in the IFN-treated control in which the expression achieved the level found in the parental TC-1 cells. In the next series of experiments, we studied the effects of 5-azaC and TSA separately. The results revealed that even treatment with TSA alone resulted in MHC class I upregulation. The data were supported by results of experiments with sodium butyrate and DAC.

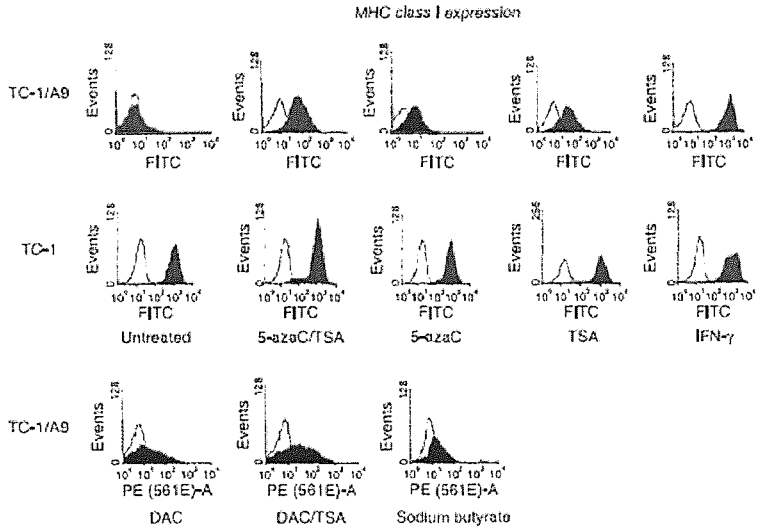

Fig.4. Epigenetic agents induce major histocompatiblity complex (MHC) class I cell surface expression on TC-1/A9 cells. MHC class I expression on TC-1/A9 and TC-1 after 5-azaC and TSA treatments was determined by flow cytometry. The efficacy of the 5-azaC and TSA treatment was compared to the effects of IFN-γ. The effects of DAC and sodium butyrate on TC-1/A9 cells were also demonstrated.

Importantly, both agents were able to upregulate MHC class I expression. Control experiments were performed on the parental MHC class I-positive cell line TC-1 in which no changes of the MHC class I surface expression were

observed. All cell lines remained MHC class II and CD86 negative (data not shown).

Proapoptotic effects of TSA and 5-azaC treatments

In order to assess proapoptotic and cytotoxic effects of the epigenetic agents, a portion of living, apoptotic and dead cells after treatment was analyzed by annexin and propidium iodide labeling (Fig. 5).The percentage of dead and live TSA-treated cells was similar to the untreated controls demonstrating that TSA at the concentrations

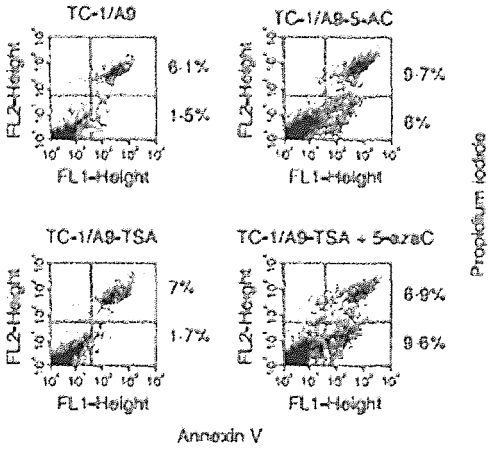

Fig.5. Determination of the proapoptotic effects of the 5-azaC and TSA. The proportions of viable, apoptotic and dead cells were determined by dual labeling with annexin V and propidium iodide; viable cells were double-negative, apoptotic cells were labeled only with annexin V and dead cells were double-positive. The percentages of the apoptotic cells (lower right quadrant) and dead cells (upper right quadrant) are depicted. The experiment was performed in duplicate with similar results.

used in the experiments had no effect on cell viability. After 5-azaC treatment, either alone or in combination, an increase in the portion of apoptotic cells was observed.

TSA-treated TC-1/A9 cells are effectively lysed with spleen cells from immunized animals

We used TSA-treated TC-1/A9 cells as targets in a chromium release micro-

cytotoxic test with the spleen cells from immunized animals immunized TC-1 cells (Fig.6). Indeed, 5-azaC and TSA-treated TC-1/A9 cells were effectively lysed in this experiment.

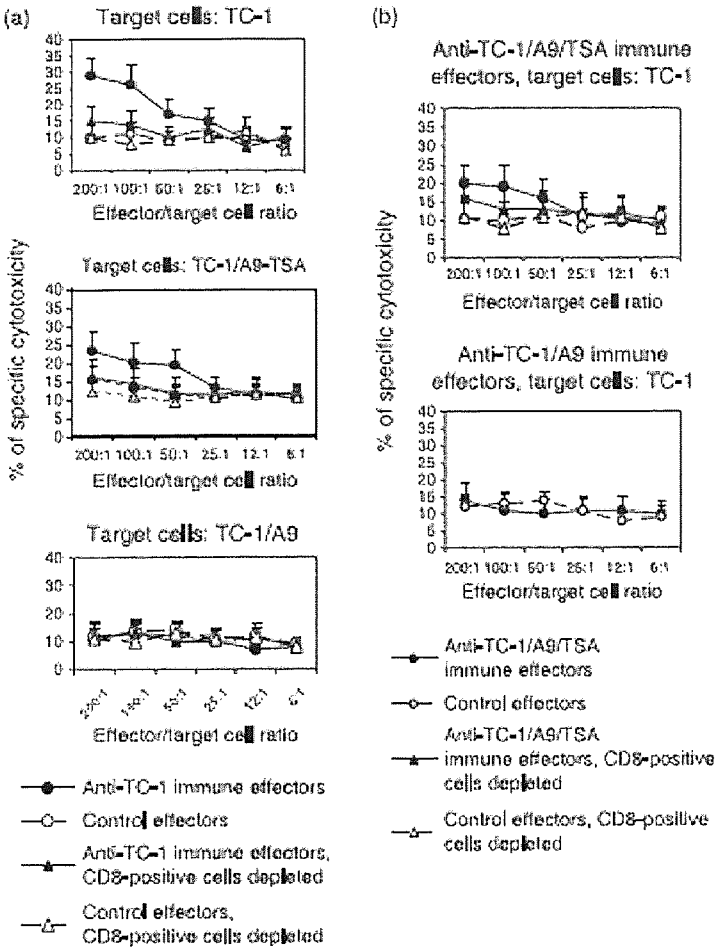

Fig.6. TSA-treated TC-1/A9 cells are susceptible to CTL-mediated lysis and TSA treatment augments the CTL-mediated immune response. (a) Spleen cells from mice immunized with TC-1 cells or control mice were used as effector cells. Significant differences observed in the following groups: target cells TC-1: $P < 0.01$ (200 : 1), $P < 0.05$ (100 : 1 and 50 : 1); anti-TC-1 immune effectors · control effectors, and anti-TC-1 immune effectors · anti-TC-1 immune effectors, CD8+ cells depleted; target cells TC-1/A9-TSA: $P < 0.05$ (200 : 1, 100 : 1 and 50 : 1); anti-TC-1 immune effectors x control effectors, and anti-TC-1 immune effectors x anti-TC-1 immune effectors, CD8+ cells depleted. (b) Spleen cells from mice immunized with TC-1/A9 or TSA-treated TC-1/A9 cells or control mice; TC-1 cells, TSA-treated TC-1/A9 cells and untreated TC-1/A9 cells were used as targets. Significant differences observed in the following groups: $P < 0.05$ (200 : 1 and 100 : 1); anti-TC-1/A9 TSA immune effectors x control effectors.

In vitro depletion of $CD8^+$ cells in the spleen cells mixture revealed that lysis was mediated by cytotoxic T cells. On the other hand, spleen cells from animals immunized with TSA-treated TC-1/A9 cells lysed MHC class I positive TC-1 cells more efficiently than spleen cells from mice immunized with untreated TC-1/A9 cells.

Expression of APM genes is upregulated by TSA and 5-azaC

The expression of MHC class I molecules does not occur on the surfaces of TC-1/A9 cells. We showed, via RT-PCR, that the two loci that produce MHC class I antigens, H-2Db and H-2Kb, were fully functional in TC-1 and TC-1/A9 cell lines (Fig. 7). On the other hand, there was a decrease in mRNA expression of *TAP1*, *TAP2*, *LMP2* and *LMP7* in TC-1/A9 cells compared to TC-1 cells. The effects of epigenetic agents on selected APM genes were studied in detail by quantitative real time RT- PCR (Fig. 7A). After administering the combination of 5-azaC and TSA to TC-1/A9 cell line, we observed an increase in the expression of the APM genes, namely *TAP2* and *LMP7*. Furthermore, we analyzed the expression of each gene after the administration of 5-azaC and TSA alone. A statistically significant upregulation of *TAP1*, *TAP2*, *LMP2* and *LMP7* was observed after the addition of TSA. In addition significant upregulation was noted in *TAP1*, *TAP2* and *LMP7* after the 5-azaC treatment. In control experiments, epigenetic agents also upregulated the expression of APM genes in MHC class I- positive TC-1 cells; TSA treatment resulted in a significant increase in expression of *TAP1*, *LMP2* and *LMP7*. *TAP2* and *LMP7* expression was significantly upregulated by 5-azaC. Notably, the effects of TSA and 5-azaC on particular genes differed and were not synergistic. The effect of the particular treatments on *TAP1* and *LMP2* were very similar.

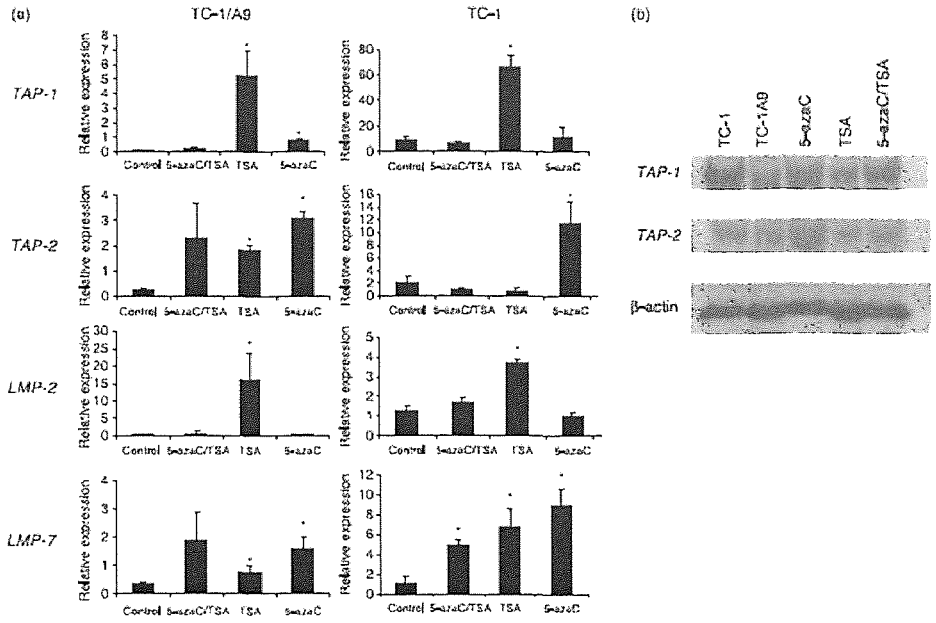

Fig.7. qPCR and western blot analysis of expression levels of APM components in TC-1/A9 and TC-1 cells. (a) qPCR: *TAP1*, *TAP2*, *LMP2* and *LMP7* mRNA expression after 5-azaC/TSA, 5-azaC and TSA treatments and in untreated cells was related to expression of β-actin. Cells were given 5-azaC in combination with TSA, 5-azaC alone, or TSA alone. *TAP1* and *LMP2* were upregulated after the administration of TSA. *TAP2* and *LMP7* were upregulated after treatment with 5-azaC and TSA and 5-azaC alone in MHC class I^+ and MHC class I^- cell lines. The samples whose relative expression exhibited a $P< 0.05$, compared with controls, were determined to be significant and are denoted by an asterisk. (b) Western blot: TAP1 and TAP2 expression at the protein level after treatment with epigenetic agents was determined in TC-1/A9 cells. β-actin expression served as a control.

Western blot analysis determined that the protein levels of TAP1 and TAP2 in cells treated with 5-azaC, TSA or the combination of the two drugs were increased when compared to the untreated TC-1/A9 control (Fig. 7b). Detectable protein expression was detected in untreated cells, however, the intensity of the band was greater in cells treated with 5-azaC alone and in combination with TSA for both proteins. TAP1 protein expression was increased in cells exposed to TSA however, TAP2 expression level appeared unchanged.

Methylated promotor regions of APM genes are demethylated after 5-azaC treatment

Bisulfite treated DNA was analyzed with primers designed to distinguish between modified and unmodified DNA. MSP analysis of the bidirectional promoter of *TAP1/LMP2* demonstrated that the promoter was partially methylated in TC-1/A9. Upon treatment with 5-azaC the promoter was demethylated (Fig. 8A). Interestingly, TSA alone appeared to induce demethylation of *TAP1*.

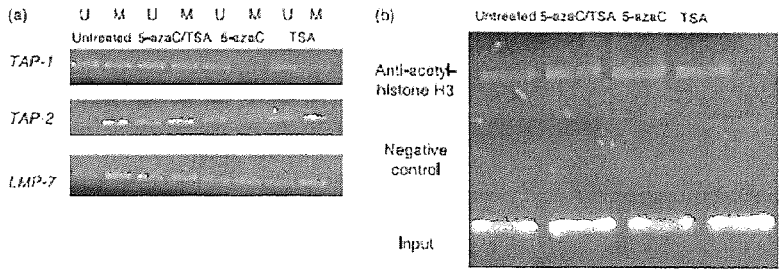

Fig.8. Epigenetic agents induce DNA demethylation and histone deacetylation. (a) DNA from the TC-1/A9 cell line treated with epigenetic agents. MSP analysis revealed partial methylation of *TAP1*, *LMP2* and *LMP7* in untreated cells. Administration of 5-azaC/TSA led to demethylation. 5-azaC alone resulted in the demethylation of *TAP1* and *LMP2*. TSA had an effect on the *TAP1* promoter but no effect on *TAP2*. *LMP7* remained methylated. U, unmethylated primer; M, methylated primer. Histone H3 is associated with the *TAP1/LMP2* bidirectional promoter. (b) ChIP analysis of chromatin isolated from TC-1/A9 treated cells demonstrated an increase in acetylated histone H3 after 5-azaC and TSA treatment.

The promoter region of *TAP2*, as well as, the upstream sequence of *LMP7* was also found to be partially methylated before treatment. However, after 5-azaC treatment, *TAP2* was demethylated while there appeared to be no change in the methylation status of *LMP7*. There were no detectable changes after treatment with the combination of drugs. Histone H3 was re-acetylated at the bidirectional promoter after 5-azaC/TSA treatment. A ChIP assay was performed to determine if the combined dose of 5-azaC/TSA, which is sufficient to reverse

the methylated *TAP1/LMP2* promoter, was able to modify the histones associated with this promoter (Fig. 8B).

The assay demonstrated that Histone H3 was re-acetylated after treatment with both epigenetic agents alone as well as in combination. Acetylated Histone H3 was detected at this region in untreated TC-1/A9 cells at a low level.

Monitoring Changes in Promoter Methylation of *p15INK4B* in MDS patients

DNA methylation in bone marrow cells was examined to monitor changes within tumor clones rather than monitoring alterations stemming from the replacement of neoplastic cells by normal cells. Genomic bisulfite sequencing of a hypermethylated *p15INK4B* promoter was done in five patients (Fig. 9A and B). Sequential sequencing of DNA from patients who had significant responses to the combined therapy, patients 13, 19, and 24 showed a significant decrease in *p15INK4B* methylation following 5-azaC treatment. Importantly, we saw a pattern of heterogeneous loss of CpG methylation within each single allele examined, strongly supporting demethylation within the tumor clone. The bisulfite sequencing of patient 19 showed evidence of remethylation before initiation of the next drug cycle. No demethylation was observed in the *p15INK4B* promoter in two clinical nonresponders.

Twelve patients evaluable for clinical response were subsequently studied by MSP for *p15INK4B*. The primers completely overlap the region of the *p15INK4B* promoter sequenced in the above studies. All twelve patients exhibited hypermethylation before treatment. Six patients developed reversal of methylation during the first cycle of treatment. The methylation status over time changed in three of four patients informative (Fig. 10)

Particularly noteworthy is patient 19: No evidence of methylation of either gene was present following 5-azaC (day 10); however, some evidence of

remethylation of *p15INK4B* was present following phenylbutyrate (day 17). Most importantly, these six patients with reversed methylation all developed

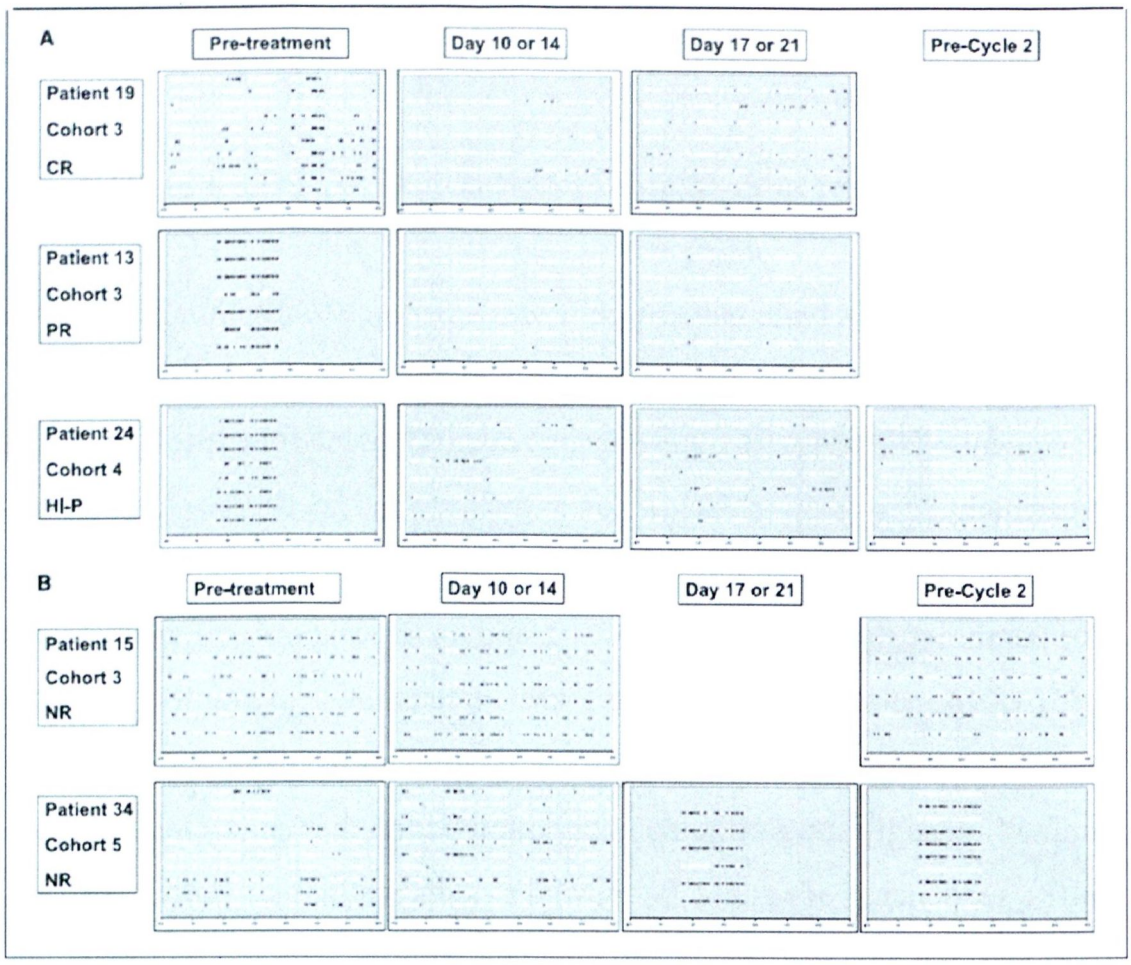

Fig.9. Genomic bisulfite sequencing of *p15INK4B* promoter region CpG island during cycle 1 Individual allele patterns: line, cloned and sequenced DNA molecule; ○ unmethylated CpG sites; ● methylated CpG sites. Numbering is according to the transcriptional start site (nucleotide 1218 from Genbank sequence AF513858), which is +1. Bone marrow mononuclear cells were procured before therapy, following the last injection of 5-azaC, following discontinuation of phenylbutyrate, and before cycle 2. A. clinical responders. B. clinical nonresponders.

hematological responses, including three complete response (CR), one partial response (PR), and two patients who achieved hematological improvement. In contrast, the six patients who did not show methylation reversal were all nonresponders. The difference in response rates between the two groups was

statistically significant (P = 0.002, Fisher's exact test).

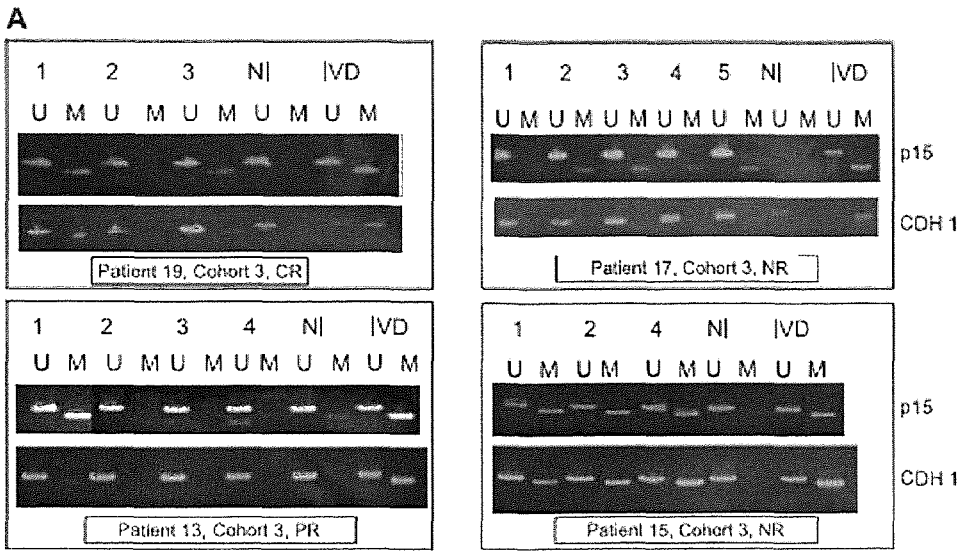

Fig.10. Changes in methylation of *p15INK4B* during cycle 1 of treatment. A, bone marrow mononuclear cells were procured before therapy (1), following the last injection of 5-azaC (2), following discontinuation of phenylbutyrate (3), and before cycle 2 (4). In one patient, a fifth time point was procured after four cycles of treatment. The four representative patients were all treated in dose cohort 3 (50 mg/m2/d 5-azaC for 10 days). Nl, normal peripheral blood lymphocytes. *In vitro* methylated DNA (IVD) was used as the positive methylation control..

H3/H4 acetylation in MDS patients treated with 5-azaC and phenylbutyrate

Changes in histone acetylation in peripheral blood and bone marrow mononuclear cells were monitored using Western blot analysis (Fig. 11). 11 of 23 evaluable patients showed increases in global acetylation of H3 and/or H4 in response to 5-azaC alone (Fig. 11A). This was observed in one of five patients in dose cohort 1, three of four in dose cohort 2, five of seven in cohort 3, and one of three each in cohorts 4 and 5 (P = not significant between cohorts). Further increases in acetylation in response to phenylbutyrate were found in 12 of the 23 patients. Overall, 17 of 23 patients developed increased histone

acetylation following either 5-azaC or phenylbutyrate dosage. Fig. 11B displays the changes in histone acetylation from patients in dose cohort 3 semi-quantitatively. Blots were stripped and reprobed with antibody to non-acetylated histone H2A.

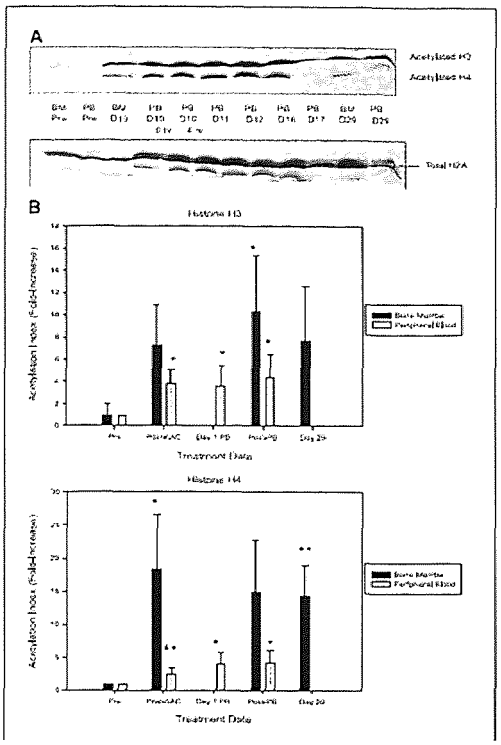

Fig.11. Changes in global histone acetylation during cycle 1 of therapy. Histones were isolated from peripheral blood and bone marrow mononuclear cells during cycle 1. Immunoblotting was done using antibodies to acetylated H3 and H4. Blots were then stripped and reprobed using antibody against nonacetylated histone H2A as a control for the efficiency of histone extraction and protein loading. A, representative patient (patient 14, cohort 3, AML-TLD, nonresponder). Pre, pretreatment. D, day of treatment. Blotting of H2A was done following stripping of the membrane after blotting for acetylated H3 and H4. Some residual Ac-H3 and Ac-H4 continues to be visualized on the H2A blot. B. changes in histone acetylation during cycle 1 in response to 5-azaC and phenylbutyrate in patients in dose cohort 3. Data reported as fold increase compared with baseline, using an acetylation index (see text). Columns, mean of six patients; bars, SE. **, $P < 0.025$, mean fold increase >1;*, $P < 0.05$, indicates mean >1.

Gels were scanned and band intensity was quantified. The data were then reported as an acetylation index: band intensity acetylated H3 or H4/band intensity non-acetylated H2A. The greatest increment in acetylation appeared following 5-azaC administration; phenylbutyrate induced a smaller increment in histone acetylation in many patients. Acetylation remained elevated compared with baseline during phenylbutyrate infusions even in patients who showed no further increment in acetylation in response to phenylbutyrate.

Microarray Analysis of mouse immune genes affected by DAC and IFN-γ

19,000 transcripts were analyzed on the Illumina Bead chip. Approximately 300 were upregulated after treatment with 5-aza-2'-deoxycytidine and interferon gamma. Genes within immune cell populations were chosen from the data. The gene expression of cells exposed to both drugs was compared to untreated cells and expressed as log fold change (logFC).

Antigen processing machinery genes showed the highest increase of logFC of those transcripts related to immune cell populations. The logFC expression of cells treated with interferon gamma was over three for the genes *Tap1*, *Tap2* and *LMP2*, *LMP7* posted changes of four to nearly six. The expression levels were nearly identical in both experimental cell sets although those cells treated with DAC exhibited a slightly lower logFC. Since the interferon gamma signal ends in interferon-sensitive response elements (ISRE) of the antigen processing genes the expression status of those transcripts were also noted. *IRF1* was upregulated more than 2 fold in IFN-γ treated cells and a slightly higher logFC was observed in DAC treated cells. Other IRFs including *IRF3*, *IRF5* and *IRF9* (data not shown) also expressed a logFC of at least three. However, the IRF gene with the greatest logFc was *IRF7* (logFC=4) in DAC treated cells and a change of three in IFN-γ treated cells. The expression of cell adhesion molecules *ICAM1* and *CD274* (*B7-H1*) was positively influenced similarly by both drugs.

Gene	Accession No.	logFC	adj.P.Val	Function
Stat1	NM_009283.3	**3,952864**	0,00334	IFN gamma path.
Stat2	NM_019963.1	**3,054803**	0,041011	IFN gamma path.
Stat3	NM_213659.2	**0,30649**	0,226536	IFN gamma path.
Psmb8 (LMP7)	NM_010724	**5,611914**	0,00334	Antigen processing
Psmb9 (LMP2)	NM_013585.2	**4,305338**	0,005232	Antigen processing
Psmb10 (LMP10)	NM_013640.2	**2,73289**	0,004374	Antigen processing
Tap1	NM_013683.1	**3,094098**	0,012775	Antigen processing
Tap2	NM_011530.2	**3,067615**	0,138684	Antigen processing
Icam1	NM_010493.2	**2,117239**	0,008417	Cell adhesion
Cd274	NM_021893.2	**4,742368**	0,004374	Cell adhesion
Cd276	NM_133983.3	**1,197923**	0,149875	Cell adhesion
H2-D1	NM_010380.3	**3,089133**	0,004374	MHC antigen
H2-K1	NM_001001892.2	**3,04777**	0,025029	MHC antigen
IRF1	NM_008390.1	**2,367095**	0,154558	IFN gamma pathway
IRF7	NM_016850.2	**4,350242**	0,016028	IFN gamma pathway

Fig.12. Microarray analysis of TC-1A/9 cells treated with DAC. Genes within immune cell populations were selected. logFC denotes the log fold change of expression compared to untreated cells. Adjusted P value is that value of duplicate samples measured.

However, another member of this family *CD276* (*B7-H3*) expressed a logFC of just over one. MHC class I antigens H2-K and H2-D expressed similarly with both drugs but, IFN-γ was slightly more effective than DAC. Both treatments yielded expression of these genes at three log fold change over untreated cells. The logFC of the selected transcripts and the p-value has been compiled in Figs.

12 and 13.

Gene	Accession No.	logFC	adj.P.Val	Function
Stat1	NM_009283.3	**3,849837**	0,00334	IFN gamma pathway
Stat2	NM_019963.1	**3,007977**	0,041011	IFN gamma pathway
Stat3	NM_213659.2	**1,838591**	0,226536	IFN gamma pathway
Psmb8 (LMP7)	NM_010724	**6,630113**	0,00334	Antigen processing
Psmb9 (LMP2)	NM_013585.2	**6,581922**	0,005232	Antigen processing
Psmb10 (LMP10)	NM_013640.2	**3,528811**	0,004374	Antigen processing
Tap1	NM_013683.1	**4,652734**	0,012775	Antigen processing
Tap2	NM_011530.2	**4,131463**	0,138684	Antigen processing
Icam1	NM_010493.2	**2,806693**	0,008417	Cell adhesion
Cd274	NM_021893.2	**5,681967**	0,004374	Cell adhesion
Cd276	NM_133983.3	**0,883169**	0,149875	Cell adhesion
H2-D1	NM_010380.3	**3,55112**	0,004374	MHC antigen
H2-K1	NM_001001892.2	**3,980846**	0,025029	MHC antigen
IRF1	NM_008390.1	**3,053495**	0,154558	IFN gamma pathway
IRF7	NM_016850.2	**3,145874**	0,016028	IFN gamma pathway

Fig.13. Microarray analysis of TC-1A/9 cells treated with the cytokine IFN-γ. Genes within immune cell populations were selected. logFC denotes the log fold change of expression compared to untreated cells. Adjusted P value is that value of duplicate samples measured.

Discussion

Our primary focus was two-fold: to determine if epigenetic changes affect the expression of MHC class I molecules on the surface of MHC class I-deficient tumor cells, the genes responsible for induction of these molecules and their influence on the immune system; to observe the epigenetic changes and tumor cell differentiation in myleodysplastic patients treated with DNA methyltransferase and histone deacetylase inhibitors.

We have shown that epigenetic agents influence MHC class I cell surface expression in tumor cells that contain reversible MHC class I expression deficiencies due to defects in antigen processing machinery components. The combination of a DNA demethylation agent and a histone deacetylase inhibitor or each agent separately induced MHC class I on the surface of the experimental tumor cell line used in our study and restored the expression of APM genes. After treatment with 5-azaC and TSA the cell line upregulated MHC class I, was subsequently recognized by $CD8^+$ cells and lysed. The level of induction of these molecules on the surface of tumor cells treated with these agents did not achieve the level of induction of the parental MHC class I positive cells but, the dosage of both drugs to MHC class I negative tumors was sufficient for lysis by effector spleen cells from mice immunized with the parental TC-1 tumor cell line. APM genes were found to be preferentially methylated in untreated tumor cells. After treatment with 5-azacytidine, methylation of the promoter region was partially removed. Although it has been long known that demethylation agents inhibit the binding of methyltransferases leading to promoters free of 5-methylcytosines, this was the first study to show that demethylation and or reacetylation of antigen processing machinery genes occurs after treatment with methyltransferase and histone deacetylase inhibitors. We also found that the HDACi affected the DNA methylation status of the *TAP1* promotor region. Although this was a surprising result, past experiments in the fungus

Neurospora (Sekler, 1998) and in Jurkat T cells (Januchowski et al., 2006) also observed the loss of methylation after treatment with TSA (On J-N et al., 2007). These findings suggest that histone acetylation may contribute to the formation of euchromatin resulting in DNA demethylation.

The effect of 5-azacytidine on MHC class I molecules leads to demethylation of promoter genes, however, a limited of number studies have been done to determine which proteins are recruited to the promoter regions of genes linked to MHC class I induction. In addition, it is unknown which epigenetic marks within histones are modified that promote the formation of euchromatin, although HLA class I heavy chain demethylation after 5-aza-2'-deoxycytidine has been described (Serrano et al, 2001). A recent study sought to examine if epigenetic mechanisms influenced the presentation of antigen on the surface of cell lines TC-1/A9 and TC1-D11 (a metastatic clone of TC-1), PA, a murine prostate cell line deficient in TAP1 expression and CMT.64 a spontaneous mouse lung carcinomas also deficient in TAP1 expression (Setiadi et al, 2007). Also, the role of transcription factors at the promoters of APM genes was analyzed to determine a link to histone acetylase activity (Setiadi et al, 2007) was performed. Earlier experiments (Setiadi et al, 2005) demonstrated that the TAP-deficient cell lines did not possess trans-acting transcription factors that target and regulate TAP1 expression. Transient transfection of a reporter gene regulated by the *TAP1* promoter into carcinomas cells restored TAP1 expression to the level of normal, TAP1 expressing cells. Additionally, stable transfection of the same promoter showed the difference in the expression of TAP-expressing versus TAP-deficient carcinomas in all cell lines studied. It was determined that the cells did not have the ability to transcribe *TAP1* in TAP-deficient cells because of low levels of the histone acetyltransferase chromatin binding protein (CBP), a well known co-activator with histone acetylase activity, at the *TAP1* promoter due to a decrease in histone H3 acetylation. The end result was the inability of RNA polymerase II to bind the *TAP1* promoter. In addition,

IFN-γ treatment of TAP-deficient cells increased levels of CBP, acetylated histone H3 and RNA polymerase II at the *TAP1* promoter, a previously unknown mechanism (Setiadi et al., 2007). It was observed that the level of expression of CBP, acetylated H3 and RNA polymerase II in the formally deficient cells was at the same level as that of TC-1 cells after IFN-γ treatment. Hence, the mechanism of *TAP1* expression seems to require CBP at the promoter which in turn stimulates histone H3 acetylation followed by the formation of euchromatin facilitating the binding of RNA polymerase II resulting in transcription of *TAP1*. This mechanism illustrated the change within chromatin when epigenetic agents are administered to MHC class I negative tumor cells. Dnmti are known to not only interfere with DNA methyl transferases and methyl binding proteins inhibiting methylation at the TAP1 promoter but also deter the binding of HDAC to methyl binding proteins thus permitting the reacetylation of histones. IFN-γ appears to take a more direct approach via the interaction with the histone rather than the gene promoting the formation of euchromatin. Our data supported the role of the HDACi and DNA demethylation agents on chromatin remodeling and DNA methylation at the *TAP1* promoter which produced a similar result (upregulation of antigen processing genes and MHC class I induction) but at a lesser intensity than IFN-γ (Fig.4). In the figure, the mean fluorescence intensity for MHC class I expression on TC-1/A9 cells treated with IFN-γ was higher than cells treated with 5-azaC all treatments. However, the expression level was above the threshold for cell lysis by CTL. This finding can be a rationale for development of therapeutical protocols for MHC class I-deficient tumors based on the combination of epigenetic drugs and vaccines.

Although we observed that the APM machinery genes were methylated and partially demethylated after drug treatment, it is important to note that numerous genes are upregulated or downregulated after treatment, some of which are found within the immune system and are linked to the induction of MHC class I

molecules. It may be the case that within the interferon gamma pathway Dnmti target important promoters that have become methylated and increases the expression of immune regulatory genes.

Preliminary microarray data of the tumor cell line treated with either 5-aza-2'-deoxycytidine and IFN-γ (Manning and Reiniš unpublished data) yielded both upregulated and downregulated transcripts. Several genes in the IFN-γ pathway were upregulated by both IFN-γ and 5-aza-2'-deoxycytidine. MHC class I antigens (*H2-D* and *H2-K*), cell adhesion molecules (*ICAM1*, *B7-H1*) were also among those genes positively affected. Microarray analysis determined that transcripts for *STAT1*, *STAT2* showed similar and significant log fold change in cells treated with 5-aza-2'-deoxycytidine and IFN-γ. These results confirmed earlier microarrays that found 5-aza-2'-deoxycytidine inhibited the growth HT29 adenocarcinoma cells and induced the expression of *STAT1*, *STAT2* and *STAT3* (Karpf et al., 1999). Overall, IFN-γ had a positive effect on the cells leading the researchers to also propose that the promoter of the inducible genes may not contain methylation but, may function once a methylated upstream gene is demethylated. However, in the case of each *STAT* cDNA analyzed, a CpG island was located in the 5'region of the gene; a prime site for promoter methylation. Additionally, a family of interferon regulatory factors (IRF) that bind the interferon-stimulated response element (ISRE) within IFN-γ inducible genes, mediating viral induced signaling, have been shown to be important in the induction of inflammatory genes in infected cells and cell development of lymphoid lineage (Pitha et al., 1998). IRF1, a transcription factor that binds to the cis-acting ISRE sequence within *TAP* and *LMP* genes as a response to IFN-γ stimulation of the JAK/STAT1 pathway was highly upregulated in our microarray experiment (Fig.12 and 13). In this regard, a recent experiment demonstrated that *IRF1* was affected by epigenetic mechanisms (Rodriguez et al., 2007). The authors concluded that two mechanisms were responsible for the non-induction of HLA class I molecules in a melanoma cell line; the absence of

STAT1 tyrosine phosphorylation early in the pathway and the epigenetic inhibition of *IRF1* leading to loss of HLA class I expression (Rodriguez et al., 2007). A related gene *IRF7*, whose transcription is also stimulated by IFN-γ, was highly upregulated in our tumor cell line after treatment with the demethylation agent and IFN-γ (Fig. 12 and 13). Noteworthy, in human 2fTGH cells, a CpG island discovered within the promoter of *IRF7* was found to be hypermethylated in which expression was restored after 5-azacytidine treatment (Lu et al., 2002). Hence, direct demethylation of *IRF7* or another gene within the pathway may be necessary to induce MHC class I surface molecules. Genes for MHC class I antigens were also found to be expressed at significantly higher levels than those found in untreated tumor cells, although the MHC class I cell surface expression was not changed. It is probable that the promoters of *H2-D* and *H-2K* in normal cells as well as tumor cells are not completely devoid of methylation and as 5- methylcytosines are removed promoter expression is increased. Since 5-azacytidine positively affected the pathway, detailed methylation analysis of the genes within the pathway would be of great interest. The effect of Dnmti must be closely examined *in vivo* because many of the genes that are normally silenced may have their status reversed causing a cascade effect resulting in severe autoimmune responses. The data obtained *in vitro* with the microarray data is positive but, genetic mechanisms in as many cells types as possible must be studied.

Recently others have presented data that corroborated our results demonstrating the effect of epigenetic agents on MHC class I induction and antigen processing machinery expression. Although the epigenetic agents of choice were histone deacetylase inhibitors TSA and valproic acid Khan et al. (2008) demonstrated that theses drugs promoted the acetylation of histones and enhanced transcription of APM genes *TAP1*, *TAP2*, *LMP2*, *LMP7* and MHC class I (*H2-D*) and *Tapasin* in mouse B16F0, B16F10 (adenocarcinoma) and Colon 26 cell lines. Although valproic acid treatment was found to be less effective that TSA

in this study, it had been previously shown to enhance cell lysis via CTL *in vitro* (Armeanu et al., 2005; Mora-Garcia et al., 2006). Little is known regarding the differences between the mechanisms of TSA and valproic acid but, it appears that TSA interacts with more HDAC than valproic acid (Johnstone and Licht, 2003; Tomasi et al., 2006), hence a more reactive HDACi. Additionally, the researchers observed the induction of MHC class I molecules and co-stimulatory molecules CD40 and CD86 on B16F10 tumor cells after treatment with both drugs. However, the induction of CD86 appears to be cell line specific since the expression of this molecule in our study of TC-1/A9 remained negative.

With so much data coming forth regarding epigenetic modifications of MHC class I molecules, Tomazou et al. (2008) set out to look for epigenetic patterns within the entire 4Mb human MHC region by employing a genomic tiling array designed to be comparable to chromatin immunoprecipitation (ChIP), methylated DNA immunoprecipitation (MeDIP), array comparative genomic hybridization (aCGH) and expression profiling including coding/noncoding RNAs. The application MeDIP was used to construct a DNA methylation profile and identified 90 tissue-specific differentially methylated regions (tDMRs) in two tissues and two cell types within the region examined. Hence, analyzing the genes that are affected by methylation in MHC just became more convenient.

The compiled data of recent experiments assessing the effect of epigenetic agents on MHC class I negative tumor cells *in vitro* has provided immunologists with a potentially new type of immunotherapy. Of course, in order to verify the result from the *in vitro* study, identical experiments should be conducted in an animal model. Current work in our laboratory has begun to analyze the affect of epigenetic agents as therapeutic agents in vivo (Šimová et al. unpublished data) yielding promising results. Mice injected with MHC class I negative tumor cells received a weekly dosage of 5-azacytidine or trichostatin A injected peritumorally over a period of 40 days. The animals injected with 5-azacytidine exhibited small, but palpable tumors after approximately 37 days. Trichostatin A

treated mice also responded well with visible tumors appearing at day 37. The cells from both experimental treatment groups were sensitive to cytotoxic T lymphocytes, however, those animals that received 5-azacytidine exhibited more effective tumor cell lysis. Although only three parameters were monitored, this preliminary data supports the results of the *in vitro* experiments. Future work will be to scrutinize of many of the genes that were discovered to be upregulated via our microarray analysis. From this work, a map of genes that are silenced by methylation either directly or indirectly can be drawn.

Clinically, since the approval of both 5-azacytidine and 5-aza-2'deoxycytidine for the treatment of cancer, these agents have demonstrated a great ability to deter or improve the outcome of more than half of patients treated (Silverman et al., 2002; Kantarjian et al., 2007). It has been observed that favorable responses required multiple cycles of therapy and actual clonal elimination (based on cytogenetic changes). Optimization of epigenetic therapy by reducing the dosage of these agents to promote hypomethylation (Issa et al., 2004), extension of administration schedules (Gore et al., 2006) and increasing the amount of the epigenetic drug after first giving the patient a lower dose of drug (Kantarjian et al., 2007) has become the focus of clinical trials. These changes have resulted in minimal side effects (hematologic) with no ensuing problems, chromosomal changes or the development of a secondary malignancy (Yang et al., 2003). On the molecular level, gene demethylation and reactivation have been observed and have been linked to patient response (Kantarjian et al., 2007; Yang et al., 2006; Mund et al., 2005). This data supports the effect of epigenetic drugs in vivo and favorable patient outcome due to clonal elimination. However, the mechanism is still not clear how the neoplastic cells are removed. Epigenetic agents have numerous effects which may affect each patient differently either by inducing cell senescence, differentiation, apoptosis and/or by engaging (or re-engaging) the immune response. The possibility arises that a combination of these mechanisms may also be responsible for the clinical response. The results

are promising, however, more work in the clinic is needed to understand why some patients present a complete response lasting months or years, while a majority of patients develop resistance to these drugs. Epigenetic therapy has been utilized against myelodysplastic syndromes with much success. The inhibitors of methylation have been effective on AML and CML. However, experiments that entail the treatment of solid tumors have not yielded similar success with some older studies suggesting that the drugs are ineffective (Aparicio and Weber, 2002). However, most of the early studies were performed with high dosages, a limited number of days of drug administration and evaluation of the effect of the drug after only one cycle. These may be crucial factors that influence a positive clinical response. Currently, changes in experimental design are being implemented as well as drug combinations that may produce positive results. Recent evidence has demonstrated that the combination of low dose 5-aza-2'-deoxycytidine with high dose interleukin-2 has shown promise in the treatment of malignant melanoma at low doses (Gollob et al., 2006). Additionally, combination therapy of 5-aza-2'-deoxycytidine with the anti-cancer agent cisplatin, a platinum-based chemodrug that binds and crosslinks DNA causing apoptosis has been shown to reverse drug resistance has also produced positive results (Plumb et al., 2000). A positive result from this data shows that epigenetic therapy can be applied to other types of cancers and biological disorders. Our study produced results that confirmed that patients stricken with myeloid neoplasms responded well to combination therapy of 5-azacytidine and phenylbutyrate. We analyzed the methylation status of the tumor suppressor *p15INK4B*, a negative regulator cyclin-dependent kinases involved in G1 to S phase transition. This gene, located on chromosome 9p21, is induced during cellular senescence (Serrano et al., 1997) as a result of the expression of the growth inhibitor TGF-β (Ekholm and Reed, 2000), binds cyclin-dependent kinases four and six hence interfering with their ability to bind cyclin D. *p15INK4B* is frequently methylated in

myleodysplastic syndromes and acute leukemias (Drexler, 1998). Our methylation specific PCR data demonstrated that patient response correlated with the demethylation and reexpression of the *p15INK4B* promoter region after treatment with 5-azaC. This was the first study to utilize bisulfite (converts unmethylated cytosine to uracil while methylated cytosines remain unchanged) sequencing as a tool that determines the number of CpG dinucleotides that are demethylated after 5-azaC treatment. This technique demonstrated that patients who responded favorably to 5-azacytidine possessed tumors whose *p15INK4B* promoter sequences were demethylated during treatment.

During the last few years, the role of epigenetic mechanisms in gene expression has become more clear but, how methylation and histone deacetylation work together is still not clear. This was the case in our study where histone acetylation was observed after the administration of 5-azaC but, before the HDACi was given to the patients. Further studies must be performed to determine if this observation was due to a Dmnt-dependent phenomenon or a direct result of the 5-azaC dose. Additionally, only half of the patients (12 of 23) treated with phenylbutyrate displayed an increase in H3/H4 acetylation. We speculate that this may be due to the maximal effect of 5-azaC. The question of how each inhibitor alone affects methylation and histone deacetylation still needs to be answered in the clinical setting. Clinically and *in vitro*, the agents responsible for reversing deacetylated histones have been utilized more regularly resulting in promising anticancer therapy. The role of trichostatin A and valproic acid were recently shown to induce TAP, LMP Tapasin and MHC class I molecules and enhance the expression of co-stimulatory molecules CD40 and B7-2 in mouse melanoma cells (Kahn et al., 2007). The effects of these inhibitors were found to be similar to those of IFN-γ. The histone deacetylase inhibitor MS-275, a benzamide derivative, that has been used in a phase I clinical trial of patients with advanced myeloid leukemias successfully induced histone H3/H4 acetylation, *p21* expression and caspase-3 activation in bone

marrow mononuclear cells (Goja et al., 2007).

Although the mechanisms involved regarding the reexpression of MHC class I and antigen processing machinery components after treatment with these inhibitors have not been completely elucidated, there are several scenarios some studies have presented to explain the action of these inhibitors. In some cases, DNA methylation may be inhibited by histone deacetylase inhibitors. A recent study supported this idea showing a decrease in global methylation after HDACi treatment however, all methylated tumor suppressor genes were not demethylated equally suggesting that the HDAC inhibitor targets specific genes (On J-N et al., 2007). Acetylation of non-histone proteins such as transcription factors, mediators of signal transduction and/or molecular chaperones may be the target of histone deacetylase inhibitors. Hence alterations of the histone code by these agents may not be the primary action that leads to apoptosis of cancer cells.

Epigenetic changes are an important phenomenon in the progression of tumors. Demethylation of cancer cells can be a positive action that leads to reexpression of silenced tumor suppression genes and enhance the interactions between immune and tumor cells.

Conclusions

In our model of C57BL/6 immunogenic cell line expressing HPV16 E6/E7 antigens we demonstrated the following:

1. Expression of MHC class I molecules on the cell surface was restored after treatment with the combination of 5-azaC and TSA and with both agents alone.
2. MHC class I induction on the tumor cell surface was associated with the upregulation of antigen processing machinery genes, *TAP1*, *TAP2*, *LMP2* and *LMP7*.
3. Cells expressing MHC class I molecules after treatment with epigenetic agents were subsequently recognized by cytotoxic T lymphocytes and lysed.

In our patient study we observed the following:

1. Patients afflicted with myeloid dysplasia suffered no unusual toxicities and exhibited a favorable response to the combination therapy 5-azacytidine and phenylbutyrate.
2. Bisulfite sequencing demonstrated that the *p15INK4B* promoter was demethylated during 5-azaC treatment.
3. Methylation specific PCR showed demethylation of the *p15INK4B* promoter in six clinical responders.
4. 5-azacytidine treatment resulted in increased acetylation of half of the patients treated. Phenylbutyrate increased the acetylation of histone H3 and H4 in half of the patients treated.

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List of publications related to thesis work

Manning J, Indrova M, Lubyova B, Pribylova H, Hejnar J, Bielblova J, Simova J, Jandlova T, Bubenik J, and Reinis M. Induction of MHC class I molecules cell surface expression by epigenetic activation of antigen processing machinery components in a murine model for human papilloma virus 16-associated tumours. Immunology 2008 Feb;123(2):218-27
(Attachment 1)

Gore SD, Baylin SB, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhou M, Smith BD, **Manning J**, Jiemjit A, Dover G, Mays A, Zweibel J, Murgo A, Weng LJ and Herman JG. Combined DNA Methyltransferase and Histone Deactylase inhibition in the treatment of Myeloid Neoplasms. Cancer Res 2006 Jun;66 (12):6361-69
(Attachment 2)

Additional materials (posters, lectures) related to the thesis

Reinis M, Simova J, Stepanek I, Indrova M, Moravcova S, Pribylova H, Hejhal T, **Manning J**, Bubenik J. Modulation of antitumour immunity and immune suppression by epigenetic agents; impacts on gene and cellular therapy. 12th International Symposium on Molecular Medicine, Loutraki, Greece, October, 2009 International Journal of Molecular Medicine, 2009; 24: S67

Vaccination against MHC class I-deficient, HPV16-associated tumours. Reinis M, Simova J, Bieblova J, Pribylova H, Stepanek I, Indrova M, **Manning J**, and Bubenik J (Poster), Recent advances in Cancer Immunotherapy, Athens, Greece, 9.-12.10, 2008

Manning J, Indrova M, Lubyova B, Pribylova H, Hejnar J, Bielblova J, Simova J, Jandlova T, Bubenik J, and Reinis M. Improvement of Vaccine Efficacy by Induction of MHC Class I Expression on Deficient Tumor Cells via Epigenetic Activation of Antigen Processing Machinery Components. (lecture) Molecular and Immunological Approaches to Vaccine Design Cold Spring Harbor Laboratory, Cold Spring Harbor, NY November 2007

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Combined DNA Methyltransferase and Histone Deacetylase Inhibition in the Treatment of Myeloid Neoplasms

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Abstract

Optimal reexpression of most genes silenced through promoter methylation requires the sequential application of DNA methyltransferase inhibitors followed by histone deacetylase inhibitors in tumor cell cultures. Patients with myelodysplastic syndrome or acute myeloid leukemia (AML) were treated with the methyltransferase inhibitor 5-azacitidine (aza-CR) followed by the histone deacetylase inhibitor sodium phenylbutyrate. Major responses associated with cytogenetic complete response developed in patients receiving prolonged dosing schedules of aza-CR. Bisulfite sequencing of the *p15* promoter in marrow DNA during the first cycle of treatment showed heterogeneous allelic demethylation in three responding patients, suggesting ongoing demethylation within the tumor clone, but no demethylation in two nonresponders. Six of six responding patients with pretreatment methylation of *p15* or *CDH-1* promoters reversed methylation during the first cycle of therapy (methylation-specific PCR), whereas none of six nonresponders showed any demethylation. Gene demethylation correlated with the area under the aza-CR plasma concentration-time curve. Administration of both drugs was associated with induction of acetylation of histones H3 and H4. This study provides the first demonstration that molecular mechanisms responsible for responses to DNA methyltransferase/histone deacetylase inhibitor combinations may include reversal of aberrant epigenetic gene silencing. The promising percentage of major hematologic responses justifies the testing of such combinations in prospective randomized trials. (Cancer Res 2006; 66(12): 6361-9)

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malignant phenotype results from a combination of genetic malities and epigenetic modifications, leading to dysregulaof critical genes controlling cell proliferation, differentiation, death. Considerable data highlight the important role that hylation of cytosine residues in promoter regions plays in the transcriptional silencing of genes (1, 2). Methylated CpG dinucleotides bind specific proteins, such as MeCP2, which recruit transcriptional corepressors (3). These transcriptional inhibitory complexes include histone deacetylases (HDAC). DNA methyltransferases (DNMT), which catalyze DNA methylation, also bind to HDACs and have the potential to target these enzymes to regions of gene silencing (1, 4–7). Removal of acetyl groups from histone lysine tails is but one of several modifications made to these proteins now known to associate with transcriptional silencing (8, 9). In fact, aberrantly hypermethylated and silenced genes in cancer are now known to have key histone modifications associated with their promoter regions (10–13). However, in the context of all of these layered silencing chromatin modifications that affect such genes, DNA methylation seems to be dominant in specifying tight heritable transcriptional repression. Thus, in cultures of cancer cells, the administration of HDAC inhibitors will not result in reexpression of densely hypermethylated genes until a DNMT inhibitor is first given—the two inhibitors are then synergistic for reexpression of the genes (14, 15).

Given the above dynamics underlying the silencing of genes with hypermethylated promoters, the sequential application of HDAC inhibitors following DNMT inhibitors could potentially increase the response rate, response duration, or percentage of complete responses (CR) in myeloid leukemias through the reexpression of silenced important cell regulatory genes. Multiple such genes are known to exist in hematopoietic neoplasms, including the key cell cycle regulating gene $p15^{INK4B}$ (16–19).

The marked clinical activity of the DNMT inhibitors 5-azacitidine (aza-CR) and 2′-deoxy-5-azacytidine (DAC) in patients with myelodysplastic syndromes (MDS) suggest that epigenetic silencing of cell regulatory genes may play important roles in the pathophysiology of this group of disorders. In addition to hematologic response rates ranging from 30% to 60% (20–23), both drugs have been shown to retard the progression of MDS to acute myeloid leukemia (AML; refs. 23, 24).

The presently described clinical trial was designed to determine whether the sequential administration of a DNMT inhibitor followed by an HDAC inhibitor could be tolerated by patients with myeloid malignancies and to explore the possibility that such combinations would increase the response rate or the quality of responses following aza-CR administration. We combined this drug with the first HDAC inhibitor available for clinical use previously studied by our group (25, 26), sodium phenylbutyrate. This short-chain fatty acid has been successfully used to induce the production of fetal hemoglobin in patients with sickle cell anemia and β-thalassemia (27, 28). In phase I studies in patients with AML

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and MDS, steady-state plasma concentrations at the maximum tolerated dose ranged between 0.3 and 0.5 mmol/L (25, 26), well within the range that induces histone acetylation *in vitro* (29).

In this first exploration of the feasibility of combined DNMT and HDAC inhibition for the treatment of myeloid malignancies, we sequenced these drugs in a dose-finding study. Modeling the study after *in vitro* models, patients were treated with the DNMT inhibitor aza-CR followed by the HDAC inhibitor phenylbutyrate. In addition to clinical toxicity and response end points, this study aimed to determine to what extent aza-CR administration was associated with reversal of promoter methylation and whether clinical administration of phenylbutyrate was associated with induction of histone acetylation.

Materials and Methods

Patient Selection

The French-American-British categorization was used to classify patients with MDS and AML; when appropriate, the WHO category of refractory cytopenias with multilineage dysplasia (RCMD) was applied. AML arising from MDS is called either AML with trilineage dysplasia (AML-TLD) or AML-MDS. Patients with MDS age ≥18 years were eligible if they had refractory anemia (RA) with excess blasts (RAEB) or chronic myelomonocytic leukemia (CMMoL). Patients with RA or RA with ringed sideroblasts were eligible if they met one of several criteria for significant hematologic compromise: absolute neutrophil count <1,000/μL, untransfused hemoglobin <8 g/dL, platelet count <20,000/μL, and anemia or thrombocytopenia requiring transfusion or if they had high-risk cytogenetic abnormalities. Patients with relapsed or refractory AML were eligible if WBC was stably <30,000/μL for at least 2 weeks. Patients with untreated AML were eligible if they met one of several high-risk criteria: age >60 years, AML arising in setting of antecedent hematologic disorder (including AML-TLD previously classified as RAEB-t), high-risk chromosomes (abnormalities of chromosome 5 or 7, or complex cytogenetic abnormalities), or medical conditions that would compromise the ability to give cytotoxic chemotherapy as the primary treatment modality. Additional eligibility requirements for both groups included Zubrod performance status <2, serum creatinine and bilirubin <2.0 mg/dL, and absence of active infections. Administration of hematopoietic growth factors was not allowed during this trial; all growth factor administration was discontinued at least 3 weeks before protocol entry. All patients signed Institutional Review Board–approved informed consent according to Department of Health and Human Services guidelines; additional specific informed consent was obtained for the laboratory research use of patient specimens. Patient characteristics are listed in Table 1.

Treatment Plan

Aza-CR and phenylbutyrate were supplied through the Cancer Therapy Evaluation Program of the National Cancer Institute. All patients received aza-CR s.c. for 5, 10, or 14 days followed by phenylbutyrate given i.v. by continuous 7-day infusion. Aza-CR was supplied as 100 mg of white, lyophilized powder with 100 mg mannitol, USP in 30 mL flint vials. The contents of each vial were dissolved in 4 mL sterile water or 0.9% sodium chloride to provide a 25 mg/mL slurry. Doses were divided so that no single injection constituted >2 mL. Patients were instructed in reconstitution and rapid self-injection of aza-CR. Phenylbutyrate was administered via continuous infusion pump (CADD Legacy Plus ambulatory infusion pump Model 6500, SIMS Deltec, St. Paul, MN). Phenylbutyrate was supplied as 50 mL of a 40% viscous solution of phenylbutyrate in sterile water (400 mg/mL); the total daily dose was diluted into 1 liter sterile water. The aza-CR dose schedules studied are listed in Table 2; all patients received phenylbutyrate at 375 mg/kg/d by continuous infusion for 7 days beginning on the final day of aza-CR administration.

Treatment cycles were repeated every 28 days unless delays were required to allow for resolution of toxicities. All patients were to receive a minimum of four cycles of therapy. MDS responses were assessed using the International Working Group Response Criteria for MDS (30); only major responses were scored for hematologic improvement. Standard response criteria were used for patients with AML (30); however, MDS IWG criteria for hematologic improvement were also applied to AML patients when appropriate. Patients showing hematologic improvement at the end of the 4th month who did not yet meet the criteria for minimal response received an additional 2 months of therapy. Responding patients continued therapy until disease progression. Bone marrow examinations were repeated every 3 months in patients treated >6 months.

Toxicity was evaluated using the NCI CTC version 2.0. Any grade 3 or 4 nonhematologic toxicity thought possibly or probably related to study drug administration was considered dose limiting. Patients who developed reversible encephalopathy during phenylbutyrate infusion (a known toxicity of phenylbutyrate administration; refs. 25, 26) could receive a dose reduction of phenylbutyrate during subsequent cycles (325 mg/kg/d IVCI × 7 days). Because many patients with AML and MDS have marked cytopenias, hematologic toxicity was only considered evaluable in patients with pretreatment neutrophils >500/μL and platelet count >20,000/μL; dose-limiting hematologic toxicity in such patients was defined as neutropenia or thrombocytopenia below those critical thresholds lasting longer than 14 days.

Correlative Studies

Sample procurement. Bone marrow aspirates were obtained before treatment, during cycle 1 of therapy following the last dose of aza-CR, and again following the last dose of phenylbutyrate and before cycle 2. Peripheral blood was obtained during cycle 1 before study; before phenylbutyrate, 4, 8, and 24 hours following phenylbutyrate; and at least once more during the infusion of phenylbutyrate as well as before cycle 2. Peripheral blood and bone marrow mononuclear cells were isolated using Ficoll-Hypaque (SG <1.077). Mononuclear cells were divided into aliquots for histone extraction before freezing (see below) and RNA and DNA preparation using standard techniques before freezing.

Bisulfite sequencing. Genomic bisulfite sequencing for the p15 promoter was done in selected patient samples as described to validate changes in promoter methylation (31). Primers used were 5′-TGAAGGAATAGAAATTTTTTGTTT-3′ and 5′-AAGCAAGCTTAAACCCTAAAACCCCAACTACCTAA-3′ for the initial amplification, followed by a nested PCR using the internal primers 5′-GGGGATTAGGAGTTGAG-3′ and 5′-ACCCTAAAACCCCAACTACC-3′. The large size of this product precluded amplification from bisulfite-treated DNA in some cases. For these samples and time points, a smaller nested PCR was done initially using the primer pair 5′-TAGGTTTTTTGGTTTAGTTGAAAA-3′ and 5′-CTCCTTCCTAAAAAACCTAAACTCAAC-3′, followed by internal primers 5′-TTTGTAGGTTGGTTTTTTATTTTGTTA-3′ and 5′-AAAACCTAAACTCAACTTCATTACCCT-3′. This product includes the central 303 bp region of the $p15^{INK4b}$ CpG island.

Methylation-specific PCR. Methylation-specific PCR (MSP) was used to detect the presence of methylated alleles for specific genes. MSP was done as previously described (32, 33). All pretreatment samples were screened for promoter methylation of $p15^{INK4B}$. In some cases, *CDH-1* (E-cadherin) methylation was also studied. Genes found to be methylated in the pretreatment specimen were monitored for changes in methylation using an MSP technique.

Histone acetylation. Histones were extracted (34) and proteins were quantified (Bio-Rad, Hercules, CA). Fifty micrograms of protein were separated by 15% SDS-PAGE gels and transferred onto nitrocellulose membranes. Primary rabbit polyclonal antibodies to human acetylated histone H3 and H4 and nonacetylated H2 (Upstate Biotechnology, Lake Placid, NY) were used for Western blot analysis, followed by horseradish peroxidase–conjugated secondary antibodies and visualization using enhanced chemiluminescence (Kodak, Scientific Imaging systems, Rochester, NY). The nonacetylated H2 band was easily resolved from the Ac-H3 and Ac-H4 band and served as a control for histone extraction and protein loading. The gels were scanned and the band density was quantified (UN-Scan-It, Silk Scientific, Orem, UT). Beginning with dose cohort 3, results for Ac-H3 and Ac-H4 were quantified as percentage of nonacetylated H2A ("acetylation index"); routine blotting for H2A was not done in all patients before this dose cohort.

F-cell quantification. Red cells containing hemoglobin F (F cells) were quantified using a standard flow cytometric technique (28).

Table 1. Patient characteristics

UPN	Age	Sex	Diagnosis	Dose cohort	Cytogenetics	International Prognostic Score	Previously treated	Comments	Response
1	58	M	AML-TLD	1	+13		No		NR
2	74	M	AML-TLD	1	WNL		No		NR
3	57	M	AML-rel	1	Del5q, −7		Induction/consolidation		HI-Nmaj
4	62	M	RA	1	WNL	Int-1	Growth factors		HI-Pmaj
5	80	F	AML-TLD	1	WNL		No		NR
6	58	M	RAEB	1	Del7q	Int-1	Induction		NR
7	66	M	RA	2	−7	Int-2	Immunosuppression		NR
8	71	M	AML-TLD	2	WNL		No		NR
9	54	F	AML-rel	2	Three clones: +8; idem t(9;12); +15		Induction/salvage		NR
10	66	M	t-RAEB	2	Complex	Int-2	Growth factors	History of adjuvant therapy for pancreatic cancer	NR
11	66	M	RA	2	WNL	Int-1	Growth factors		NR
12	76	M	AML-TLD	2	2 clones: +14; idem, t(X;9)		No		NR
13	47	F	RCMD	3	WNL	Int-1	No		PR
14	73	F	AML-TLD	3	WNL		Growth factors		NR
15	81	F	RAEB	3	WNL	Int-1	Growth factors		NR
16	78	M	AML-TLD	3	+8		Growth factors	Never completed first cycle	NE
17	64	M	AML-TLD	3	+8		No		NR
18	71	M	AML-TLD	3	WNL		No		NR
19	63	F	AML-TLD	3	Del9q		No		CR
20	72	M	AML-TLD	1	Complex		Growth factors	Never treated	NE
22	51	F	AML-TLD	4	Del11q		No		NR
23	67	M	RAEB	4	Complex	High		Never completed first cycle	NE
24	71	M	RAEB	4	+8	High	Growth factors		HI-Pmaj
25	85	M	AML-TLD	4	del5q, +11, i14		No		HI-Pmaj, HI-Nmaj
26	64	M	RAEB	3	Del5q	Int-1	Growth factors, bryostatin		CR
27	77	M	RA	3	−Y	Low	Growth factors, corticosteroids	Treatment initiated due to steroid-refractory vasculitis attributed to MDS	CR
29	53	F	t-RAEB	5	−7	Int-2	No	History of autologous stem cell transplantation for non-Hodgkin's lymphoma	HI-Nmaj,
30	73	F	AML-TLD	5	+8		Growth factors, cytarabine, gemtuzumab		HI-Pmaj, HI-Emin
33	72	F	AML-TLD	5	Complex		Growth factors, thalidomide		CR
34	47	M	RAEB	5	Two clones: +21; idem +8	Int-2	Growth factors		NR
35	41	F	AML-Rel	5	Inv(16)		Induction/consolidation/ autologous SCT/salvage		NR
36	66	M	CMMoL	5	Two clones: del7q; idem, +21	Int-1	Growth factors		NR

NOTE: WNL indicates normal cytogenetic analysis. Patients who consented to study but were screen failures or withdrew consent before treatment are not displayed. Four screen failures were not included.

Abbreviations: NR, no response; HI-Nmaj, hematologic improvement neutrophils, major; HI-Pmaj, hematologic improvement, platelet, major; HI-Emajor, hematologic improvement, erythroid, major.

Table 2. Accrual to dose-finding trial of aza-CR plus phenylbutyrate in MDS and AML

Cohort	Aza-CR dose ($mg/m^2/d$)	Days of aza-CR	Patients assigned to cohort	Number not evaluable	Number reduced to this cohort	Number increased to this cohort	No. patients evaluated at cohort*	Responses total/major*
1	75	5	7	1	1†	0	6	1/0
2	50	5	6	0	1‡	0	7	1/0
3	50	10	9	1	2§	1‖	9¶	5/4
4	50	14	4	1	0	0	1	1/0
5	25	14	6	0	0	0	6	3/1
Total			32	3			29	11/5

*For patients who underwent dose escalation or dose reduction, responses are tallied in their final dose cohort.
†Patient originally in dose cohort 4, reduced to dose cohort 3, then reduced to cohort 1.
‡Patient reduced from dose cohort 1.
§Both patients reduced from dose cohort 4; one of these patients was subsequently further reduced to dose cohort 1.
‖Patient dose increased from cohort 5 following lack of response after four cycles.
¶Only nine patients were evaluated at this dose cohort because the patient dose reduced from dose cohort 4 was further dose reduced to cohort 1 and is tabulated in that cohort. The patient dose escalated from cohort 5 was a nonresponder after an additional four cycles at cohort 3, but is not included in this primary analysis of response.

Aza-CR pharmacokinetics. Samples were obtained before treatment and at 0.25, 0.5, 1, 2, 4, 8, and 24 hours after aza-CR administration. Samples were processed and frozen within 30 minutes by centrifugation at 3,000 × *g* for 5 minutes in a refrigerated centrifuge. Plasma was divided into multiple aliquots to avoid freeze/thaw cycling and was stored at −80°C until analysis. Initially, samples were collected without tetrahydrouridine. Following observation of samples in which aza-CR could not be detected, presumably due to presumed breakdown by cytidine deaminase, tetrahydrouridine was added to the plasma supernatant at a final concentration of 100 μmol/L to increase the stability of drug in plasma during storage in the freezer. Tetrahydrouridine increased freezer stability from 7 to ~21 days when frozen at −80°C (35, 36). Aza-CR was quantified in plasma using a previously described validated liquid chromatography/tandem mass spectrometry method over the range of 5 to 500 ng/mL (20.4 to 2,041.6 nmol/L; ref. 36).

Individual concentration-time data were analyzed using noncompartmental methods using WinNonlin Professional version 3.1 as reported elsewhere (Pharsight, Mountain View, CA; ref. 35). Pharmacokinetic variables were summarized using descriptive statistics.

Phenylbutyrate pharmacokinetics. Samples were obtained before treatment and on days 4 and 7 of phenylbutyrate infusion. Samples were collected in heparinized tubes (5 mL) and centrifuged at 3,000 rpm for 5 minutes; plasma was aliquoted and stored at −80°C until the time of analysis. Samples were analyzed for phenylbutyrate and two metabolites, phenylacetylglutamine and phenylacetic acid, using a high-performance liquid chromatography method using UV detection (25, 26, 37–39). The linear calibration curves were generated over the range of 10 to 3,000 μmol/L.

Average steady-state concentrations (C_{ss}) were calculated for phenylbutyrate, phenylacetylglutamine, and phenylacetic acid based on graphical presentation of concentration-time profiles.

Statistical considerations. The primary end points of this trial were to evaluate the toxicities and responses associated with the different dosing regimens. Toxicities were recorded for each individual. The responses were graded as described above and response rates were calculated as percentages with 95% confidence intervals both for the entire cohort as well as within each treatment group. Among the responders, duration of individual responses and the median response time were calculated. In addition, median cycle times and ranges are reported for durational outcomes of interest, such as time until response.

Changes in biological variables over time were represented graphically with additional tests for associations. For each dose group, the association between the mean percentage of F cells and time was tested using a one-way ANOVA. ANOVA was used to determine the association between aza-CR or phenylbutyrate exposure and clinical response expressed as a categorical variable [i.e., 1, a major response (CR or partial response, PR); 2, minor response (i.e., hematologic improvement/lineage response); 3, no response]. A paired *t* test was used to determine the association between aza-CR or phenylbutyrate exposure and methylation changes, methylation reversal–induced F cells, and acetylation changes expressed as a categorical variable (i.e., response or no response). The aza-CR exposure variables explored were maximum plasma concentrations (C_{max}), area under the concentration-time curve (AUC) from time 0 to 2 hours ($AUC_{0\text{-}2h}$), AUC from time 0 to 4 hours ($AUC_{0\text{-}4h}$), AUC from time 0 to the last quantifiable time point ($AUC_{0\text{-}tf}$), and AUC extrapolated to infinity ($AUC_{0\text{-}\infty}$). The phenylbutyrate exposure variables explored were C_{ss} for phenylbutyrate, phenylacetylglutamine, and phenylacetic acid. Statistical analysis was done using JMP Statistical Discovery Software version 3.2.6 (SAS Institute, Cary, NC). The *a priori* level of significance was $P < 0.05$.

Results

Study Population

Thirty-six patients signed consent; four patients were screen failures. Characteristics of treated patients are listed in Table 1. Median age was 66 years (41-85 years). Thirteen patients had MDS (including two treatment-induced patients), 1 CMMoL, 15 AML-TLD (MDS-AML), and 3 relapsed AML. Ten of fourteen (71%, 95% confidence interval, 41-92) patients with MDS or CMMoL and 14 of 18 (78%, 95% confidence interval, 52-94) patients with AML had clonal cytogenetic abnormalities. International Prognostic Score categories among MDS/CMMoL patients were as follows: low, 1; Int-1, 7; Int-2, 4; high, 2 (40).

Toxicity

No unusual toxicities were discovered in this trial. Three patients withdrew consent before completing a single cycle: one because of needle phobia, one because of poor performance status, and one for undisclosed personal reasons. Mild nausea was frequent in patients receiving aza-CR and occasional in patients receiving phenylbutyrate; nausea was alleviated in all cases with either 10 mg prochlorperazine or 100 mg dolasetron. Injection site reactions were common in response to aza-CR. One patient in dose cohort 1 developed severe asthenia requiring a dose reduction of aza-CR to 50 $mg/m^2/d \times 5$ days. Encephalopathy attributed to phenylbutyrate occurred in nine patients. As in the previous phase I studies of phenylbutyrate, encephalopathy completely reversed within 24 to 48 hours of stopping the infusion. In four cases,

encephalopathy was mild and the 7-day infusion could be successfully completed. Three patients received dose reductions of phenylbutyrate in subsequent treatment cycles (325 mg/kg/d). Two patients tolerated the lower dose; one patient developed severe recurrent encephalopathy and was removed from the trial following his third cycle. The final patient decided not to resume treatment upon recovery from initial symptoms of encephalopathy.

Dose-limiting hematologic toxicity was observed in dose cohort 4 (50 mg/m^2/d × 14 days). These toxicities include dose-limiting neutropenia (one patient), dose-limiting thrombocytopenia (two patients), and septic death during neutropenia in cycle 2 (one patient). One surviving patient tolerated dose reduction to 50 mg/m^2/d × 10 days; the other patient had recurrent dose-limiting neutropenia at both 50 mg/m^2/d × 10 days and 75 mg/m^2/d × 5 days and was removed from study. No dose-limiting toxicities occurred in dose cohort 5.

Clinical Outcomes

Three patients received less than one complete cycle of therapy and are not considered evaluable for response. Overall, 11 of 29 evaluable patients responded, including 5 of 9 evaluable patients in dose cohort 3 (one patient reduced from dose cohort 4) and 3 of 6 evaluable patients in dose cohort 5. Five were major responders (four CR/one PR); these responders were all treated in dose cohorts 3 (four patients) and 5 (one patient). Seven of 11 responders, including three of the major responders, had clonal cytogenetic abnormalities before treatment. The three major responders had complete cytogenetic responses; none of the minor responders developed cytogenetic remission. Major responses developed in two patients with AML-TLD, one patient with RAEB, one patient with RA with steroid-refractory MDS-associated vasculitis (complete hematologic and rheumatologic response), and one patient with RCMD (complete hematologic response with evidence of ongoing dysplasia in bone marrow). Response duration among major responders was 8, 10, 15, and 19+ months. The patient considered to have "progressed" at 8 months showed cytologic evidence of recurrent MDS with recurrent neutropenia following AML-TLD; complete cytogenetic response persisted.

Minor responses (IWG hematologic improvement-major) occurred in four patients with RAEB (including a patient with therapy-related MDS) and two patients with AML-TLD including four monolineage and two bilineage response (Table 2). Median duration of response among minor responders was 3 months; four patients were removed from protocol with ongoing response because of persistent cytopenias in other lineages or persistent increases in blasts. Among patients who achieved major hematologic improvement, the median number of treatment cycles to observe response (range) were as follows: platelets 1.5 (1-4, n = 7); reticulocytes 3 (2-3, n = 6); hemoglobin 3 (2-4, n = 6); neutrophils 1 (1-5, n = 7). No trend was observed in time to response according to the aza-CR dose cohort.

Changes in Promoter Methylation

DNA methylation in bone marrow cells was examined during the first course of drug administration in dose cohorts 3 to 5 to monitor changes within tumor clones rather than monitoring alterations stemming from replacement of neoplastic cells by normal cells. Genomic bisulfite sequencing of a hypermethylated *p15* promoter was done in five patients (Fig. 1). Sequential sequencing of DNA from patients who had significant responses to the combined therapy, patients 13 (PR, cohort 3), 19 (CR, cohort 3), and 24 (hematologic improvement, cohort 4, reduced to dose cohort 3) showed significant decrement in *p15* methylation following aza-CR treatment. Importantly, we saw a pattern of heterogeneous loss of CpG methylation within each single allele examined, strongly supporting demethylation within the tumor clone. The bisulfite sequencing of patient 19 showed evidence of remethylation before initiation of the next drug cycle. No demethylation was observed in the *p15* promoter in two clinical nonresponders (patient 15, cohort 3; patient 34, cohort 5).

Twelve patients evaluable for clinical response were subsequently studied by MSP, a sensitive PCR assay for the methylation of multiple CpG sites in a CpG island (33) for *p15* and/or *CDH-1*. The primers completely overlap the region of the *p15* promoter sequenced in the above studies and a region of the *CDH-1* gene, which we have used in multiple previous studies. All 12 patients were informative for pretreatment hypermethylation of either or both genes. Six patients developed reversal of methylation for either or both genes during the first cycle of treatment. The methylation status over time of the two genes changed in parallel in three of four patients informative at both loci (Fig. 2*A*). Particularly noteworthy is patient 19: No evidence of methylation of either gene was present following aza-CR (day 10); however, some evidence of remethylation of both genes was present following phenylbutyrate (day 17). Most importantly, these six patients with reversed methylation of either gene all developed hematologic responses, including three CR patients, one PR, and two patients who achieved hematologic improvement. In contrast, the six patients who did not show methylation reversal were all nonresponders. The difference in response rates between the two groups was statistically significant (P = 0.002, Fisher's exact test).

Histone acetylation. Changes in histone acetylation in peripheral blood and bone marrow mononuclear cells were monitored using Western analysis. A representative example is displayed in Fig. 3*A*. Eleven of 23 evaluable patients showed increases (minimum 50%) in global acetylation of H3 and/or H4 in response to aza-CR alone, similar to the example shown in Fig. 3*A*. This was observed in one of five patients in dose cohort 1, three of four in dose cohort 2, five of seven in cohort 3, and one of three each in cohorts 4 and 5 (P = not significant between cohorts). Further increases in acetylation in response to phenylbutyrate (minimum 50%) were found in 12 of 23 patients. Overall, 17 of 23 patients developed increased histone acetylation following either aza-CR or phenylbutyrate. Figure 3*B* displays the changes in histone acetylation from patients in dose cohort 3 semiquantitatively. Blots were stripped and reprobed with antibody to nonacetylated histone H2A. Gels were scanned and band intensity was quantified. The data were then reported as an acetylation index: band intensity acetylated H3 or H4/band intensity nonacetylated H2A. The greatest increment in acetylation appeared following aza-CR administration; phenylbutyrate induced a smaller increment in histone acetylation in many patients. Acetylation remained elevated compared with baseline during phenylbutyrate infusions even in patients who showed no further increment in acetylation in response to phenylbutyrate.

F cells. Changes in percentage of RBC containing hemoglobin F (F cells) were monitored as an indirect measure of induction of expression of a surrogate gene silenced through epigenetic mechanisms (*γ globin*). Baseline F-cell data were obtained on 26 patients who consented to study. The mean percentage F cells was 7.9% (median 4.9%, range 0.9-48.3%). There was no statistical difference between the mean percentage F cells of patients with the diagnoses of MDS or AML nor was there a statistical difference in the proportion of patients with each diagnosis in patients whose F cells were above or below the mean.

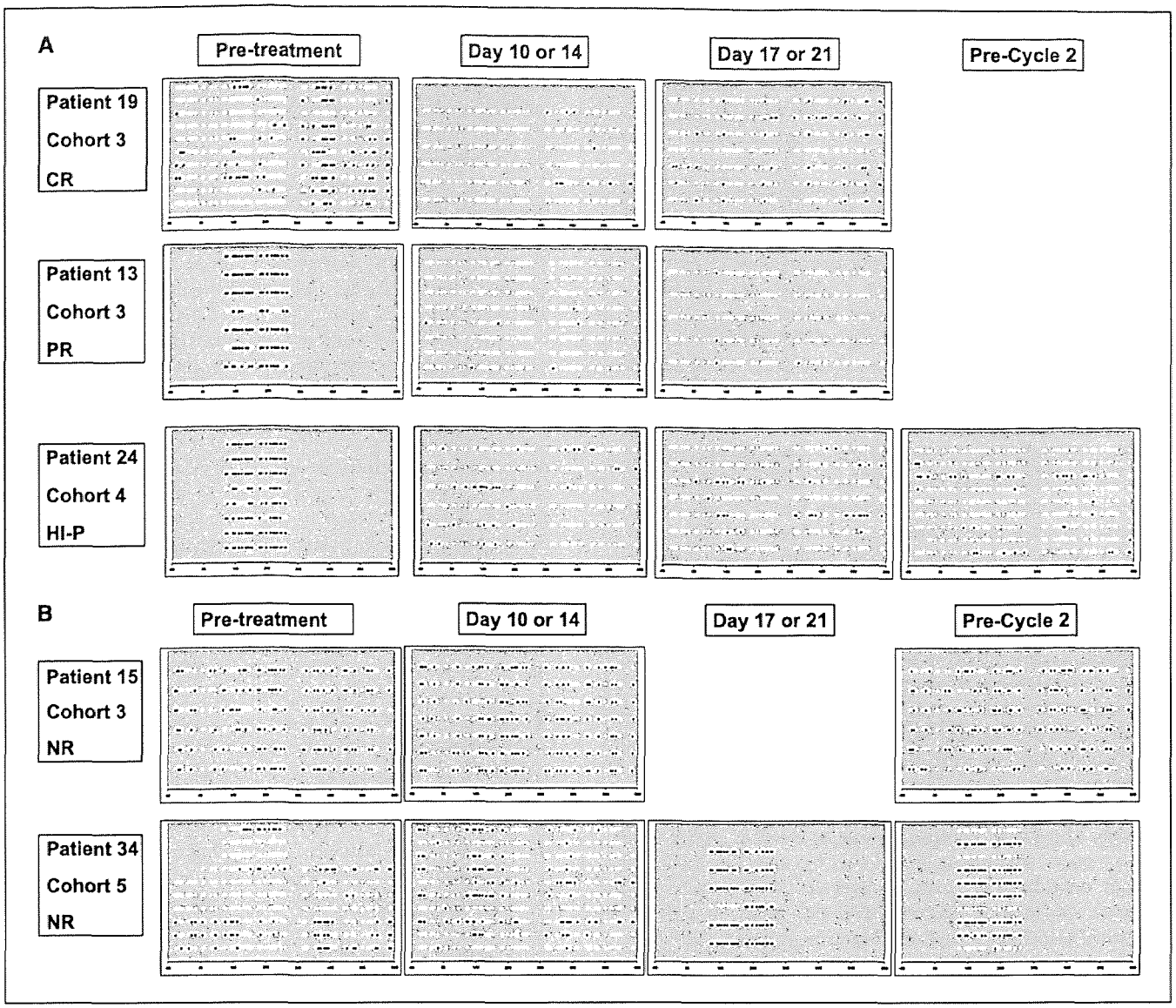

Figure 1. Genomic bisulfite sequencing of *p15* promoter region CpG island during cycle 1. Individual allele patterns: *line*, cloned and sequenced DNA molecule; ○, unmethylated CpG sites; ●, methylated CpG sites. Numbering is according to the transcriptional start site (nucleotide 1218 from Genbank sequence AF513858), which is +1. Bone marrow mononuclear cells were procured before therapy, following the last injection of aza-CR, following discontinuation of phenylbutyrate, and before cycle 2. *A*, clinical responders. *B*, clinical nonresponders.

Sequential F-cell data were obtained on 24 patients (Fig. 4). Six patients increased percentage of F cells over time. Three patterns of F-cell induction were observed: a gradual induction throughout the first cycle of therapy (three patients, all in dose cohort 3); delayed induction, coincident with clinical response (one patient in dose cohort 3 and one in dose cohort 5); and transient increase in F-cell production in response to phenylbutyrate (one patient in dose cohort 2). Each cohort was examined for a correlation between time point and F-cell percentage by ANOVA. An association between F cell and treatment time, indicating an increase in F cells over time, was found only dose cohort 3. Three of four clinical responders in cohort 3 who were evaluated for F-cell response developed significant increases in F cells. The other patient in this cohort with an F-cell response did not respond clinically. The patients in cohorts 2 and 5 who developed significant increases in F cells were clinical nonresponders. No significant correlation between changes in F cells and clinical response was found when all evaluable patients were examined.

Aza-CR pharmacokinetics. The results of the aza-CR pharmacokinetics, combined with results from a trial in nonhematologic tumors, have been reported elsewhere (35). The current analysis includes data beginning with patient 18; tetrahydrouridine, required for stability of aza-CR, was added to the plasma starting with this patient. Aza-CR was rapidly absorbed with the mean T_{max} occurring at 0.50 hour. Average maximum concentration (C_{max}) and area under the curve ($AUC_{0\text{-infinity}}$) values increased in a dose-proportionate manner with increasing dose from 25 to 75 mg/m^2/d; the mean ± SD C_{max} and $AUC_{0\text{-}8}$ at 25 mg/m^2/d were 1,612 ± 1,042 nmol/L and 1,941 ± 980 h × nmol/L, respectively, and at 75 mg/m^2/d was 6,207 nmol/L and 5,831 h × nmol/L, respectively. Aza-CR has a short terminal half-life of 1.04 ± 0.88 hours.

A trend to correlation between aza-CR $AUC_{0\text{-}8}$ and reversal of methylation was detected among the small number of patients

with complete methylation and pharmacokinetic data (n = 9, P = 0.0658; Fig. 2*B*). Aza-CR exposure was not correlated with clinical response, changes in histone acetylation, or induced F cells.

Phenylbutyrate pharmacokinetics. The median steady-state phenylbutyrate plasma concentration was 322.5 μmol/L (mean 562.7 ± 818.9, range 19.5-4,371.2). For phenylacetic acid, the median steady-state plasma concentration was 723.9 μmol/L (mean 1,617.1 ± 1987.6, range 250.2-6,769.2). For phenylacetylglutamine, the median steady-state plasma concentration was 440.3 μmol/L (mean 677.6 ± 457.1, range 153.4-1,520.9). A total of six patients displayed an unidentified phenylbutyrate metabolite in the plasma samples, which was quantified from the phenylbutyrate standard curve. For the unknown metabolite, the median steady-state plasma concentration was 187.1 μmol/L (mean 192.8 ± 71.6, range 84.6-292.9). The median steady-state plasma concentration of patients who showed an increase in acetylation following phenylbutyrate was 237 μmol/L (mean 317.1 ± 100.3). The median steady-state plasma concentration of patients who did not increase acetylation during phenylbutyrate was 343.4 μmol/L (mean 976.4 ± 457.5). Phenylbutyrate and metabolite exposure was not correlated with clinical response, changes in histone acetylation, induced F cells, or reversal of methylation.

Discussion

The DNMT inhibitors aza-CR and DAC comprise two of the most active agents for the treatment of MDS. Despite data demonstrating an ~50% hematologic response rate (23, 41, 42), these agents were developed empirically without careful dose finding, and the molecular mechanism underlying their clinical activity remains unclear. Issa et al. (22) studied DAC administered from 5 to 20 mg/m^2/d i.v. for 10 to 20 doses in patients with a variety of hematologic malignancies. The data suggested a potentially improved response rate (65%) in the patients treated at 15 mg/m^2/d for 10 doses. Responses were not correlated with baseline p15 methylation or changes in *p15* methylation in peripheral blood mononuclear cells during treatment (22).

Daskalakis et al. (43) monitored *p15* methylation during treatment of MDS patients with a more dose-dense schedule of DAC (45 mg/m^2/d × 3 days). This group detected decreased methylation in 9 of 12 patients studied investigated at a median time following 3.5 cycles of DAC (range 1-6 cycles). Mund et al. (44) showed decreased methylation in three of four MDS patients treated with DAC, determined following the first cycle of DAC in only two patients. Significantly decreased methylation could be detected after cycle 2 in one patient, cycle 4 in a second patient, and cycle 5 in the third patient. Karyotype normalization preceded changes in genomic methylation; thus, the methylation changes measured likely occurred in normal cells and were irrelevant to proximal molecular changes induced by DAC.

The current study is the first to perform bisulfite sequencing of hypermethylated gene promoters in bone marrow cells during the first cycle of aza-CR. Immediately following the first 10- to 14-day exposure to aza-CR in three clinically responding patients, bone marrow cells possess heterogeneously methylated alleles and an incomplete demethylation pattern. This observation confirms that changes in methylation detected following aza-CR do not arise from the replacement of the abnormal (methylated) clone with normal (unmethylated) cells following a cytotoxic event. The precise parallels between the bisulfite sequencing data and MSP results allowed us to use the latter procedure to monitor methylation of two gene promoters in clinical samples. The results, requiring confirmation in a larger series, revealed a tight correlation between initial tumor clone changes in hypermethylation of the p15 and CDH-1 promoters and clinical response. The development of cytogenetic complete remission in a patient in whom partial demethylation developed in malignant cells during cycle 1 suggests that these agents, which target epigenetically silenced transcription, may activate genetic programs leading to terminal differentiation, apoptosis, or senescence. More normal clones can then replace the starting population. Partial demethylation fits well with the possibility that HDAC inhibitors may increase therapeutic efficacy by contributing to reexpression of

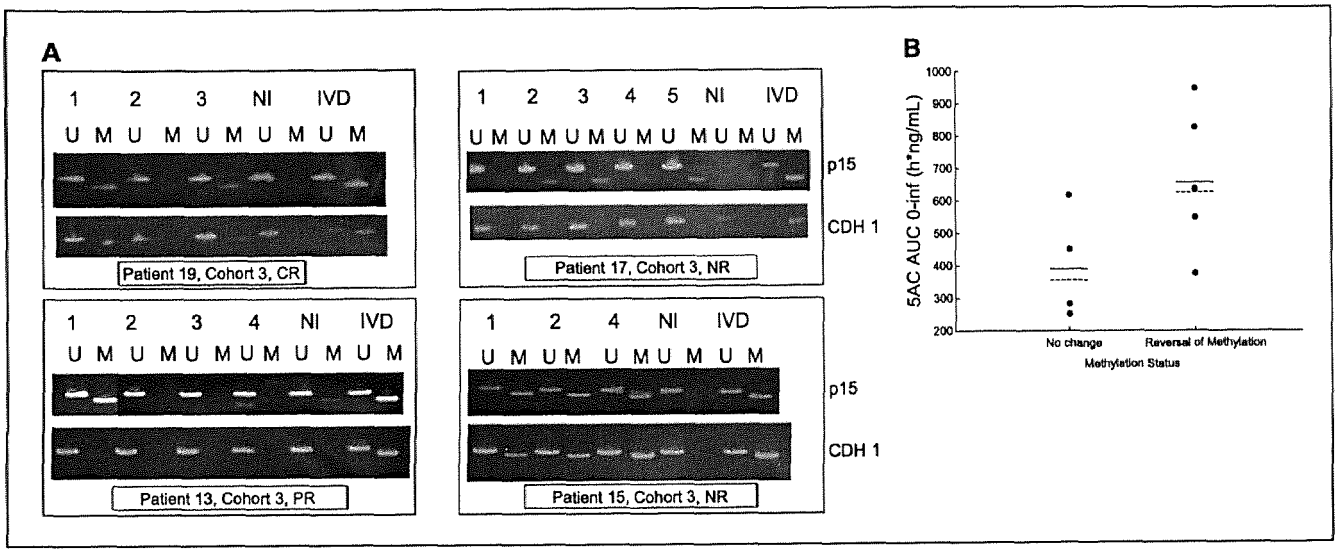

Figure 2. Changes in methylation of *p15* and *CDH-1* promoters during cycle 1 of treatment. *A*, bone marrow mononuclear cells were procured before therapy (*1*), following the last injection of aza-CR (*2*), following discontinuation of phenylbutyrate (*3*), and before cycle 2 (*4*). In one patient, a fifth time point was procured after four cycles of treatment. Methylation of p15 and CDH-1 promoters was determined using MSP. The four representative patients were all treated in dose cohort 3 (50 mg/m^2/d aza-CR for 10 days). *NI*, normal peripheral blood lymphocytes. *IVD*, positive methylation control. *B*, reversal of promoter methylation for either *p15* and or *CDH-1* after one cycle of therapy is plotted against the aza-CR AUC for the first dose of drug for nine patients with complete set of methylation and pharmacokinetic data. Correlation approached statistical significance (P = 0.0658) *Dotted line*, median concentration. *Solid line*, mean concentration.

densely hypermethylated genes once some demethylation is initiated by drugs, such as aza-CR, as shown *in vitro* (14, 15).

The pharmacokinetic/pharmacodynamic studies suggest that reversal of promoter methylation may correlate with AUC for aza-CR. Nonresponse may be due to failure of adequate exposure of the target to drug, raising hopes that adjusted dosing schedules might improve clinical outcomes further. The relationship between aza-CR AUC and methylation reversal could be due to genetic polymorphisms affecting drug metabolism; one candidate would be differences in cytidine deaminase, the primary catabolic enzyme for aza-CR. Polymorphisms and differences in expression of cytidine deaminase and other catabolizing enzymes have been associated with *in vivo* resistance to cytarabine (45–48). These data suggest that more easily administered formulations of aza-CR could be developed, with potential for an adaptive control mechanism to increase the percentage of patients with promoter methylation reversal.

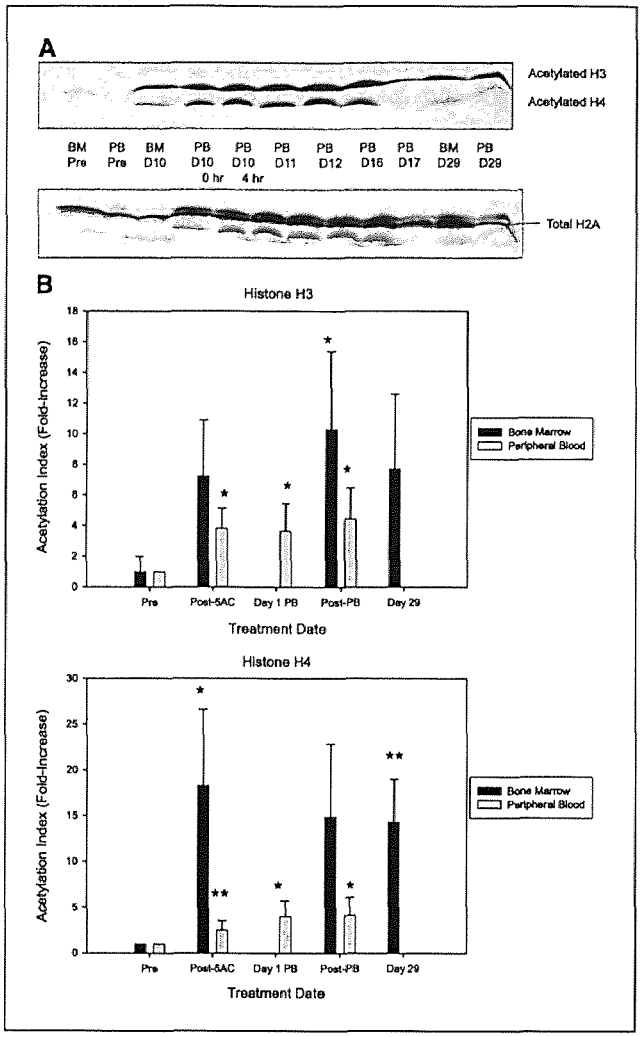

Figure 3. *Changes in global histone acetylation during cycle 1 of therapy.* Histones were isolated from peripheral blood and bone marrow mononuclear cells during cycle 1 of therapy. Immunoblotting was done using antibodies to acetylated H3 and H4. Blots were then stripped and reprobed using antibody against nonacetylated histone H2A as a control for the efficiency of histone extraction and protein loading. *A*, representative patient (patient 14, cohort 3, AML-TLD, nonresponder). *Pre*, pretreatment. *D*, day of treatment. Blotting of H2A is done following stripping of the membrane after blotting for acetylated H3 and H4. Some residual Ac-H3 and Ac-H4 continues to be visualized on the H2A blot. *B*, changes in histone acetylation during cycle 1 in response to aza-CR and phenylbutyrate in patients in dose cohort 3. Data are reported as fold increase compared with baseline, using an acetylation index (see text). *Columns*, mean of six patients; *bars*, SE. **, $P < 0.025$, mean fold increase >1; *, $P < 0.05$, indicates mean >1.

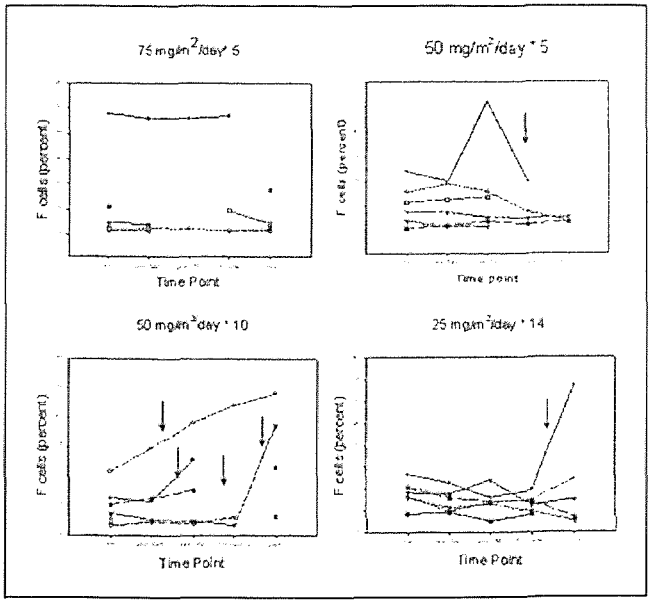

Figure 4. Changes in F cells over time. The percentage of F cells in patients' peripheral blood was monitored using flow cytometry before treatment, after aza-CR, after phenylbutyrate, before cycle 2, and at later time points (either before cycle 3 or 4). The patient data are displayed by dose cohort. In patients in whom a value is missing, the trend lines are discontinuous. *Arrows*, patients with significant increase.

The current study design does not allow assessment of the specific biochemical contribution of the HDAC inhibitor. Ultimately, a randomized trial of a DNMT inhibitor with and without an HDAC inhibitor will be required. Increases in global histone acetylation were observed in the majority of patients studied. The induction of histone acetylation in response to aza-CR before the HDAC inhibitor was unexpected. The mechanism by which aza-CR led to histone acetylation is currently unknown; it is not clear whether this is a DNMT-dependent phenomenon and whether similar induction of histone acetylation occurs in response to *in vivo* administration of DAC. Increases in acetylation could be specifically attributed to phenylbutyrate in only 12 of 23 patients. This may be due to maximal induction of acetylation in response to aza-CR alone. The median steady-state plasma concentration of phenylbutyrate was consistent with previous phase I studies and would be predicted to be adequate for the induction of acetylation (25, 26, 37–39). However, the large percentage of patients evaluable for acetylation who developed increments in this end point (17 of 23) precludes assessment of the contribution of HDAC inhibition to clinical response.

From a clinical perspective, larger trials will be required to assess the efficacy of combining aza-CR with HDAC inhibitors in the treatment of MDS and AML. The current trial shows the clinical feasibility of combining a DNMT and HDAC inhibitor. Our current study also suggests that aza-CR in combination with phenylbutyrate yields a potential enrichment in major responses in patients who received aza-CR at 50 mg/m^2/d × 10 days followed by phenylbutyrate. In the definitive Cancer and Leukemia Group B study of aza-CR, the CR plus PR rate was 23% (23). In dose cohort 3, four of nine evaluable patients achieved CR or PR. These numbers are small and require confirmation in a phase II setting; however, they suggest the possibility that prolonged exposure to lower doses of a DNMT inhibitor and/or the addition of an HDAC inhibitor may increase the major response rate.

In summary, we have developed a well-tolerated combination schedule of aza-CR and HDAC inhibitor, which induces promoter methylation reversal, global histone acetylation, F-cell production, and clinical responses. This first study provides a recommended phase II dose schedule for aza-CR (50 mg/m^2/d × 10 days s.c.) in studies involving comparisons without an HDAC inhibitor to fully evaluate the efficacy of combining such drugs. We have also shown the first solid evidence that demethylation of hypermethylated genes in tumor clones is a target effect of aza-CR in patients who respond to therapy with this drug. This provides a possible assay to make early judgments about efficacy in individual patients and may be evidence that demethylation may underlie the responses themselves.

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Induction of MHC class I molecule cell surface expression and epigenetic activation of antigen-processing machinery components in a murine model for human papilloma virus 16-associated tumours

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Summary

Epigenetic events play an important role in tumour progression and also contribute to escape of the tumour from immune surveillance. In this study, we investigated the up-regulation of major histocompatibility complex (MHC) class I surface expression on tumour cells by epigenetic mechanisms using a murine tumour cell line expressing human E6 and E7 human papilloma virus 16 (HPV16) oncogenes and deficient in MHC class I expression, as a result of impaired antigen-presenting machinery (APM). Treatment of the cells with the histone deacetylase inhibitor Trichostatin A, either alone or in combination with the DNA demethylating agent 5-azacytidine, induced surface re-expression of MHC class I molecules. Consequently, the treated cells became susceptible to lysis by specific cytotoxic T lymphocytes. Further analysis revealed that epigenetic induction of MHC class I surface expression was associated with the up-regulation of APM genes [transporter associated with antigen processing 1 (*TAP-1*), *TAP-2*, low-molecular-mass protein 2 (*LMP-2*) and *LMP-7*]. The results demonstrate that expression of the genes involved in APM are modulated by epigenetic mechanisms and suggest that agents modifying DNA methylation and/or histone acetylation have the potential to change the effectiveness of antitumour immune responses and therapeutically may have an impact on immunological output.

Keywords: antigen processing; cancer; human papilloma virus; major histocompatibility complex class I; epigenetics

Introduction

Tumour cells are able to escape specific immune responses mediated by cytotoxic T cells (CTLs) by down-modulation of the surface expression of major histocompatibility complex (MHC) class I molecules.[1] Reversible MHC class I down-modulation is frequently mediated by inhibition of the transcription of genes involved in the antigen-processing machinery (APM),[2,3] namely genes encoding proteasome subunits, low-molecular-mass proteins LMP-2 and LMP-7, and transporters associated with antigen processing TAP-1 and TAP-2. Transcription of these genes can be induced by cytokines [interferon (IFN)-γ and tumour necrosis factor (TNF)-α], leading to restoration of surface expression of MHC class I molecules, which makes tumour cells susceptible to lysis by CTLs.[4,5] In general, agents that increase the expression of MHC class I molecules can be of therapeutic significance.[6]

Epigenetic modifications of the human genome, namely altered histone acetylation or aberrant DNA methylation, represent tumorigenic events that are functionally equivalent to genetic changes.[7] Re-expression of genes down-modulated by DNA methylation can be achieved by exposure to inhibitors of DNA methyltransferase, 5-aza-2′-deoxycytidine (DAC) or 5-azacytidine (5-azaC). The

Abbreviations: 5-azaC, 5-azacytidine; APM, antigen-processing machinery; ChIP, chromatin immunoprecipitation; c.p.m., counts per minute; C_t, threshold cycle; CTL, cytotoxic T lymphocyte; DAC, 5-aza-2′-deoxycytidine; HPV, human papilloma virus; IFN-γ, interferon gamma; MHC, major histocompatibilty complex; MSP, methylation-specific polymerase chain reaction; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; RT-PCR, reverse transcription–polymerase chain reaction; TSA, Trichostatin A.

effect of these inhibitors can be increased by inhibitors of histone deacetylases, for example Trichostatin A (TSA)[8] or sodium butyrate,[9] which can also be used for treatment without DNA demethylation agents. The antitumour therapeutic potential of these compounds has been tested in clinical studies, and the targets of choice in the experimental setting are usually epigenetically silenced tumour suppressor genes.[10] The expression of immunoactive (e.g. MHC class I or II), costimulatory and adhesive molecules on tumour cells, cytokine production and the expression of tumour-rejecting antigens can also be directly or indirectly modulated by epigenetic mechanisms.[11–15] Hence, the reversal of the immune escape phenotype of tumour cells represents one of the possible modalities by which 'epigenetic agents' can inhibit growth of the tumour.[16]

Down-modulation of MHC class I expression by hypermethylation of MHC class I genes has been documented in melanoma cells, and the combination of 5-azaC and TSA promoted the re-expression of HLA class I antigens and subsequent restoration of the antigen-specific immune response after demethylation.[17] Histone deacetylase inhibitors have been shown to up-regulate MHC class I expression on several tumour cell lines, including melanoma B16/BL6,[18] human neuroblastoma SK-N-MC and mouse adenocarcinoma Colon 26-L5.[11] Notably, little is known about the role of the epigenetic mechanisms involved in APM regulation and the associated regulation of MHC class I cell surface expression. An interesting question is whether up-regulation of MHC class I cell surface expression can be mediated by epigenetic induction of the expression of APM genes and how the particular APM components (TAP-1, TAP-2, LMP-2 and LMP-7) are regulated by histone deacetylase inhibitors and DNA demethylation agents.

Cervical neoplasms are mostly attributed to infection with human papilloma virus (HPV), mainly its 'high risk' oncogenic types.[19] Early viral antigens of the high-risk HPV types, E6 and E7, are necessary for the maintenance of the malignant phenotype and are expressed in all tumour cells. A number of experimental E6/E7 targeting immunotherapeutic protocols designed to elicit a specific CTL-mediated response are currently under development.[20] However, their efficiency against E6/E7-expressing tumours with down-modulated MHC class I expression is questionable. Therefore, the problem of the mechanisms of MHC class I up-regulation on HPV-associated tumours is of particular interest. As promotor hypermethylation of multiple genes in carcinoma of the uterine cervix has been described,[21,22] the possible modulation of MHC class I gene expression by demethylation is of relevance and has therapeutic potential in cervical carcinoma.[23] Considering that the deficiency of surface MHC class I on tumours associated with HPV is frequently caused by APM defects,[24] we asked the question of whether epigenetic modifications can modulate surface MHC class I expression indirectly through the silencing or activation of APM genes. To address this problem, we employed an animal model for human HPV16-associated tumours. Using a model of tumour cells expressing E6/E7 antigens with MHC class I down-modulation caused by defects in APM, we examined the impact of histone deacetylase inhibitors and demethylation agents on the surface expression of MHC class I molecules and on the activation of APM genes.

Materials and methods

Cell lines

TC-1 is a malignant, immunogenic cell line of C57BL/6 mouse origin expressing HPV16 E6/E7 antigens.[25] The TC-1/A9 cell line is a TC-1 MHC class I-deficient derivative.[26] All cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine and antibiotics.

In vitro *experiments*

Cells were cultured in fresh medium for 24 hr, after which the medium was removed and the cells were grown in one of several RPMI/drug groups: 50 μM 5-azaC, 1 μM DAC, 5 μM DAC, 10 ng/ml TSA or 35 μM sodium butyrate (all chemicals were purchased from Sigma, St Louis, MO). The combination of 50 μM 5-azaC and 10 ng/ml TSA was also analysed. The medium was replaced with fresh medium after 48 hr and the cells were cultured for an additional 24 hr in drug-free medium, giving a total of 72 hr, and harvested.

Flow cytometry

Cell surface MHC class I expression of the cell lines was determined by two-step cytoflourometric analysis using anti-mouse H-2K^b/H-2D^b monoclonal antibody (clone 28-8-6) and fluorescein isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin G (IgG) secondary antibody or by one-step labelling using phycoerythrin (PE) anti-H-2D^b (KH95) and PE anti-H-2K^b (AF-88·5) antibodies. The following antibodies were used for detection of CD86 and MHC class II molecules: PE anti-CD86 (GL1), FITC anti-I-A^b (AF6-120·1). The cells were initially preincubated with anti-CD16/CD32 to prevent non-specific binding. Analysis of 10 000 cells was carried out with a FACSCAN ELITE cytometer (Coulter, Miami, FL). All antibodies used, including the mouse IgG$_{2a}$ isotope-matching control, were obtained from Pharmingen (San Diego, CA). The proportions of living, dead and apoptotic cells were determined with propidium iodide and the Annexin V-FITC apoptosis detection kit (Sigma, St Louis, MO).

Reverse transcription–polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR

Total RNA was extracted using the High Pure RNA isolation kit (Roche, Basel, Switzerland). RNA (200 ng) was reverse-transcribed to cDNA using random hexamer primers from the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA) in a 20-μl reaction volume at 42° for 30 min. PCR analysis was performed using Amplitaq polymerase (Applied Biosystems). cDNA was amplified under the following conditions: 95° for 2 min, followed by 25 cycles of denaturation at 95° for 30 seconds, annealing at 60° for 1 min, elongation at 72° for 1 min and incubation at 72° for 5 min. Quantification of PCR products was performed in 25 μl of SYBR Green Super Mix (Bio-Rad, Hercules, CA) using an iCycler thermocycler (Bio-Rad). DNA was denatured at 95° for 2 min, followed by 35 cycles of denaturation at 95° for 30 seconds, annealing at 60° for 1 min, elongation at 72° for 1 min and incubation at 72° for 5 min. cDNAs were amplified with specific primers for β-actin, H-2D^b, H-2K^b, TAP-1, LMP-2,[26,27] TAP-2[28] and LMP-7.[29] A list of the genes and their primer sequences is given in Table 1. Fold changes in transcript levels were calculated using threshold cycle (C_t) values standardized to β-actin, which was used as the endogenous control (reference gene), in order to normalize the quantification of mRNA. All samples were run in triplicate. For statistical analysis the Student *t*-test was used. Differences between experimental and control samples at $P < 0{\cdot}05$ were considered to be statistically significant.

Animals

Two-month-old C57BL/6 mice, purchased from Anlab (Prague, Czech Republic), were used for these experiments. They were maintained in the animal care facility at the Institute of Molecular Genetics according to approved protocols of the Institutional Animal Care Committee at the Institute of Molecular Genetics, Prague.

^{51}Cr microcytotoxicity assay

Mice (three per goup) were immunized with irradiated (150 Gy) TC-1 or TC-1/A9 and either untreated or treated with 10 ng/ml TSA. In all immunization protocols, the dosage was 10^7 cells per mouse, and the mice were immunized twice at a 3-week interval as previously described.[30] Eight days after the second immunization, spleen cells from immunized and control mice were extracted and used as effector cells. After lysis of erythrocytes with Tris-NH_4Cl buffer, the effector cells were mixed with the ^{51}Cr-labelled tumour target cells and incubated at four different target-to-effector cell ratios (1 : 25, 1 : 50, 1 : 100 and 1 : 200) for 18 hr in triplicate in 96-well round-bottom microtitre plates (Nunc, Roskilde, Denmark). The medium was also enriched with 10^{-5} M mercaptoethanol. To assess the role of CTLs, CD8-positive cells were depleted with specific monoclonal antibody 2·43[31] and complement (Baby Rabbit Complement; Cederlane, Hornby, Ontario, Canada) prior to the mixing of effector cells with targets. The percentage of specific ^{51}Cr release was expressed according to the formula [(c.p.m. experimental release – c.p.m. control release)/(c.p.m. maximum release – c.p.m. control release)] × 100, where c.p.m. is counts per minute.[30,32] For statistical analyses, Student's *t*-test was used.

Chromatin immunoprecipitation assay

The assay was performed using a chromatin immunoprecipitation assay kit (ChIP; Upstate Biotechnologies Inc., Billerica, MA) as per the manufacturer's instructions, with some modifications. Briefly, proteins were cross-linked to DNA by adding 1% formaldehyde directly to the medium for 10 min at 37°. Cross-linking was stopped by the addition of 0·125 M glycine at 37° for 15 min. The medium was removed and the cells were washed with phosphate-buffered saline (PBS) containing protease inhibitors [1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml aprotinin and 1 μg/ml pepstatin]. The cells were then resuspended in sodium dodecyl sulphate (SDS) and sonicated. The DNA/protein mixture was precleared with salmon sperm DNA/protein A agarose/50% slurry and then incubated with the antibody for acetylated histone H3 (Upstate Biotechnologies Inc.). After several washes, DNA bound to the immunocomplexes was obtained and then incubated with proteinase K. DNA was recovered via

Table 1. Primers used for polymerase chain reaction (PCR)/quantitative PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
TAP-1	GAC AAG AGC CGC TGC TAT TTG G	TGA TAA GAA GAA CCG TCC GAG A
TAP-2	GCC TGT GCT GTT CTC GGG TTC TGC	TGT ACC AGG TGG GCG TAG
LMP-2	CTC TGC ACC AGC ACA TCT T	AGA GTG ATG GCA TCT GTG GT
LMP-7	ATG GCG TTA CTG GAT CTG TGC GGT GC	TCA CAG AGC GGC CTC TCC GTA CTT GTA
β-actin	CCA GAG CAA GAG AGG TAT CC	GAG TCC ATC ACA ATG CCT GT

LMP, low-molecular-mass protein; *TAP*, transporter associated with antigen processing.

Table 2. Methylation-specific polymerase chain reaction (MSP) primers

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
TAP-1 (unmeth)	GTA AGT TAG TTT TAG AAG GAG GTG T	ATC CTA ACC TAA AAC TCT CAA CAT C
TAP-1 (meth)	GTA AGT TAG TTT TAG AAG GAG GCG T	ATC CTA ACC TAA AAC TCT CGA CGT
TAP-2 (unmeth)	TTT TTT AAA ATA TGT TTG GAG GTT G	CCA ACA AAT AAC ACC TAT CAA TTT ACA
TAP-2 (meth)	GTT TTT TAA AAT ATG TTC GGA GGTC	AAC AAA TAA CGC CTA TCA ATT TAC G
LMP-7 (unmeth)	TTT TGA TTT GTT TTT TAT TAG ATG G	CCT TTC TCT CTA TAC ACT TTA AAC ATT
LMP-7 (meth)	TTT TTC GAT TCG TTT TTT ATT AGA	CCT TTC TCT CTA TAC ACT TTA AAC GTT

LMP, low-molecular-mass protein; meth, methylated; *TAP*, transporter associated with antigen processing; unmeth, unmethylated.

phenol/chloroform extraction. For promoter analysis, we designed the following PCR primers which span the *TAP-1*, *LMP-2* bidirectional promoter: TAPsh-F GGC AAA TCT GCC CAG AGA and TAPbd R CCT AGC CTG GGA CTC TCG AC.

Bisulphite modification and methylation-specific PCR (MSP)

DNA from TC-1/A9 cells was treated with sodium bisulphite using a previously established protocol.[33] In order to identify CpG islands within the promoter region of the antigen-processing genes, MSP analysis was performed with primers designed with the program MethPrimer,[34] which spanned the 570-bp region of the bidirectional promoter *TAP-1*/*LMP-2*,[35] 1000 bp of the promoter region of *TAP-2*[36] and the 1622-bp upstream region of *LMP-7* (Table 2).

Western blot analysis

Whole-cell protein extracts were prepared from cell lines TC-1 and TC-1/A9 using lysis buffer containing 20 mM HEPES (pH 7·9), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, 10% glycerol, 1 mM dithiotreitol, 1 mM phenylmethylsulphonyl fluoride and 0·2 mM protease inhibitor cocktail (Sigma). Proteins (30 μg) were separated on a 7·5% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was blocked in 0·1%/tris buffered saline tween (TBST)/5% skimmed milk for 1 hr at room temperature and incubated overnight at 4° with goat polyclonal antibody against TAP-1 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1 : 1000). After incubation with anti-goat-horseradish peroxidase (HRP) (Santa Cruz Biotechnology), followed by extensive washing, the bound antibodies were visualized using enhanced chemiluminescence (West Femto; Pierce, Rockford, IL). Levels of actin were analysed using goat polyclonal antibody (Santa Cruz Biotechnology; dilution 1 : 1000) and were used as a control for equal loading.

Results

Epigenetic agents up-regulate MHC class I expression on the surface of MHC class I-deficient TC-1/A9 cells

The effect of 5-azaC and TSA on MHC class I expression in MHC class I-deficient TC-1/A9 was studied. Treatment of this cell line with 5-azaC and TSA induced expression of MHC class I molecules on the cells (Fig. 1). The effect was weaker than that observed in the IFN-γ-treated control, in which expression reached the level found in the parental TC-1 cells. In the next series of experiments, we studied the effects of TSA and 5-azaC separately. The results revealed that even treatment with TSA alone resulted in MHC class I up-regulation. The data were supported by results of experiments with sodium butyrate and DAC. Importantly, both agents were able to up-regulate MHC class I expression. Control experiments were performed on the parental MHC class I-positive cell line TC-1, in which no changes in MHC class I surface expression were observed. All cell lines remained MHC class II and CD86 negative (data not shown).

Proapoptotic effects of TSA and 5-azaC treatments

In order to assess the proapoptotic and cytotoxic effects of the epigenetic agents, a percentage of living, apoptotic and dead cells after treatment was analysed using annexin and propidium iodide labelling (Fig. 2). The percentages of dead and living TSA-treated cells were similar to those of the untreated controls, demonstrating that TSA at the concentrations used in the experiments had no effect on cell viability. After 5-azaC treatment, either alone or in combination, an increase in the portion of apoptotic cells was observed.

TSA-treated TC-1/A9 cells are effectively lysed with spleen cells from immunized animals

To determine whether MHC class I up-regulation by TSA was sufficient for the recognition of tumour cells by CTLs, we used TSA-treated TC-1/A9 cells as targets in a

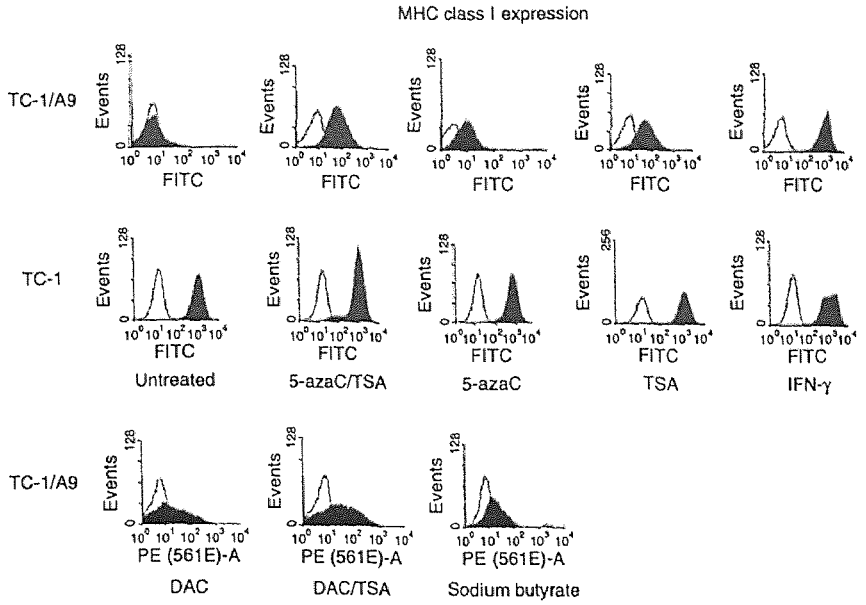

Figure 1. Epigenetic agents induce major histocompatibility complex (MHC) class I cell-surface expression on TC-1/A9 cells. MHC class I expression on TC-1/A9 and TC-1 after 5-azacytidine (5-azaC) and Trichostatin A (TSA) treatments was determined by flow cytometry. The efficacy of the 5-azaC and TSA treatment was compared to the effects of interferon (IFN)-γ. In the control experiments the effects of sodium butyrate and 5-aza-2′-deoxycytidine (DAC) on TC-1/A9 cells were demonstrated. All experiments were performed at least three times with similar results.

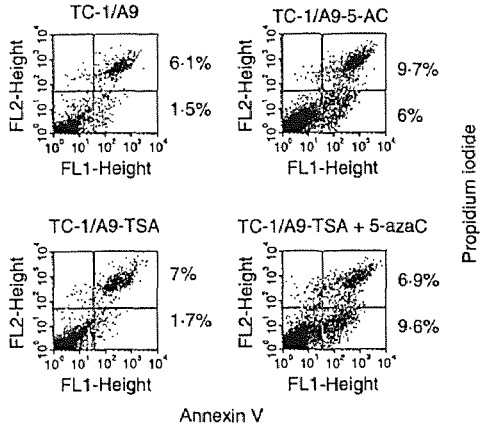

Figure 2. Determination of the proapoptotic effects of the 5-azacytidine (5-azaC) and Trichostatin A (TSA) treatments. The proportions of viable, apoptotic and dead cells were determined by dual labelling with annexin V and propidium iodide; viable cells were double-negative, apoptotic cells were labelled only with annexin V and dead cells were double-positive. The percentages of the apoptotic cells (lower right quadrant) and dead cells (upper right quadrant) are depicted. The experiment was performed twice with similar results.

chromium-release microcytotoxic test with the spleen cells from animals immunized with TC-1 cells (Fig. 3). 5-azaC- and TSA-treated TC-1/A9 cells were effectively lysed in this experiment. *In vitro* depletion of $CD8^+$ cells in the spleen cell mixture revealed that lysis was mediated by cytotoxic T cells. However, spleen cells from animals immunized with TSA-treated TC-1/A9 cells lysed MHC class I-positive TC-1 cells more efficiently than spleen cells from mice immunized with untreated TC-1/A9 cells.

Expression of APM genes is up-regulated by TSA and 5-azaC

The expression of MHC I molecules does not occur on the surface of TC-1/A9 tumour cells. We showed, via RT-PCR, that the two loci that produce MHC class I antigens, *H-2D*^{b} and *H-2K*^{b}, were fully functional in TC-1 and TC-1/A9 cell lines (Fig. 4). However, there was a decrease in mRNA expression of *TAP-1*, *TAP-2*, *LMP-2* and *LMP-7* in TC-1/A9 cells compared with TC-1 cells.

The effects of epigenetic agents on selected APM genes were studied in detail by quantitative real-time RT-PCR (Fig. 5a). After administering the combination of 5-azaC and TSA to the TC-1/A9 cell line, we observed an increase in the expression of APM genes, namely *TAP-2* and *LMP-7*. Furthermore, we analysed the expression of each gene after the administration of 5-azaC and TSA alone. Statistically significant up-regulation of *TAP-1*, *TAP-2*, *LMP-2* and *LMP-7* was observed after the addition of TSA, and statistically significant up-regulation of *TAP-1*, *TAP-2* and *LMP-7* after the 5-azaC treatment. In control experiments, epigenetic agents also up-regulated the expression of APM genes in MHC class I-positive TC-1 cells; TSA treatment resulted in a significant increase in expression of *TAP-1*, *LMP-2* and *LMP-7*. *TAP-2* and *LMP-7* expression was

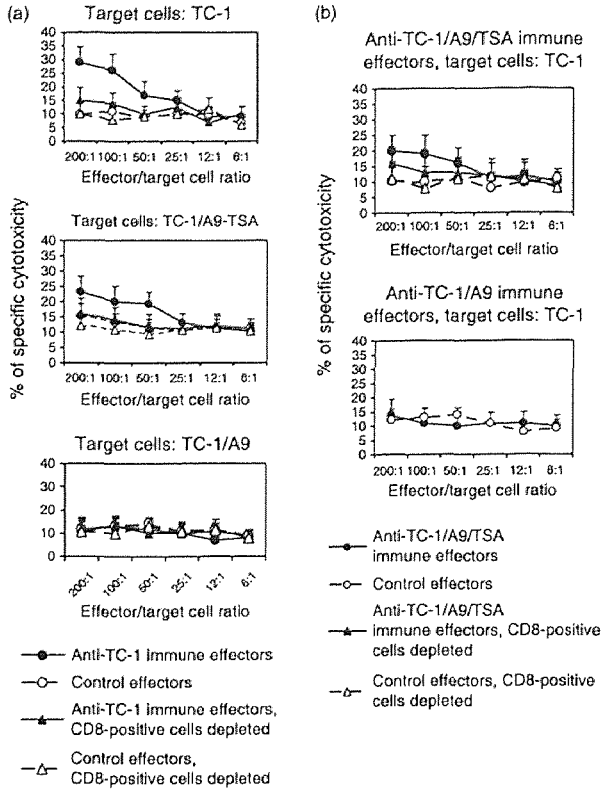

Figure 3. Trichostatin A (TSA)-treated TC-1/A9 cells are susceptible to cytotoxic T lymphocyte (CTL)-mediated lysis and TSA treatment augments the CTL-mediated immune response. (a) Spleen cells from mice immunized with TC-1 cells or control mice (pooled from three mice) were used as effector cells. Significant differences were observed between the following groups: target cells TC-1: $P < 0{\cdot}01$ (200 : 1), $P < 0{\cdot}05$ (100 : 1 and 50 : 1); anti-TC-1 immune effectors × control effectors, and anti-TC-1 immune effectors × anti-TC-1 immune effectors, $CD8^+$ cells depleted; target cells TC-1/A9-TSA: $P < 0{\cdot}05$ (200 : 1, 100 : 1 and 50 : 1); anti-TC-1 immune effectors × control effectors, and anti-TC-1 immune effectors × anti-TC-1 immune effectors, $CD8^+$ cells depleted. (b) Spleen cells from mice immunized with TC-1/A9 or TSA-treated TC-1/A9 cells (pooled from three mice) or control mice were used, and TC-1 cells, TSA-treated TC-1/A9 cells and untreated TC-1/A9 cells were used as targets. Significant differences were observed between the following groups: $P < 0{\cdot}05$ (200 : 1 and 100 : 1); anti-TC-1/A9 TSA immune effectors × control effectors. All experiments were performed twice with similar results.

significantly up-regulated by 5-azaC. Notably, the effects of TSA and 5-azaC on particular genes differed and were not synergistic. The effects of the particular treatments on *TAP-1* and *LMP-2* were very similar.

Western blot analysis determined that the protein levels of TAP-1 and TAP-2 in cells treated with 5-AzaC, TSA or a combination of the two drugs were increased when compared with the untreated TC-1/A9 control (Fig. 5b). Detectable protein expression was detected in untreated cells; however, the intensity of the band was greater in cells treated with 5-azaC alone and in combination with

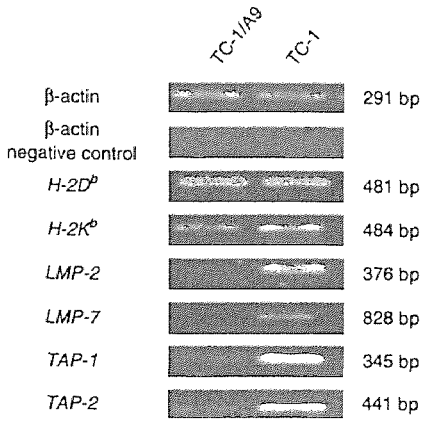

Figure 4. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of the expression of major histocompatibility (MHC) class I $H\text{-}2D^b$ and $H\text{-}2K^b$ genes and antigen-processing machinery (APM) components transporter associated with antigen processing 1 (*TAP-1*), *TAP-2*, low-molecular-mass protein 2 (*LMP-2*) and *LMP-7*. Twenty-five PCR cycles were performed to distinguish the different patterns of gene expression between particular cell lines.

TSA for both proteins. TAP-1 protein expression was increased in cells exposed to TSA; however, the TAP-2 expression level appeared to be unchanged.

Antigen-processing genes are demethylated after 5-azaC and TSA treatment

Bisulphite-treated DNA was analysed with primers designed to distinguish between modified and unmodified DNA. MSP analysis of the bidirectional promoter of *TAP-1/LMP-2* demonstrated that the promoter was partially methylated in TC-1/A9. Upon treatment with 5-azaC the promoter was demethylated (Fig. 6a). Interestingly, TSA alone appeared to induce demethylation of *TAP-1*. The promoter region of *TAP-2*, as well as the upstream sequence of *LMP-7*, was also found to be partially methylated before treatment. However, after 5-azaC treatment, *TAP-2* was demethylated while there appeared to be no change in the methylation status of *LMP-7*. There were no detectable changes after treatment with the combination of drugs.

Histone H3 is re-acetylated at the bidirectional promoter after 5-azaC/TSA treatment

A ChIP assay was performed to determine whether the combined dose of 5-azaC/TSA, which is sufficient to reverse the methylation of the *TAP-1/LMP-2* promoter, was able to modify the histones associated with this promoter (Fig. 6b). The assay demonstrated that histone H3 was re-acetylated after treatment with both epigenetic agents alone as well as in combination. Acetylated histone H3 was detected at this region in untreated TC-1/A9 cells at a low level.

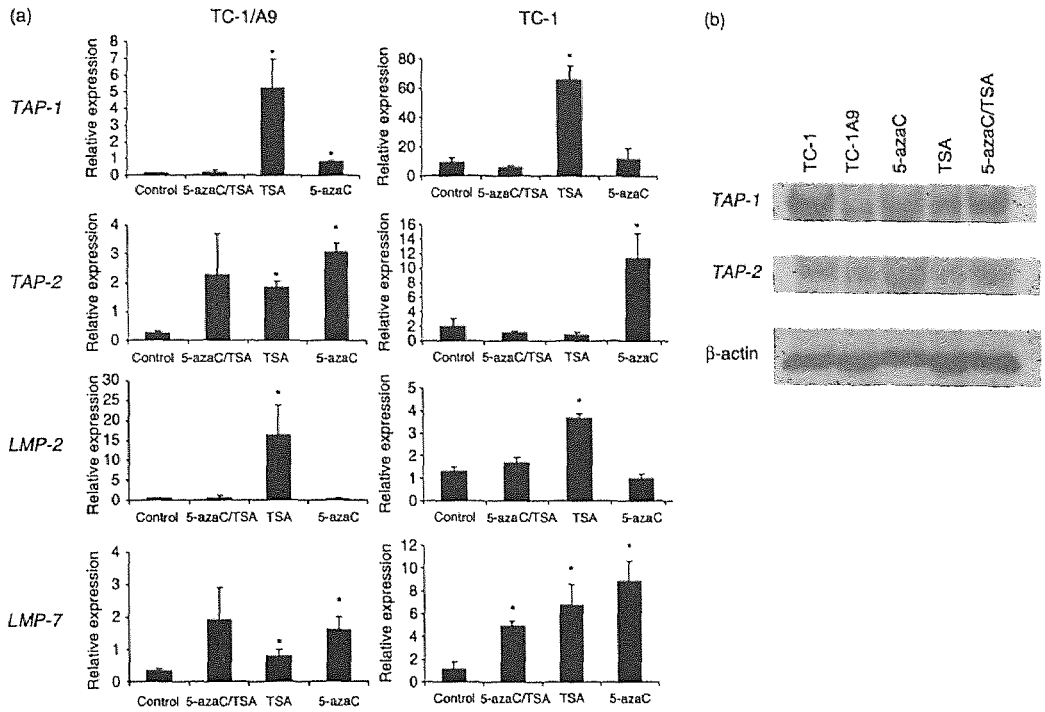

Figure 5. Quantitative polymerase chain reaction (PCR) and western blot analysis of expression levels of antigen-processing machinery (APM) components in TC-1/A9 and TC-1 cells. (a) Quantitative PCR: transporter associated with antigen processing 1 (*TAP-1*), *TAP-2*, low-molecular-mass protein 2 (*LMP-2*) and *LMP-7* mRNA expression after 5-azacytidine (5-azaC)/Trichostatin A (TSA), 5-azaC and TSA treatments and in untreated cells was related to expression of β-actin. Cells were given 5-azaC in combination with TSA, 5-azaC alone, or TSA alone. In both major histocompatibility complex (MHC) class I^+ and MHC class I^- cell lines, *TAP-1* and *LMP-2* were up-regulated after the administration of TSA. *TAP-2* and *LMP-7* were up-regulated after treatment with 5-azaC and TSA and 5-azaC alone. The samples whose relative expression exhibited a *P*-value less than 0·05, compared with controls, were determined to be significant and are denoted by an asterisk. (b) Western blot: TAP-1 and TAP-2 expression at the protein level after treatment with epigenetic agents was determined in TC-1/A9 cells. β-actin expression served as a control.

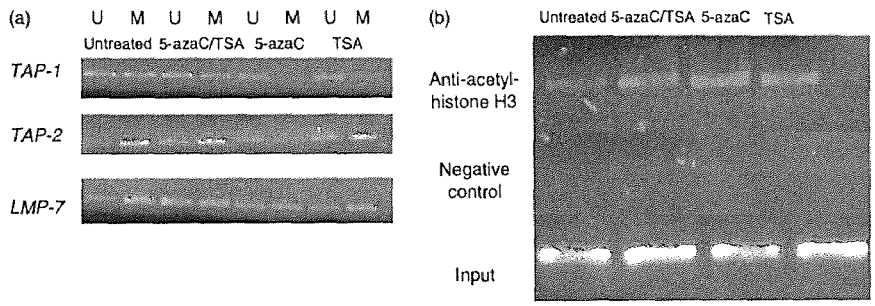

Figure 6. Epigenetic agents induce DNA demethylation and histone deacetylation. (a) DNA from the TC-1/A9 cell line treated with epigenetic agents was treated with bisulphite. Methylation-specific polymerase chain reaction (MSP) analysis revealed partial methylation of transporter associated with antigen processing 1 (*TAP-1*), low-molecular-mass protein 2 (*LMP-2*) and *LMP-7* in control cells not treated with epigenetic agents. Administration of 5-azacytidine (5-azaC)/Trichostatin A (TSA) led to demethylation. 5-azaC alone resulted in the demethylation of *TAP-1* and *LMP-2*. TSA had an effect on the *TAP-1* promoter but no effect on *TAP-2*. *LMP-7* remained methylated. U, unmethylated primer; M, methylated primer. Histone H3 is associated with the *TAP-1/LMP-2* bidirectional promoter. (b) ChiP analysis of chromatin isolated from TC-1A9 treated cells demonstrated an increase in acetylated histone H3 after 5-azaC and TSA treatment. Experiments were repeated at least twice with similar results.

Discussion

The aim of this study was to elucidate whether epigenetic modifications influence MHC class I cell-surface expression in tumour cells that contain reversible MHC class I expression deficiencies caused by defects in APM components Histone deacetylase inhibitors and DNA demethylatio agents partially restored the expression of APM compo

nents and induced the expression of MHC class I molecules on the surface of the tumour cells. The level of expression did not reach that of the parental MHC class I-positive cells, but was sufficient for lysis by effector spleen cells from mice immunized with parental TC-1 tumour cells.

A plausible mechanism of the MHC class I up-regulation is activation of the APM components. Induction of the expression of these genes was observed in the TC-1/A9 cell line, although not all investigated APM genes were up-regulated in the same manner. We also cannot exclude the possibility of a role for other factors in MHC class I up-regulation, as a number of other genes are affected by epigenetic agents. MHC class I up-regulation was also induced by TSA, suggesting a role in the modification of histone H3 associated with TAP-1 and LMP-2, resulting in the transformation from heterochromatin to euchromatin. However, 5-azaC appears to have a greater influence with regard to histone re-acetylation, probably as a result of its action on the methylated promoter inhibiting DNA methyltransferases and promoting an environment in which histone acetyltransferases can act on chromatin. In tandem, changes in DNA demethylation and histone acetylation lead to the re-expression of the epigenetically silent gene. *TAP-1* and *LMP-2* genes were influenced by the two epigenetic agents similarly. This is not surprising as these genes are regulated by the same promoter.[34]

Interestingly, the APM-related genes analysed were also significantly epigenetically up-regulated in MHC class I-positive TC-1 cells, with no effects on the levels of MHC class I cell-surface expression. The results demonstrated that, in this case, the APM components were epigenetically up-regulated in the cells with functional antigen processing and presentation, but their up-regulation was not the limiting factor for a further increase in MHC class I expression.

We also investigated the effect of 5-azaC/TSA on other immunoactive molecules. Interestingly, in neither of our cell lines was MHC class II cell surface expression up-regulated, contrary to what has been found in some other cell lines.[12,15,37] MHC class II and also MHC class I induction can be associated with up-regulation of MHC class II transactivator (CIITA) coactivators,[38,39] which has been reported to be modulated by histone acetylation.[15]

We must also take into consideration the possibility that the MHC class I induction could be related to the proapoptotic effects of the agents or a result of their effects on the cell cycle. This possibility cannot be excluded, as TAP-1 and TAP-2 expression has been found to be cell-cycle dependent.[40] However, unlike 5-azaC, TSA was used in our experiments in concentrations that did not induce apoptosis of the treated cells compared with untreated controls. These mechanisms, as well as the problem of possible involvement of the IFN-γ pathway in epigenetic induction of APM components, should be studied in more detail.

It is now generally accepted that epigenetic non-mutational changes represent important mechanisms in the multistep process of tumour development. There is mounting evidence that epigenetic modifications are important factors driving the tumour cell to escape from immune surveillance. However, understanding of the epigenetic regulation of APM modulation, which is a frequent mechanism of reversible inhibition of MHC class I cell surface molecules in cervical carcinoma, is limited.[41] We have demonstrated that MHC class I re-expression in APM-defective tumour cells can be induced by treatment with TSA either alone or in combination with 5-azaC. This up-regulation was associated with the activation of down-modulated genes of the APM. The mechanisms of this activation remain to be elucidated in future studies. It remains to be determined whether these genes are regulated directly, independently or, as in the case of IFN-γ treatment, by upstream regulatory elements. It is also noteworthy that MHC class I reconstitution on tumour cells has not been as effective as, for instance, with IFN-γ treatment. These results indicate that epigenetic mechanisms can substantially contribute to MHC class I expression but are not the primary mechanism.

The increased sensitivity of the treated tumour cells to a specific immune response is an important mechanism of antitumour effects in addition to tumour-suppressor gene activation and proapoptotic effects, and the usage of epigenetic agents can improve the efficacy of immunotherapeutic protocols.

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