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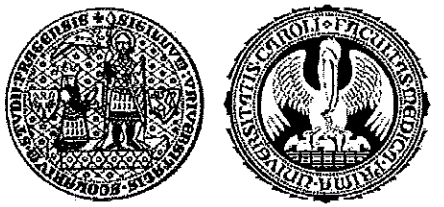

Ph.D. Thesis Summary

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

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Souhrn

Epigenetické mechanismy hrají důležitou roli během růstu nádorů a také mohou přispívat k úniku nádorových buněk imunitnímu dozoru. V této práci byla sledována indukce exprese MHC glykoproteinů I. třídy na MHC I-deficientních nádorových buňkách pomocí epigenetických agens. K těmto experimentům byla využita MHC I-deficientní myší nádorová linie buněk exprimující *E6* a *E7* onkoproteiny lidského papillomaviru, u nichž byla snížena exprese genů kódující proteiny nutné pro úpravu a presentaci antigenu (antigen presenting machinery; APM) *TAP1*, *TAP2*, *LMP2* a *LMP7*. Bylo zjištěno, že inhibitor histondeacetyláz Trichostatin A (HDACi, TSA) a také inhibitor DNA metyltransferáz 5-azacytidin (Dnmti, 5-azaC), indukují expresi MHC molekul I. třídy na povrchu buněk, což bylo spojeno s indukcí APM genů, jejichž exprese je nutná pro expresi MHC glykoproteinů I. třídy na membránách buněk. Pomocí metylačně specifické RT-PCR (MSP) bylo prokázáno, že vlivem 5-azaC i TSA došlo k demetylaci částečně metylovaných promotorových sekvencí APM genů. Pomocí chromatinové imunoprecipitace byla prokázána reacetylace histonů H3 vázaných na bifunkční promoter *TAP1/LMP2*. ^{51}Cr microcytoxický test ukázal, že nádorové buňky se vlivem TSA staly citlivé ke specifickým cytotoxickým T lymfocytům. Tyto výsledky prokázaly epigenetickou regulaci genů nutných pro presentaci antigenu a s tím spojenou povrchovou expresí MHC glykoproteinů I. třídy.

V druhé části práce bylo u pacientů s myelodisplastickým syndromem nebo s akutní myeloidní leukemií pomocí MSP prokázáno, že terapie pomocí 5-azaC vedla k demetylaci promotoru genu *p15INK4B*, který je při leukémiích často metylován. Pomocí bisulfitové reakce a následné sekvenaci DNA bylo toto zjištění potvrzeno a byla kvantifikován rozsah demetylace tohoto genu během léčby. Terapie pomocí 5-azaC and fenylbutyrátu indukovala reacetylaci histonů H3 a H4. Tato studie ukazuje na možné spojení mezi demetylací DNA a acetylací histonů s terapeutickými účinky epigenetických agens.

Summary

Epigenetic events play a crucial role in tumor progression and can also contribute to escape of the tumor from immune surveillance. We investigated the induction of MHC class I surface expression on MHC class I-deficient tumor cells by epigenetic mechanisms using a murine tumor cell line expressing human *E6* and *E7* HPV16 oncogenes and downregulated antigen presenting machinery (APM) genes *TAP1*, *TAP2*, *LMP2*, and *LMP7*. After treatment with the histone deacetylase inhibitor Trichostatin A (HDACi, TSA), and the DNA methyltransferase inhibitor 5-azacytidine (Dnmti, 5-azaC), FACS analysis revealed the induction of MHC class I molecules on the surface of the cells. Quantitative RT-PCR determined that the APM genes responsible for the induction of MHC class I molecules were significantly upregulated by both 5-azaC and TSA. Methylation specific PCR (MSP) analysis showed that 5-azaC demethylated the partially methylated promoters of the APM genes. Chromatin immunoprecipitation (ChIP) studies of the bidirectional promoter *TAP1/LMP2* found that both 5-azaC and TSA was involved in the re-acetylation of Histone H3. ^{51}Cr microcytoxicity assay demonstrated that tumor cells treated with TSA were lysed by cytotoxic T lymphocytes (CTL). These results demonstrate that APM gene expression is influenced by epigenetic mechanisms.

In our study of patients with myeloid neoplasms, MSP data provided evidence that 5-azaC treatment led to the demethylation of the *p15INK4B* promoter, often methylated in leukemia. Upon closer examination of this promoter, bisulfite sequencing confirmed this finding and demonstrated demethylation of specific CpG dinucleotides within the promoter over time. The administration of 5-azaC and phenylbutyrate promoted histones H3 and H4 reacetylation.

This study demonstrates a link between DNA demethylation and histone acetylation and the therapeutic effects of epigenetic agents.

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 with 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 the immune response.

Tumor cells have the ability to evade specific immune responses mediated by CTL by several mechanisms including downmodulation of major histocompatibility complex (MHC) class I molecules on their surface (reviewed by 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 inhibition of transcription of the genes involved in antigen processing (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 expression 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 expression which are subsequently recognized and lysed by CTL (Lu et al., 2001; Mikyskova et al., 2005).

The mechanism linking antigen processing machinery gene regulation and cell surface MHC class I expression in relation to the administration of IFN-γ to tumor cells with downregulated MHC class I is known but, not completely understood. The possibility that epigenetic mechanisms may also be involved in the silencing of APM genes has 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., 2010). 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).

This thesis focuses on the effect of DNA methyltransferase and histone deacetylase inhibitors on MHC class I-deficient tumor cells, the genes responsible for the induction of these molecules on the surface of these cells and on the tolerance/response of patients treated with a combination of these drugs.

Hypothesis and project aims

We proposed that histone deacetylase inhibitors trichostatin A, sodium butyrate, Dnmti 5-azacytidine and 5-aza-2'deoxycytidine upmodulate MHC class I molecules and reactivate the expression of antigen processing machinery genes. TC-1(MHC class I^+; Lin et al., 1996) a malignant, immunogenic cell line expressing HPV16 E6/E7 antigens of C57BL/6 mice origin and TC-1A9 (Šmahel et al., 2003), a TC-1 MHC class I-deficient derivative with downmodulated MHC class I molecules due to reversible defects in antigen processing machinery genes were used for the study.

To answer the question of the effect of these epigenetic agents in patients suffering from myelodyplastic syndrome (MDS) or acute myeloid leukemia (AML), we attempted to demonstrate within a clinical phase I trial that sequential treatment with a DNA methyltransferase inhibitor followed by an histone deacetylase inhibitor would reverse promoter methylation of *p15INK4B*, a gene often methylated in leukemia and induce histone reacetylation.

Specific Aims

1. To determine the effect of epigenetic agents on the induction of MHC class I molecules on the surface of tumor cells.
2. To determine if the expression of antigen processing machinery genes is increased.
3. To demonstrate that cytotoxic T lymphocytes recognize treated tumor cells.
4. To demonstrate that epigenetic drugs target *p15INK4B* in myeloid dysplasia and reverse its methylation/histone deacetylation status.

Materials and Methods

Cell lines

TC-1 (MHC I^+/MHC II^-) (Lin et al., 1996) is a malignant, immunogenic cell line expressing HPV16 E6/E7 antigens of C57BL/6 mice origin. TC-1/A9 cell line represents a TC-1 MHC class I-deficient derivative (Šmahel et al., 2003).

Patient samples

Bone marrow samples were obtained from patients who exhibited either myeloid dysplastic syndrome (MDS) or acute myeloid leukemia (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).

Flow cytometry

Cell surface MHC class I expression on the cell lines was determined by two-step cytoflourometric analysis. 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

RNA from the APM genes was amplified via qRT-PCR. Fold changes in transcript levels were calculated using CT values standardized to β-actin, used as the endogenous control, in order to normalize the quantitation of mRNA.

^{51}Cr microcytotoxicity assay

Mice were immunized with irradiated (150 Gy) TC-1 cells. Spleen cells from immunized and control mice were used as effector cells which were mixed with ^{51}Cr-labelled tumor target cells (TC-1/A9 cells, either untreated or treated with

10 ng/ml TSA). To assess the role of CTL, CD8-positive cells were depleted with the specific antibody from the effector/target cell mix. The percentage of specific ^{51}Cr release 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)

Histone acetylation of the *TAP1/LMP2* bidirectional promoter was analyzed via chromatin immunoprecipitation (Millipore, Billerica, MA, USA).

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). CpG islands within the promoter region of the antigen processing genes (*TAP1/LMP2*, *TAP2* and *LMP7*) were analyzed with MSP primers.

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).

Western Blot analysis

Whole cell protein extracts were prepared from cell lines TC-1 and TC-1/A9. Goat polyclonal antibody against TAP1 was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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.

Results

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

Treatment of this cell line with 5-azaC and TSA induced expression of MHC class I molecules on the cells (Fig.1). IFN-gamma-treated cells exhibited levels comparable to MHC class I positive TC-1 cells. Induction was also observed with epigenetic drugs sodium butyrate and 5-aza-2'deoxycytidine (DAC). TC-1 exhibited no change in MHC class I surface expression after treatment.

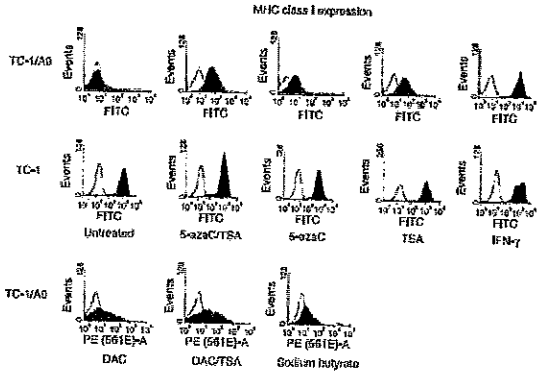

Fig.1. Epigenetic agents induce MHC class I cell surface expression on TC-1/A9 cells. MHC class I expression on TC-1/A9 and TC-1 after 5-azaC and TSA treatments was determined by flow cytometry. The efficacy of the 5-azaC and TSA treatment was compared to the effects of IFN-γ. The effects of sodium butyrate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

Proapoptotic effects of TSA and 5-azaC treatments

A portion of living, apoptotic and dead cells after treatment was analyzed by annexin and propidium iodide labeling to determine cytotoxic effects. The percentage of dead and live TSA-treated cells was similar to the untreated controls. 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 microcytotoxic test with spleen cells from animals immunized with TC-1 cells. TSA-treated TC-1/A9 cells were effectively lysed however, upon depletion of $CD8^+$ fraction from the spleen cells tumor cell lysis was not observed.

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

mRNA expression of *TAP1*, *TAP2*, *LMP2* and *LMP7* was increased after treatment with 5-azaC, TSA or combination of the drugs in both TC-1/A9 and TC-1 cells. Increased levels of TAP1 and TAP2 protein was increased in TC-1/A9.

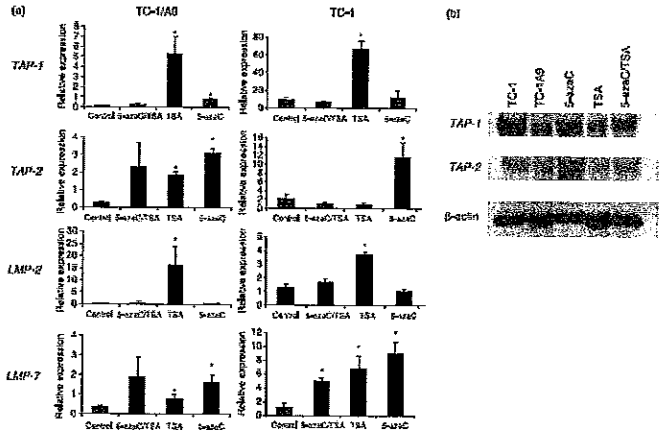

Fig.2. qPCR and western blot analysis of expression levels of APM components in TC-1/A9 and TC-1 cells. (a) qPCR: *TAP1*, *TAP2*, *LMP2* and *LMP7* mRNA expression after 5-azaC/TSA, 5-azaC and TSA treatments and in untreated cells was related to expression of *β-actin*. The samples whose relative expression exhibited $P< 0.05$, 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.

Methylated APM genes are demethylated after 5-azaC treatment

Bisulfite treated DNA was analyzed with primers designed to distinguish between unmethylated and methylated DNA. *TAP1/LMP2* and *TAP2* promoters were partially methylated in TC-1/A9 and demethylated with 5-azaC. The methylation status of *LMP7* remained unchanged. TSA alone appeared to induce demethylation of TAP1. ChIP assay demonstrated reacetylation of the *TAP1/LMP2* promoter at Histone H3 with 5-azaC, TSA and in combination.

Changes in Promoter Methylation of *p15INK4B* in MDS patients

p15INK4B promoter of twelve patients was analyzed for changes in methylation using MSP. Six patient responders developed reversal of methylation for during the first cycle of treatment; six patient nonresponders did not have a change in their methylation (Fig. 2 examples of responder/nonresponder). The difference in response rates between the two groups was statistically significant ($P = 0.002$, Fisher's exact test).

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. 11 of 23 evaluable patients showed increases in global acetylation of H3 and/or H4 in response to 5-azaC alone. 12 of the 23 patients had further increases in acetylation with phenylbutyrate. Overall, 17 of 23 patients developed increased histone acetylation following either 5-azaC or phenylbutyrate treatment.

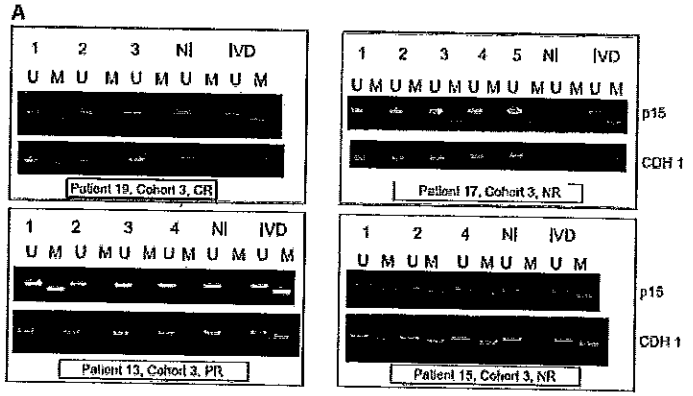

Fig.3. Changes in methylation of *p15INK4B* during cycle 1 of treatment. A, bone marrow mononuclear cells were procured before therapy (1), following the last injection of 5-azaC (2), following discontinuation of phenylbutyrate (3), and before cycle 2 (4). The four representative patients were all treated 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.

Discussion

Our primary focus was two-fold: to determine if epigenetic changes affect the expression of MHC class I molecules on the surface of human papilloma virus E6/E7 expressing 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 with 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 expression 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, MHC class I molecules were upregulated on the tumor cell surface, was subsequently recognized by $CD8^+$ cells and lysed. 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/reacetylation of antigen processing machinery genes occurs after treatment with DNA methyltransferase and histone deacetylase inhibitors and that these genes are regulated epigenetically.

Although we observed that the APM genes were partially methylated and 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 IFN-gamma pathway, Dnmti target important promoters that have become methylated and increases the expression of immune regulatory genes.

Epigenetic therapy has been utilized against myelodysplastic syndromes with much success. The inhibitors of methylation have been effective on acute

myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). Patients stricken with myeloid neoplasms have responded well to combination therapy of 5-azaC and the HDACi, phenylbutyrate. We analyzed the methylation status of the tumor suppressor *p15INK4B*, a negative regulator of cyclin-dependent kinases involved in G1 to S phase transition. *p15INK4B* is frequently methylated in myleodysplastic syndromes and acute leukemia (Drexler, 1998). Our MSP 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 sequencing as a tool in patient samples determined that CpG dinucleotides were demethylated after 5-azaC treatment over time.

Clinically, since the approval of both 5-azaC and DAC for the treatment of cancer, epigenetic therapy has demonstrated the 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.

It is clear that Dnmti block DNA methyltransferases from promoter regions, however, the mechanism of HDACi *in vitro* or *in vivo* is not well understood. There are several scenarios some studies have presented to explain the action of these inhibitors. There are differences between the activities of HDACi such as TSA and valproic acid. It appears that TSA interacts with more HDACs than valproic acid (Johnstone and Licht, 2003; Tomasi et al., 2006), hence a more

reactive HDACi. 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 mechanism that leads to reexpression of silenced tumor suppression genes and enhances the interactions between immune and tumor cells.

Conclusions

In our model of MHC class I-deficient TC-1/A9 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 was associated with the upregulation of antigen processing machinery genes *TAP1*, *TAP2*, *LMP2* and *LMP7* as well as with the epigenetic changes in their promoter regions.
3. Cells expressing MHC class I molecules after treatment with epigenetic agents were subsequently recognized by cytotoxic T lymphocytes and lysed.

In our MDS and AML 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-azaC and phenylbutyrate.
2. Bisulfite sequencing demonstrated that CpG dinucleotides of *p15INK4B* in tumor cells were demethylated over time.
3. MSP showed demethylation of the *p15INK4B* promoter in six clinical responders.
4. 5-azaC treatment resulted in reacetylation of half of the patients treated. Phenylbutyrate reacetylated histone H3 and H4 in half of the patients treated.

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List of publications related to thesis work

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Additional materials (posters, lectures) related to the thesis

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