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To the editor:

5-Aza-2′-deoxycytidine (decitabine) treatment of hematopoietic malignancies: a multimechanism therapeutic approach?

We read with great interest the report by Daskalakis et al[1] on the regulation of the expression of cyclin-dependent kinase inhibitor *p15*, an inhibitor of G_1/S progression, in patients with myelodysplastic syndrome (MDS) treated with the DNA hypomethylating agent 5-Aza-2′-deoxycytidine (decitabine). The authors detected a baseline hypermethylation in the 5′ region of the *p15* gene in bone marrow (BM) mononuclear cells (MNCs) from 15 of 23 MDS patients and demonstrated that systemic administration of at least one course of low-dose decitabine decreased *p15* hypermethylation in 9 of 12 of patients investigated. Based on these findings, which correlated with a restoration of *p15* protein expression and associated with clinical response, the authors envisaged pharmacologic demethylation as a possible mechanism for generating hematologic response in MDS patients treated with decitabine.

We certainly agree with the consolidating notion that the restoration of proliferation-associated genes silenced by aberrant DNA hypermethylation,[2] including *p15*,[1] represents a possible relevant mechanism for the antineoplastic activity of decitabine. Nevertheless, we also are learning about the pleiotropic biologic activities of decitabine on transformed cells that exceed a pure growth inhibitory role and that may act as synergistic layers of therapeutic efficacy in the clinical setting. In this respect, the in vitro ability of decitabine to promote the de novo expression on transformed cells of different histotypes of cancer testis antigens (CTAs),[3,4] a recently identified category of immunogenic tumor–associated antigens (TAAs) that is comprehensively regulated by DNA methylation,[5] is highly intriguing. In fact, due to their tissue distribution, which is restricted to neoplastic cells,[6] CTAs represent optimal candidates for therapeutic vaccination of cancer patients, and several ongoing studies are testing their clinical efficacy.[7]

To provide preliminary evidence on the potential immunologic role of in vivo administration of decitabine and to corroborate the idea that multiple mechanisms of action could be involved in its antineoplastic activity, we investigated the effect of decitabine on the expression of the CTAs designated MAGE-1, NY-ESO-1, and SSX in peripheral blood (PB) or BM MNCs of patients affected by acute myeloid leukemias (AML) or MDS before treatment (T0) and 15 (T15) and 30 (T30) days after the beginning of the first 3-day cycle of systemic administration of decitabine.[8,9] Sex and age of patients, French-American-British (FAB) assignment of diseases, doses of decitabine administrated, as well as indication on previous treatments, are summarized in Table 1.

Table 1. Clinical features of AML and MDS patients treated with decitabine

Patient no.	Sex	Age	Diagnosis	FAB	DAC mg/m²/day (× 3 days)	Previous treatment
1	F	65	de novo AML	M1	60	No*
2	F	83	de novo AML	M1	30	No
3	M	69	de novo AML	M1	90	No
4	F	60	de novo AML	M4	60	No
5	M	62	de novo AML	M4	60	No
6	F	62	de novo AML	M4	30	No
7	M	64	de novo AML	M5	30	No
8	M	74	Secondary AML†	M1	30	HU
9	F	79	MDS	AREB-t	15	No
10	M	72	MDS	AREB-t	15	No
11	M	67	MDS	CMMoL	15	No

Decitabine was administrated at the indicated daily dosages, divided into three 4-hour infusions for 3 days.

DAC indicates decitabine; F, female; M, male; RAEB-t, refractory anemia with excess blasts in leukemic transformation; HU, hydroxyurea; and CMMoL, chronic myelomonocytic leukemia.

*Decitabine was used as first-line therapy due to the low performance status (less than 60%, Karnofsky scale) of patients.

†Secondary AMLs evolved from a previous myeloproliferative disorder.

According to the known constitutive hypermethylation of hematopoietic malignancies,[10] reverse transcriptase–polymerase chain reaction (RT-PCR) analyses demonstrated no expression of the investigated CTAs in most (31 of 33) of the investigated patients at T0 (Table 2). In contrast, a de novo expression of MAGE-1, NY-ESO-1, and SSX was detected in all but one (32 of 33) samples studied at T15; noteworthy, this neoexpression of CTAs in vivo induced by a single cycle of decitabine administration was persistent, as it was still detectable at T30 in most patients, and even at T40 in a single case studied (Table 2). This finding is particularly relevant in that it stands for a more general event common to neoplastic cells of different histology. In fact, we have previously shown that the de novo expression of several CTAs induced by decitabine persists after the end of treatment, not only in cultured melanoma and renal carcinoma cells[3,11] but also in human melanoma xenografts in nude mice (MM et al, unpublished data, September 2002).

Altogether, these findings suggest that CTA genes, which are constitutively silent in transformed cells of hematopoietic origin, represent additional promising molecular targets for the DNA hypomethylating activity of decitabine. In this setting, it is intriguing to envisage a multistep, likely subsequent, mechanism of therapeutic activity of decitabine in hematopoietic malignancies in which the initial cytotoxic/cytostatic effects, mediated by the neoexpression of cell cycle regulators,[1] along with a proapoptotic and/or differentiation-inducing activity, are followed by a longer term disease control sustained by the activation of a CTA-specific immune response.

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Table 2. De novo expression of CTA in AML and MDS patients treated with decitabine

Patient no.	Diagnosis	FAB	T	BM blasts, %	PB blasts, %	MAGE-1	SSX	NY-ESO-1	β-actin
1	de novo AML	M1	0	90	87	−	−	−	+
			15	nd	66	+	+	+	+
			30	nd	64	+	−	+	+
2	de novo AML	M1	0	80	86	−	+	−	+
			15	nd	75	+	+	+	+
3	de novo AML	M1	0	95	90	−	−	−	+
			15	nd	18	+	+	+	+
			30	nd	5	−	+	+	+
4	de novo AML	M4	0	90	75	−	−	+	+
			15	nd	63	+	+	+	+
			30	nd	67	+	+	+	+
			40	nd	66	+	+	+	+
5	de novo AML	M4	0	65	58	−	−	−	+
			15	nd	12	−	+	+	+
6	de novo AML	M4	0	70	44	−	−	−	+
			15	nd	25	+	+	+	+
7	de novo AML	M5	0	85	67	−	−	−	+
			15	nd	45	+	+	+	+
8	secondary AML*	M1	0	60	58	−	−	−	+
			15	nd	45	+	+	+	+
			30	nd	49	+	+	+	+
9	MDS	AREB-t	0	25	2	−	−	−	+
			15	20	1	+	+	+	+
10	MDS	AREB-t	0	22	0	−	−	−	+
			15	7	1	+	+	+	+
11	MDS	CMMoL	0	15	12†	−	−	−	+
			15	nd	10†	+	+	+	+
			30	nd	13†	−	−	−	+

Total RNA was extracted from PB or BM (patients no. 9 and 10) MNCs using the TRIzol Reagent (Life Technologies, Milan, Italy), according to the manufacturer's instructions. RT was performed on 2 μg total RNA using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and oligo $dT_{(12\text{-}18)}$ as primer. PCR was performed on 100 ng retrotranscribed total RNA, using 50 pmol each sense and antisense primer. Oligonucleotide primer sequences and gene-specific PCR amplification programs used have been previously reported.[5] The integrity of RNA and oligo(dT)-synthesized cDNA was confirmed by amplification of all cDNA samples with β-actin-specific primers. Then 10 μL each amplified product was run on a 2% agarose gel and visualized by ethidium bromide staining.

T indicates time from the beginning of decitabine therapy (days); RAEB-t, refractory anemia with excess blasts in leukemic transformation; CMMoL, chronic myelomonocytic leukemia; and nd, not determined.

*Secondary AMLs evolved from a previous myeloproliferative disorder.

†Figures refer to the percentage of PB atypical monocytes.

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Response:

Multiple hypermethylated genes are potential in vivo targets of demethylating agents

In their interesting and timely report of the in vivo antileukemic effects of the demethylating agent 5-aza-2′-deoxycytidine (decitabine, DAC), Sigalotti et al observed changes in mRNA expression of 3 cancer testis antigens (CTAs), namely MAGE-1, SSX, and NY-ESO-1. Since CTAs are hypermethylated and suppressed in different cancers,[1] they represent potential targets for the cytosine demethylating activity of azanucleosides. Eleven older patients with acute myeloid leukemia (AML) and high-risk myelodysplastic syndrome (MDS) (median age 67 years;

range, 62-83 years) in whom standard induction treatment was not feasible were treated with low to intermediate doses of DAC (135-810 mg/m^2 over 72 hours, intravenously). Fifteen to thirty days after treatment start, blood or bone marrow blast percentage was decreased in 10 of 11 patients. mRNA expression of all 3 genes was undetectable in blasts from 9 patients before treatment, and following treatment was up-regulated in 8 of 9 cases. This was unlikely to be due to selection of normal hematopoietic cells via immediate cytotoxicity: in most patients, peripheral blood leukemic blasts were examined. These cleared slowly, over 2 to 4 weeks after treatment, as well-described for demethylating agents.[2,3] In each patient (independent of the dose level), up-regulation of the genes was detected at day 15 after treatment start, and for at least 2 of the CTAs, persisted for up to 30 days in 4 of 8 samples, indicating a stable effect upon their methylation status. The authors demonstrate that these genes may reasonably be considered possible targets of a demethylating agent: a T-cell response may be elicited by de-repression of CTAs, which thus provide interesting targets for future methylation studies in vivo.

Since multiple genes are hypermethylated in cancers, up-regulation of several targets by demethylating agents in vivo is very likely. This hypothesis is reinforced by the diverse effects observed in hematologic diseases and solid tumors treated with azanucleosides. For instance, an early, sometimes dramatic, platelet increase in responding MDS patients is followed by stabilization of erythropoiesis and granulopoiesis, in addition to an often-delayed blast clearance.[2-4] Hematologic responses to low-dose azanucleosides may be associated with cytogenetic normalization or with persistence of the cytogenetically abnormal clone in MDS[5,6]; re-expression of fetal hemoglobin has been noted in hemoglobinopathies,[7,8] and platelet increases are seen also in patients not suffering from hematopoietic stem cells disorders.[8,9] Since many genes, including surface and nuclear receptors, are known to be silenced by methylation,[10] the search for potential target genes of demethylating activity mediating these diverse effects is an intriguing goal for further translational studies. For instance, RNA microarray analyses of AML blasts treated ex vivo with DAC allow identification of large numbers of potential target genes.[11]

Tumor-associated antigens represent very interesting candidates, since they may mediate an immune response when de-repressed. Early studies by Dr Pinto and colleagues had already shown up-regulation of HLA-DR on AML blasts treated ex vivo with DAC,[12] providing a rationale for using DAC to increase the immunogenic potential of conditioning regiments prior to allogeneic bone marrow transplantation.[13] Proteinase(PR)3/myeloblastin, the autoantigen of Wegener granulomatosis, is also regulated by DNA methylation in myeloid cells,[14] and a 9 amino acid peptide derived from PR3 may be a powerful antigen for T lymphocytes directed specifically against myeloid leukemia cells.[15]

In addition to the presently used schedules of azanucleosides aimed at hematologic normalization or at least improvement in MDS and AML,[3,4,6] maintenance treatment with even lower doses may offer prolongation of response duration. Similarly, following allogeneic stem cell transplantation, modulation of a graft-versus-leukemia or graft-versus-tumor effect might be achieved by the up-regulation of suppressed CTAs via demethylation in residual malignant cells. In summary, T-cell responses elicited by presentation of tumor-assocated antigens may be a later consequence of demethylation, and complement gene re-expression effects modulating apoptosis and/or differentiation, which may be key mechanisms during the early phase of response to azanucleosides.

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