5-aza-2′-Deoxycytidine-induced Expression of Functional Cancer Testis Antigens in Human Renal Cell Carcinoma: Immunotherapeutic Implications[1]

Sandra Coral, Luca Sigalotti, Maresa Altomonte, Arne Engelsberg, Francesca Colizzi, Ilaria Cattarossi, Eugene Maraskovsky, Elke Jager, Barbara Seliger, and Michele Maio[2]

Cancer Bioimmunotherapy Unit, Centro di Riferimento Oncologico, Istituto di Ricovero e Cura a Carattere Scientifico, 33081 Aviano, Italy [S. C., L. S., M. A., F. C., I. C., M. M.]; Johannes Gutenberg University, III. Department of Internal Medicine, 55131 Mainz, Germany [A. E., B. S.]; Ludwig Institute for Cancer Research, Austin and Repatriation Medical Centre, Heidelberg, Victoria 3084, Australia [E. M.]; and Nordwest Krankenhaus, Department of Internal Medicine, 60488 Frankfurt, Germany [E. J.]

ABSTRACT

Purpose: **Limited therapeutic options are presently available for advanced renal cell carcinoma (RCC). This study was designed to define the clinical potential of the DNA hypomethylating agent 5-aza-2′-deoxycytidine (5-AZA-CdR) in human RCC, through its control of the expression of "therapeutic targets" of the cancer testis antigen (CTA) family, and of the tumor-associated antigen RAGE-1, in RCC cells.**

Experimental Design: **Reverse transcription (RT)-PCR assays of a panel of RCC cells treated with 5-AZA-CdR, investigated the induction of the expression of several CTAs and of RAGE-1. Immunoprecipitation and Western blotting assessed whether the expression of CTA-specific mRNA induced by 5-AZA-CdR resulted in a translated protein of appropriate molecular weight. The functional activity of *de novo* expressed CTA was evaluated using ^{51}Cr release cytotoxicity assays of 5-AZA-CdR-treated HLA-A2-positive RCC cells using HLA-A2-restricted NY-ESO-1-specific CTLs.**

Results: **Exposure to 5-AZA-CdR invariably induced the expression of the CTA MAGE-1, -2, -3, and -4, GAGE 1-6, and NY-ESO-1 in all of the RCC cells investigated. *De novo* expression of NY-ESO-1 was persistent, being still detectable 60 days after the end of treatment, and generated a functional protein efficiently recognized by HLA-A2-restricted NY-ESO-1-specific CTLs. 5-AZA-CdR also induced RAGE-1 expression in RAGE-1-negative RCC and sarcoma cells but not in neoplastic cells of different histology.**

Conclusions: **This study provides the scientific rationale to establish new strategies of chemoimmunotherapy in RCC patients. The well-defined immunogenicity of the investigated CTAs and of RAGE-1 suggest that systemic administration of 5-AZA-CdR represents a promising strategy to enhance the constitutively poor immunogenic potential of RCC cells, and to propose that virtually all RCC patients receive active and/or adoptive CTA- or RAGE-1-based immunotherapy.**

INTRODUCTION

Metastatic RCC[3] remains highly resistant to treatment and current therapeutic options for advanced disease include surgery, cytotoxic chemotherapy, and/or nonspecific immunotherapy (1, 2). The occurrence of spontaneous tumor regressions in RCC patients, the presence of a systemic T-cell-mediated immune response, and the tumor responses obtained in patients receiving cytokine therapy, indicate the potential immunogenicity of RCC cells and suggest that immunotherapeutic approaches represent a promising strategy to treat RCC patients (3, 4). However, the thus far poor therapeutic advantage of immunological treatment of RCC (5) suggests that additional information is required to improve its clinical efficacy.

Ongoing preclinical studies are evaluating the potential of TAAs expressed by RCC cells as therapeutic targets. Among these, RCC tissues exhibit a more frequent expression of the TAA RAGE-1 than do other tumors; however, its expression was restricted to 21% of investigated RCC samples (6). Noteworthy, the expression of RAGE-1 induced a significant cytotoxicity of RCC cells by anti-RAGE-1-specific CTLs (6). Besides additional TAAs such as PRAME and gp75 (6), RCC cells and tissues exceptionally express the "therapeutic" antigens belonging to the CTA family (6). Therefore, the identification of new strategies to circumvent this very limited expression of CTA in RCC tissues, and the poor expression of RAGE-1, might allow treating the majority of RCC patients with CTA- or RAGE-1-based immunotherapeutic approaches.

Previous evidence has clearly defined the regulatory role of DNA methylation in the constitutive expression of CTAs by cutaneous melanoma cells, and showed that *in vitro* treatment

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[2] To whom requests for reprints should be addressed, at Cancer Bioimmunotherapy Unit, Centro di Riferimento Oncologico, I.R.C.C.S., Via Pedemontana Occ.le 12, 33081 Aviano, Italy. Phone: 39-0434-659342; Fax: 39-0434-659566; E-mail: mmaio@cro.it.

[3] The abbreviations used are: RCC, renal cell carcinoma; TAA, tumor-associated antigen; CTA, cancer testis antigen; 5-AZA-CdR, 5-aza-2′-deoxycytidine; RT, reverse transcription; mAb, monoclonal antibody.

Table 1 RT-PCR analysis of TAAs expressed in RCC cells treated with 5-AZA-CdR

	MAGE-1		MAGE-2		MAGE-3		MAGE-4		GAGE 1-6		NY-ESO-1		RAGE-1	
Cells	Ctrl[a]	5-AZA-CdR	Ctrl	5-AZA-CdR	Ctrl	5-AZA-CdR	Ctrl	5-AZA-CdR	Ctrl	5-AZA-CdR	Ctrl	5-AZA-CdR	Ctrl	5-AZA-CdR
LE9104RC	−[b]	+	−	+	−	+	−	++	−	++	−	++	−	++
LE9211RC	++	++	++	++	++	++	−	++	++	++	−	++	++	++
MZ1257RC	−	+	−	+	−	+	−	++	−	+	−	++	+	+
MZ1846RC	−	+	−	+	−	+	−	++	−	++	−	++	−	+
MZ1851RC	−	+	−	+	−	+	−	+	−	+	−	+	+	+
MZ1879RC	−	+	−	+	−	+	−	++	−	++	−	+	−	−
MZ1940RC	−	+	−	+	−	+	−	++	−	+	−	+	+	+
MZ1973RC	−	+	−	+	−	+	−	++	−	++	−	+	+	+
HEARD	−	+	−	+	−	+	−	+	−	+	−	+	−	+

[a] Ctrl, control.
[b] Intensity of RT-PCR products: −, not detectable; +, weak; ++, strong.

with the DNA hypomethylating agent 5-AZA-CdR induced and/or up-regulated their expression in neoplastic cells (7–9).

In light of this evidence, and of the potential therapeutic implications of CTA and RAGE-1 expression, in this study we analyzed the role of 5-AZA-CdR in the control of the expression of different CTAs and of RAGE-1 in RCC cells. Furthermore, the functional role of *de novo* expressed CTAs was investigated. The results of these studies provide the basis to establish new strategies to enhance the immunogenic potential of RCC and to design novel immunotherapeutic approaches in RCC patients.

MATERIALS AND METHODS

Cells. Cells were grown in RPMI 1640 (Flow Laboratories, Inc., McLean, VA) supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. The RCC cells were obtained as described previously (6). The human cell lines COLO 201 (colon adenocarcinoma), Caco-2 (colon adenocarcinoma), MCF7 (breast adenocarcinoma), SK-BR-3 (breast adenocarcinoma), A549 (lung carcinoma), HT-1080 (fibrosarcoma), MG-63 (osteosarcoma), and SK-UT-1 (leiomyosarcoma) were purchased from American Type Culture Collection (Rockville, MD). The human cell line GLL-19 (ocular melanoma) was obtained as described previously (10). Cutaneous melanoma cell lines were generated from surgically removed metastatic melanoma lesions from patients who were in different clinical stages with no history of chemotherapy or immunotherapy and who had been admitted for surgery at the National Cancer Institute of Aviano, Italy, as described previously (11).

***In Vitro* Treatment of Tumor Cells with 5-AZA-CdR.** Treatment with 5-AZA-CdR (Sigma Chemical Co., St. Louis, MO), was performed as described previously (7). Briefly, cells were seeded at a density of $3–4 \times 10^5$ cells/ml in a T175 tissue culture flask. When cells became firmly adherent to plastic, the medium was replaced with fresh medium containing 1 μM 5-AZA-CdR, every 12 h for 2 days (four pulses). At the end of treatment, the medium was replaced with fresh culture medium without 5-AZA-CdR, cells were cultured for an additional 48 h (T_0), and used for molecular and functional assays. Control cultures were treated under similar experimental conditions in the absence of 5-AZA-CdR.

RT-PCR Analysis. Total RNA was extracted from control and 5-AZA-CdR-treated cells, using the TRIzol Reagent (Life Technologies, Inc., Milan, Italy) according to the manufacturer's instruction. RT was performed on 2 μg of total RNA, and PCR amplifications were then performed starting from 5 μl of the cDNA solution, using CTA-specific primer pairs, as described previously (7). Oligonucleotide primer sequences and the gene-specific PCR amplification programs that were used have been defined for MAGE-1, -2, -3, and -4 (12); NY-ESO-1 (13); GAGE 1-6 (14); and RAGE-1 (6). The integrity of RNA and oligo(dT)-synthesized cDNA was confirmed by the amplification of all cDNA samples with β-actin-specific primers, as described previously (7).

Ten μl of each RT-PCR sample were run on a 2% agarose gel and visualized by ethidium bromide staining.

The level of expression of distinct antigens was scored according to the intensity of the specific RT-PCR product, which was obtained by densitometric analysis of ethidium bromide-stained agarose gels using a Gel Doc 2000 documentation system and the QuantityOne densitometric analysis software (Bio-Rad, Milan, Italy). The intensity of RT-PCR products were compared with that of the reference human melanoma cell line Mel 142 (MAGE-1-, -2-, -3-, and -4-positive and GAGE 1-6-positive) or human fibrosarcoma cell line HT1080 (NY-ESO-1-positive) or human RCC cell line LE9211 (RAGE-1-positive). Samples were scored as follows: −, no RT-PCR product detectable; +, expression level <10% of that of the appropriate reference cell line; and ++, expression level >10% of that of the appropriate reference cell line.

Monoclonal Antibodies, Antisera, and Reagents. The anti-NY-ESO-1 mAb ES121 used in this study had been published previously (15). To produce the full-length NY-ESO-1 recombinant protein, the coding sequence for the protein was amplified by PCR from cDNA of the NY-ESO-1-positive HT-1080 fibrosarcoma cell line and subsequently cloned into the plasmid vector pQE30 containing histidine tags (Qiagen, Milan, Italy). The PCR primers for NY-ESO-1 amplification were ESO1-S 5′-CATCACGGATCCATGCAGGCCGAAGGCCGG-3′ and ESO1-AS 5′-ACCCGGGGTACCGCGCCTCTGCCCTGAGGG-3′. After transformation into *Escherichia coli* strain M15[pREP4], positive transformants were confirmed to contain the appropriate insert by restriction mapping and DNA sequencing. Recombinant NY-ESO-1 protein was then produced by isopropyl β-D-thiogalactoside induction and purified by Ni^{2+} affinity chroma-

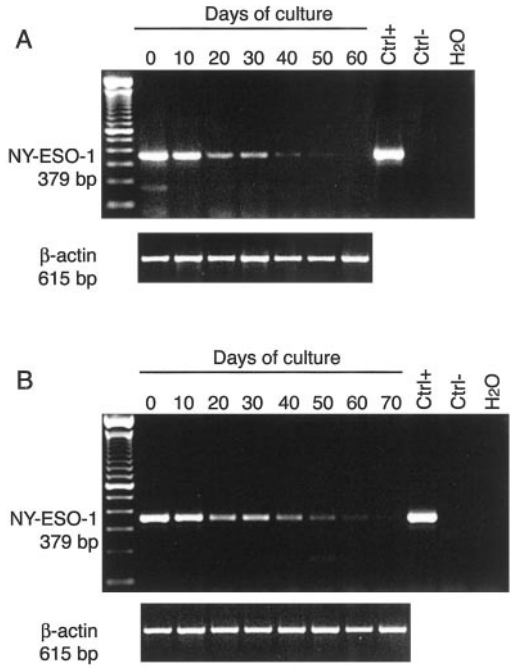

Fig. 1 Time-dependency of NY-ESO-1 induction in LE9104RC or MZ1257RC RCC cells by 5-AZA-CdR. Total RNA was extracted from MZ1257RC (*A*) or LE9104RC (*B*) RCC cells treated with 5-AZA-CdR and *in vitro* cultured for the indicated time in the absence of additional treatment. RT-PCR analysis was performed using NY-ESO-1- or β-actin-specific primer pairs. PCR products were then separated on a 2% agarose gel. One hundred-bp markers were run in the flanking lanes of each gel.

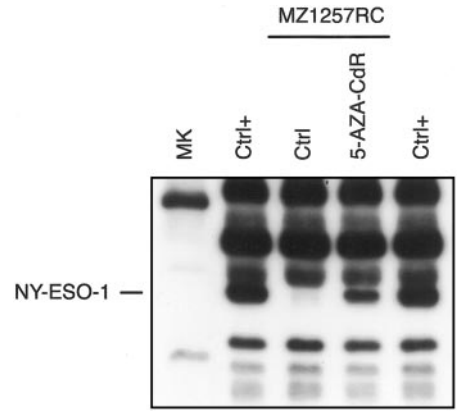

Fig. 2 Expression of NY-ESO-1 protein in MZ1257RC RCC cells treated with 5-AZA-CdR. Cell lysates of MZ1257RC cells, treated (*5-AZA-CdR*) or not (*Ctrl*) with 5-AZA-CdR, and of NY-ESO-1-positive fibrosarcoma cells HT1080 used as positive control (*Ctrl+*), were immunoprecipitated by an anti-NY-ESO-1 rabbit antiserum, size-fractionated by a 13% one-dimensional SDS-PAGE under reducing conditions, and blotted onto Hybond-C super-transfer nitrocellulose membranes. Then, membranes were incubated with 1 μg/ml ES121 anti-NY-ESO-1 mAb and were developed by the enhanced chemiluminescence technique.

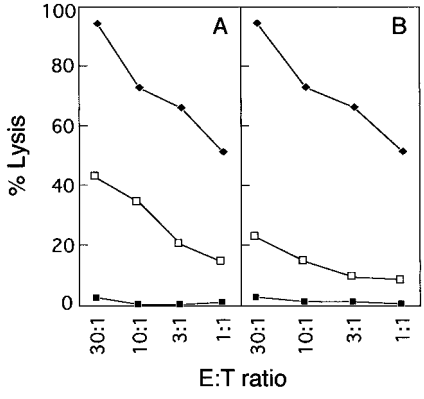

Fig. 3 CTL recognition of MZ1257RC or LE9104RC RCC cells treated with 5-AZA-CdR. Cytolytic activity of HLA-A2-restricted NY-ESO-1-specific MZ19-CTL-3/27 was tested by a standard 4-h ^{51}Cr release assay against MZ1257RC (*A*) or LE9104RC (*B*) RCC cells, before (■) and after (□) treatment with 5-AZA-CdR. RCC cells, pulsed with 10 μg/ml NY-ESO-1-encoded peptide p157-167, were used as control target cells (◆).

tography, following the procedures recommended by the manufacturer (Qiagen). The concentration of the purified protein was determined by colorimetric protein quantification assay (Bio-Rad, Milan, Italy).

The anti-NY-ESO-1 rabbit antiserum was obtained from a 20-week-old New Zealand White female rabbit immunized at weekly intervals with four s.c. injections of 1 mg of recombinant NY-ESO-1 protein. The rabbit was bled before immunization and 3 days after the last immunization.

Immunoprecipitation, SDS-PAGE, and Western Blotting. Immunoprecipitation, SDS-PAGE, and Western blotting were performed essentially as described previously (16). Briefly, cell lysates were incubated for 4 h with anti-NY-ESO-1 rabbit antisera bound to protein A-Sepharose (Amersham Pharmacia Biotech, Milan, Italy). Subsequently, immunoprecipitated components were size-fractionated by one-dimensional SDS-PAGE on 13% polyacrylamide slab gels under reducing conditions and were electroblotted onto Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were than incubated with 1 μg/ml of the anti-NY-ESO-1 mAb ES121, and blots were developed by the enhanced chemiluminescence technique, using the ECL kit (Amersham Pharmacia Biotech).

Cytotoxicity Assays. The HLA-A2-restricted anti-NY-ESO-1 CTL clone MZ19-CTL-3/27 was generated as described previously (17, 18). The sensitivity of the untreated and 5-AZA-CdR-treated RCC target cell lines to lysis by the HLA-A2-restricted anti-NY-ESO-1 CTL clone MZ19-CTL-3/27 was evaluated by a standard 4-h ^{51}Cr release assay, using different E:T ratios, and the results are expressed as percentage of specific lysis (17, 18).

RESULTS

Modulation of CTA Expression in RCC Cells by 5-AZA-CdR. The effects of 5-AZA-CdR on the constitutive expression of CTA in RCC were investigated by RT-PCR analyses. Exposure to 5-AZA-CdR invariably induced the ex-

Table 2 RT-PCR analysis of TAAs expressed in malignant cells of different histotype treated with 5-AZA-CdR

		RAGE-1		MAGE-1		MAGE-3		NY-ESO-1	
Cells	Histotype	Ctrl[a]	5-AZA-CdR	Ctrl	5-AZA-CdR	Ctrl	5-AZA-CdR	Ctrl	5-AZA-CdR
COLO 201	Colon carcinoma	−[b]	−	−	++	−	++	−	++
Caco-2	Colon carcinoma	−	−	−	++	−	++	−	++
MCF7	Breast carcinoma	−	−	+	++	−	+	−	++
SK-BR-3	Breast carcinoma	−	−	++	++	−	++	−	++
A549	Lung carcinoma	−	−	+	++	−	++	−	++
HT-1080	Fibrosarcoma	++	++	+	+	++	++	++	++
MG-63	Osteosarcoma	−	++	−	++	−	++	−	++
SK-UT-1	Leiomyosarcoma	−	++	−	++	−	++	−	++
GLL-19	Ocular melanoma	−	−	++	++	++	++	++	++
Mel 97	Cutaneous melanoma	−	−	++	++	++	++	−	++
Mel 116	Cutaneous melanoma	−	−	++	++	++	++	−	++
Mel 158	Cutaneous melanoma	−	−	++	++	++	++	−	++
Mel 195	Cutaneous melanoma	−	−	++	++	−	++	−	++
Mel 200	Cutaneous melanoma	−	−	++	++	++	++	++	++
Mel 275	Cutaneous melanoma	−	−	−	++	++	++	−	++
Mel 335	Cutaneous melanoma	−	−	++	++	++	++	−	++

[a] Ctrl, control.
[b] Intensity of RT-PCR products: −, not detectable; +, weak; ++, strong.

pression of MAGE-1, -2, -3, and -4, GAGE 1-6, and NY-ESO-1, in all of the CTA-negative RCC cells under study (Table 1). Additionally, the expression of the TAA RAGE-1 was induced in all of the RAGE-1-negative RCC cells, except MZ1879RC cells (Table 1).

Time-course analysis of cultured MZ1257RC and LE9104RC RCC cells demonstrated that the expression of NY-ESO-1 induced by the treatment with 5-AZA-CdR was persistent (Fig. 1). In fact, although it progressively decreased with time, it was still detectable after 50 and 60 days of culture in 5-AZA-CdR-free medium in MZ1257RC (Fig. 1*A*) and LE9104RC (Fig. 1*B*) RCC cells, respectively.

Biochemical Analysis of NY-ESO-1 Expressed by MZ1257RC RCC Cells Treated with 5-AZA-CdR. To assess whether the induction of CTA expression observed at mRNA level was followed by the production of the respective protein, immunoprecipitation and Western blotting for NY-ESO-1 were performed on untreated and 5-AZA-CdR-treated MZ1257RC cells. As shown in Fig. 2, the expression of NY-ESO-1 protein was induced in 5-AZA-CdR-treated RCC cells. Noteworthy, the molecular weight of *de novo* expressed NY-ESO-1 protein was identical to that of NY-ESO-1 constitutively expressed by HT-1080 fibrosarcoma cells, used as a positive control (Fig. 2).

Functional Analysis of *de Novo* Expressed NY-ESO-1 on RCC Cells Treated with 5-AZA-CdR. To evaluate the functional activity of *de novo* expressed NY-ESO-1, the HLA-A2-positive, NY-ESO-1-negative MZ1257RC and LE9104RC RCC cells were left untreated or treated with 5-AZA-CdR for the indicated time, and then challenged with the HLA-A2-restricted anti-NY-ESO-1 CTL MZ19-CTL-3/27 clone. Cytotoxic activity of MZ19-CTL-3/27 was exclusively observed on 5-AZA-CdR-treated RCC cells. The percentages of ^{51}Cr release for 5-AZA-CdR-treated MZ1257RC (Fig. 3*A*) and LE9104RC (Fig. 3*B*) RCC cells ranged, depending on different E:T ratios, from 42 to 14% and from 22 to 8%, respectively. In contrast, untreated MZ1257RC and LE9104RC cells were fully resistant to the lysis by HLA-A2-restricted NY-ESO-1-specific CTL MZ19-CTL-3/27 clones at all of the E:T ratios investigated (Fig. 3).

Modulation by 5-AZA-CdR of RAGE-1 Expression in Tumor Cell Lines of Different Histotype. To further investigate whether the induction of RAGE-1 by 5-AZA-CdR was restricted to RCC cells (Table 1) or whether it represents a general phenomenon of transformed cells, neoplastic cells of different histological origin were exposed to 5-AZA-CdR and assessed for RAGE-1 expression using RT-PCR. Noteworthy, the induction of RAGE-1 expression was restricted to sarcoma cells that, in selected cases, constitutively expressed the antigen (Table 2). In contrast, no RAGE-1 induction was observed in colon, breast, or lung carcinomas nor in cutaneous or ocular melanomas (Table 2). However, this lack of RAGE-1 induction (Table 2) was not attributable to ineffective exposure to 5-AZA-CdR because the expression of other CTAs was induced/up-regulated on this treatment.

DISCUSSION

In this study, we provide the first evidence on the immunological role of the DNA hypomethylating agent 5-AZA CdR in human RCC, and we foretell its potential immunotherapeutic implications in RCC patients. In general, RCC cells (Table 1) and tissues (6) very rarely express genes of the CTA family that are used as therapeutic targets of vaccination in different solid malignancies (19, 20), whereas we found that the exposure to 5-AZA-CdR invariably induced the expression of the CTAs MAGE-1, -2, -3, and -4, GAGE 1-6 and NY-ESO-1 in RCC cells (Table 1). In addition to that of the other CTAs investigated, the induction of NY-ESO-1 appears to be of major relevance because it represents the most immunogenic CTA defined to date, by eliciting both cellular and humoral responses (13). Noteworthy, *de novo* expression of NY-ESO-1 induced by 5-AZA-CdR was found to be persistent, inasmuch as its mRNA was still detectable by RT-PCR in MZ1257RC and LE9104RC

RCC cells 50 to 60 days after the end of treatment with 5-AZA-CdR (Fig. 1). Furthermore, the effect of 5-AZA-CdR on NY-ESO-1 expression by RCC cells was not limited to the transcriptional level but resulted in the expression of a NY-ESO-1 protein identical to that constitutively detectable in other neoplastic cell types (Fig. 2).

The efficient recognition of 5-AZA-CdR-treated MZ1257RC and LE9104RC RCC cells by the HLA-A2-restricted NY-ESO-1-specific CTL clone MZ19-CTL-3/27 demonstrated that (*a*) *de novo* synthesized NY-ESO-1 is functional, and (*b*) it allows specific CTL recognition of RCC cells that are otherwise completely resistant to NY-ESO-1-specific CTL-mediated lysis. The induction of CTL response appears to be most likely attributable to the *de novo* induced NY-ESO-1 protein rather than to the enhanced expression of HLA class I antigens on the cell surface of RCC cells. In fact, at variance with our previous observations demonstrating that 5-AZA-CdR up-regulates the constitutive expression of HLA class I antigens in melanoma cells (7), exposure to 5-AZA-CdR did not affect the baseline levels of HLA class I antigens and/or HLA class I allospecificities on RCC cells (data not shown). Thus, the CTL-mediated lysis of RCC cells induced by 5-AZA-CdR appears to represent a direct and exclusive consequence of *de novo* expressed NY-ESO-1, which generates sufficient amounts of immunogenic peptides to be loaded on preexisting HLA-A2 molecules, which provides cell surface copies of HLA class I-peptide complexes above the threshold level required for the efficient CTL recognition of target cells (21). Nevertheless, the relative contribution of the components of the antigen-processing machinery (22), whose quantitative expression and functional activity might be modified by 5-AZA-CdR, remains to be investigated in detail.

The consistent induction and the persistent expression of functional CTAs activated by 5-AZA-CdR in RCC cells provide new therapeutic options to RCC patients. In fact, 5-AZA-CdR is currently used as a cytotoxic/differentiating agent in ongoing Phase I/II clinical trials in hemopoietic malignancies (23). Thus, it can be foreseen that systemic administration of 5-AZA-CdR in metastatic RCC, in addition to exerting a direct antineoplastic activity, may be useful in reverting the CTA-negative phenotype of neoplastic lesions. Such an approach would, in fact, qualify virtually all RCC patients to be vaccinated or to receive adoptive T-cell-based and/or humoral immunotherapy against multiple therapeutic CTAs. Furthermore, the systemic use of 5-AZA-CdR could prove useful to up-regulate the constitutive immunogenicity of RCC cells through the neo-expression of different CTAs by neoplastic cells as well as through the increased expression of costimulatory molecules. Preliminary support to these hypotheses is provided by the demonstration that i.p. injection of 5-AZA-CdR induced or up-regulated the expression of different CTAs in nude mice xenografts of human melanoma cells.[4]

The immunotherapeutic potential of 5-AZA-CdR in RCC is further supported by its ability to induce the expression of RAGE-1 in the majority of the RAGE-1-negative RCC cells analyzed (Table 1). In fact, RAGE-1 appears to be a promising therapeutic target in RCC patients, because autologous and allogeneic HLA-B7-positive RCC cells that expressed minimal levels of RAGE-1 were highly susceptible to the cytotoxic activity of anti-RAGE-1 CTLs (6). The notion that RAGE-1 expression in RCC cells is regulated by DNA methylation allowed an additional intriguing observation. Whereas the exposure to 5-AZA-CdR invariably induced or up-regulated the expression of different CTAs in the neoplastic cells of different histology investigated, the expression of RAGE-1 was exclusively induced in RCC and sarcoma cells that, in selected cases, constitutively expressed the antigen (Table 2). Thus, in analogy to CTA, the expression of RAGE-1 in neoplastic tissues is clearly methylation regulated, but, in contrast to CTA, its distribution is restricted to transformed cells of selected histology, which may be attributable to the requirement of the expression of tissue-specific transcription factors.

Altogether, the data reported in this study define immunological activities of 5-AZA-CdR in RCC. This evidence strongly suggests that 5-AZA-CdR represents a useful pharmacological agent to design and establish new therapeutic strategies in RCC patients, either using the compound alone or using it in combination with CTA- and/or RAGE-1-based immunotherapeutic approaches.

[4] S. Coral and M. Maio, unpublished observations.

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