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The frequent expression of cancer/testis antigens provides opportunities for immunotherapeutic targeting of sarcoma

Maha Ayyoub[1], Robert N. Taub[2], Mary-Louise Keohan[2], Mary Hesdorffer[2], Genevieve Metthez[1], Lorenzo Memeo[3], Mahesh Mansukhani[3], Hanina Hibshoosh[3], Charles S. Hesdorffer[2], and Danila Valmori[1]

[1]Ludwig Institute Clinical Trial Center, Division of Medical Oncology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY, USA
[2]Division of Medical Oncology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY, USA
[3]Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY, USA

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Abstract

Sarcomas are rare but aggressive malignant tumors associated with high mortality, for which the efficacy of standard therapies remains limited. In order to develop immunotherapeutic approaches for the treatment of sarcoma, we studied the relevance of cancer/testis antigens (CTAs), a group of antigens whose expression is developmentally regulated and that is specifically found in some tumor types, as sarcoma vaccine targets. CTA expression was assessed by PCR and/or immunohistochemistry (IHC) in sarcoma tumor samples that included different histological subtypes and sarcoma cell lines. Expression of HLA class I was assessed by IHC in tumor samples and by FACS® analysis in cell lines. More than 70% of the tumor samples expressed at least one CTA. The majority of tumors and cell lines expressed normal levels of HLA class I. HLA class I expression in cell lines was enhanced upon treatment with IFN-gamma. CTA expression was enhanced or induced by treatment with the demethylating agent 5-aza-2'-deoxycytidine, resulting in recognition by specific CTLs. Interestingly, a spontaneous humoral and CD8+ T cellular response to the CTA NY-ESO-1 was detected in a synovial sarcoma patient. Together, these findings strongly support the implementation of CTA-based immunotherapy of sarcoma as a means to improve the efficacy of the standard therapy.

Introduction

Sarcomas are a heterogeneous group of malignant mesenchymal tumors. Although rare, there are approximately 9800 new cases of sarcoma each year in the United States. These include the two main groups of sarcomas, namely soft tissue sarcomas (7800 new cases each year) and bone tumors (2000 new cases each year). They

are often aggressive diseases characterized by a large local invasion, a tendency to metastasize, and a high mortality rate. Current standard therapy includes surgical resection of the tumor and adjuvant radiation therapy and/or chemotherapy to attempt to prevent recurrence. Adjuvant therapy has been shown to decrease local recurrence in some instances, but no convincing effect on overall survival has been demonstrated (1). Thus, alternative treatments that may increase the poor survival rate of sarcoma patients are eagerly awaited.

The identification of antigens expressed predominantly or exclusively by tumor cells as compared to normal tissues has recently led to the implementation of a growing number of clinical trials aimed at eliciting or enhancing immune responses to such antigens in cancer patients (2). Identification of tumor antigens, however, has mostly been achieved for tumors of known high spontaneous immunogenicity, such as malignant melanoma. In contrast, in the case of sarcomas, the identification of relevant target antigens has remained elusive.

The group of so-called CTAs comprises nonmutated self-antigens whose expression is mostly limited to germ-line cells and to cancer cells but is not found in the cells of healthy adults (3). CTAs include 20 genes or gene families identified to date (3). These genes are frequently members of multigene families and often map to chromosome X. The biological function of CTAs is unknown for most group members, with the exception of a few proteins involved in gametogenesis and fertilization. In the case of other family members, the information available suggests that they could correspond to regulators of gene transcription or to factors involved in cell cycle progression and apoptosis. The molecular mechanisms leading to CTA expression in cancer cells are still poorly defined. An engaging hypothesis is that it may be due to the activation of a gametogenic protein expression program (4). However, both the genes involved in the activation of this program, as well as the relationship between these events and the oncogenic process, remain to be defined.

In contrast to other antigens expressed only by a certain type of tumors (for example, melanocyte differentiation antigens in melanoma), CTAs are frequently expressed in tumors of some histological types but infrequently in others. As CTA-specific immune responses selectively target cancer cells, CTAs are ideal candidates for the development of cancer vaccines. In this study, we have addressed the relevance of CTAs as vaccine targets for immunotherapy of sarcoma. Together, our results indicate that the frequent expression of CTAs in sarcoma provides opportunities for the implementation of novel immunotherapeutic approaches that could improve treatment outcomes.

Results

CTA expression in sarcoma tumors and cell lines

We used RT-PCR to analyze tumor tissues from 36 sarcoma patients, including 9 histological subtypes, for the expression of *SSX*, *MAGE-A1*, *-A3*, *-A4*, *-A10*, *NY-ESO-1*, *LAGE-1*, *CT-7*, *CT-10*, *BAGE*, *GAGE*, and *SCP-1*. CTA expression was also analyzed in 8 sarcoma cell lines. The results we obtained for all antigens and patients are summarized in Table 1. Some of the CTAs most frequently expressed in melanoma (for example, *MAGE-A3* was expressed in about 40% of primary and 70% of metastatic melanoma tumor samples) (5) were also frequently expressed in sarcoma. *MAGE-A3* expression was found in 44% of the tumor samples. Other *MAGE-A* family members were also expressed in a smaller, but still significant, fraction of the samples (*MAGE-A1*, 27%; *MAGE-A4*, 30%; and *MAGE-A10*, 14%). Other CTAs frequently expressed in sarcomas included *NY-ESO-1* (36%) and the highly homologous gene, *LAGE-1* (27%). *NY-ESO-1* and *LAGE-1* have been shown to encode common CTL epitopes (6, 7, 8). Consistent with our previous data on melanoma tumors (9), expression of *NY-ESO-1* and *LAGE-1* was not completely overlapping in tumor samples: One of the two genes was expressed in 47% of the samples, whereas simultaneous expression of both genes was found in only 16% of the samples.

GAGE and SSX antigens were also frequently expressed in sarcoma (in 44% and 39% of samples, respectively). The remaining genes (*CT-7*, *CT-10*, *BAGE*, and *SCP-1*) were expressed in a lower proportion of samples (14%, 28%, 17%, and 5%, respectively). Overall, we found detectable expression of at least one antigen in 27/36 (75%) of tumor samples and 7/8 cell lines. CTA expression was not limited to a given histological subtype. As was documented in previous studies, expression was often clustered, with multiple CTA being expressed simultaneously in the same tumor (3). Indeed, whereas only 7 of the positive tumors expressed a single antigen, the remaining 20, as well as all positive sarcoma cell lines, coexpressed 2 to 9 antigens (Table 1). Unlike other CTAs, the expression of *SCP-1* was not clustered with that of the other antigens. Indeed, of the two positive tumor samples, both expressing high levels of the antigen, one did not express any of the other antigens tested and the other only coexpressed low levels of *SSX*. Similarly, the only *SCP-1*-expressing line (SK-ES-1) only coexpressed intermediate levels of *MAGE-A3*. To confirm CTA expression in sarcoma at the protein level, we performed a parallel analysis of the tumor samples by IHC using the NY-ESO-1-specific monoclonal antibody, ES121. The results of this analysis are summarized in Table 2. In general, there was an excellent correlation between the two sets of results, with the exception of a few samples showing low levels of expression by PCR (1% of the reference line) that read negative by IHC.

Table 1. CTA expression in sarcoma tumor samples and cell lines.

Histological subtype	Case no./ Cell line	SSX	NY-ESO-1	LAGE-1	MAGE-A1	MAGE-A3	MAGE-A4	MAGE-A10	CT-7	CT-10	SCP-1	GAGE	BAGE
Tumor samples													
Gastrointestinal stromal tumor	1	+	+++	+++	++	++	+	++	-	+	-	++	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	+	-	-	+	-	-	-	+++	-	+	+
	4	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-
Synovial sarcoma	1	-	+++	++	+	+	+++	-	-	-	-	+	-
	2	-	+++	-	+	-	+++	-	-	-	-	-	-
Uterine leiomyosarcoma	1	+++	+	++	+	++	+	+	-	+	-	++	+
	2	-	-	-	-	-	-	-	-	-	-	++	-
	3	+++	-	+++	+++	+++	+++	+++	+++	+	-	+++	-
	4	+	+	-	+	+	-	-	-	+	-	++	+
	5	++	+++	-	-	++	+++	-	-	-	-	++	-
Leiomyosarcoma	1	-	-	-	-	-	-	-	-	-	-	+	-
	2	-	-	-	-	-	++	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	++	-	-	+	-	++	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-
	6	+	-	++	-	++	-	-	+	++	-	+	-
	7	-	-	-	-	-	-	-	-	-	-	-	+
Angiosarcoma	1	-	+	-	-	+++	+++	-	-	-	-	-	-
	2	-	++	-	-	+	-	-	-	-	-	+	-
	3	-	-	-	-	++	-	-	-	-	-	-	-
Malignant fibrous histiocytoma	1	+	++	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	+++	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	+++	-	-	+	+++	-	+++	+	++	-	+++	+++
	5	-	-	-	-	-	-	-	-	-	-	+	-
Liposarcoma	1	+++	-	++	-	++	-	-	+	+++	-	+++	+++
	2	++	+++	+++	-	+	++	+	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	+++	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-
Osteosarcoma	1	+	-	-	-	-	-	-	-	-	+++	-	-
Chondrosarcoma	1	+++	+	+++	+++	+++	+++	-	+	++	-	+++	-
	2	+++	+	++	+	+	-	-	-	+	-	+	-
Tumor cell lines													
Ewing's sarcoma	SK-ES-1	-	-	-	-	++	-	-	-	-	+++	-	-
Leiomyosarcoma	SK-LMS-1	++	-	-	-	+	-	-	-	+++	-	++	-
Uterine leiomyosarcoma	SK-UT-1	-	-	-	-	-	-	-	-	-	-	-	-
Liposarcoma	SW 872	++	-	-	++	+++	++	+++	++	+++	-	+++	++
Osteosarcoma	Saos-2	++	+	+++	++	+++	-	-	-	+++	-	++	-
Osteosarcoma	HOS	-	-	-	-	-	-	-	-	-	-	+	-
Uterine sarcoma	MES-SA	-	-	-	-	++	-	-	-	-	-	-	+++
Fibrosarcoma	HT-1080	+++	+++	+++	++	+++	-	-	-	+++	-	+++	+++

Table 2. Comparison of NY-ESO-1 expression in sarcoma tumors as assessed by PCR or IHC.

Histological subtype	Case no.	NY-ESO-1	
		PCR [a]	IHC [b]
Gastrointestinal stromal tumor	1	+++	3/pos
	2	-	0/neg
	3	+	2/pos
	4	-	0/neg
	5	-	0/neg
Synovial sarcoma	1	+++	2-3/pos
	2	+++	3/pos
Uterine leiomyosarcoma	1	+	1/neg
	2	-	1/neg
	3	-	0/neg
	4	+	2/pos
	5	+++	3/pos
Leiomyosarcoma	1	-	0/neg
	2	-	1/neg
	3	-	0/neg
	4	-	1/neg
	5	-	0/neg
	6	-	0/neg
	7	-	1/neg
Angiosarcoma	1	+	1/neg
	2	++	2/pos
	3	-	1/neg
Malignant fibrous histiocytoma	1	++	2/pos
	2	-	1/neg
	3	-	1/neg
	4	-	1/neg
	5	-	0/neg
Liposarcoma	1	-	1/neg
	2	+++	3/pos
	3	-	0/neg
	4	-	0/neg
	5	-	0/neg
	6	-	nd
Osteosarcoma	1	-	0/neg
Chondrosarcoma	1	+	0/neg
	2	+	1/neg

[a] NY-ESO-1 expression at the RNA level was assessed by semiquantitative RT-PCR.
[b] NY-ESO-1 expression at the protein level was assessed by IHC using monoclonal antibody ES121 to NY-ESO-1. Scoring: 0, no positive cells; 1, rare and scattered positive cells; 2, ≤50% positive cells; 3, >50% positive cells; nd, not done. Interpretation: pos, positive; neg, negative.

HLA class I expression in sarcoma cell lines and tumors

Targeting of tumors by specific CTLs requires sufficient expression of HLA class I by tumor cells. Defective HLA class I expression has been documented in variable proportions in different tumors (10). The sarcoma tumor cell lines analyzed in this study expressed clearly detectable levels of HLA class I, as assessed by FACS® analysis using the anti-HLA class I mAb W6/32, although the expression levels were variable depending on the cell line (Table 3). Four of the six sarcoma cell lines that were HLA-A2+ by molecular typing expressed high levels of HLA-A2 antigen as assessed using the mAb BB7.2 (Table 3). In addition, in all cases, both total HLA class I and HLA-A2 (in the case of HLA-A2+ cells) antigen expression levels were substantially increased after treatment with IFN-gamma. It is noteworthy that the sarcoma tumor cell lines did not express detectable levels of HLA class II antigens, even after treatment with IFN-gamma (not shown). We also performed an IHC analysis using the W6/32 mAb to assess total HLA class I expression in a fraction of sarcoma tumors for which frozen tumor sections were available. As summarized in Table 4, for the majority of the tumor samples analyzed (20 of 26 = 77%) we found normal levels of total MHC class I antigen expression in a large fraction of tumor cells, indicating that lack of MHC class I expression does not represent an inherent obstacle for the development of a CTL-based vaccine for sarcoma.

Table 3. MHC class I and HLA-A2 expression in tumor cell lines.

Cell line	HLA-A2 typing	MHC class I expression [a]			HLA-A2 expression [b]		
		Isotype control	- IFN-γ	+ IFN-γ	Isotype control	- IFN-γ	+ IFN-γ
SK-ES-1	+	5	20	325	2	6	134
SK-LMS-1	+	7	184	543	6	107	551
SK-UT-1	+	6	263	412	4	5	8
SW 872	+	6	84	121	5	187	630
Saos-2	+	6	118	150	4	84	150
HOS	+	11	183	230	7	244	558
MES-SA	-	6	28	245	3	5	6
HT-1080	-	5	36	330	4	8	8
SK-MEL-37	+	4	78	158	4	38	63
Mel 275	+	5	76	+ [c]	4	75	+ [c]

[a]MHC-class-I expression was assessed by staining with mAb W6/32. The mean fluorescence intensity is reported.
[b]HLA-A2 expression was assessed by staining with mAb BB7.2. The mean fluorescence intensity is reported.
[c]Cells died upon treatment with IFN-γ.

Table 4. MHC class I expression in sarcoma tumors.

Histological subtype	Case no.	MHC class I expression	
		W6/32 [a]	Interpretation [b]
Gastrointestinal stromal tumor	1	C1	+
	2	C2	+
	3	A1	-
	4	B1	+
	5	C2	+
Synovial sarcoma	1	A0	-
Uterine leiomyosarcoma	1	C2	+
	3	C2	+
	4	C2	+
Leiomyosarcoma	1	A0	-
	2	A0	-
	3	C2	+
	4	C2	+
	5	C2	+
	6	C2	+
Angiosarcoma	1	C2	+
	3	C2	+
Malignant fibrous histiocytoma	1	C2	+
	2	A0	-
	4	C2	+
	5	B2	+
Liposarcoma	1	C2	+
	2	A0	-
	5	C2	+
	6	A2	+
Chondrosarcoma	1	C2	+

[a]W6/32 scoring based on intensity (score from 0 to 3) and percentage of positive cells (A: <25% positive cells, B: 25-75% positive cells, C: >75% positive cells).
[b]Cases were considered negative if the immunohistochemical staining had a score of 0 to 1 in ≤25% tumor cells (A0 or A1). All other cases were considered positive.

Impact of DNA demethylation on CTA expression in sarcoma cells and recognition by specific CTLs

Aberrations in DNA methylation, including genomic hypomethylation, are a common feature of human cancer (11, 12) and may provide a common mechanism for the expression of CTAs in cancer cells. In line with this hypothesis, it has been shown that treatment with DNA demethylating agents can induce the expression of CTA encoding genes in cancer cells (13), suggesting a strategy to augment antitumor immunity in cancer patients. To analyze the impact of DNA demethylation on the expression of CTAs by sarcoma cells, the expression of selected antigens in sarcoma cell lines was analyzed after treatment with 5-aza-2'-deoxycytidine (5-AZA-CdR) for 72 h. As illustrated in Figure 1A for *MAGE-A10*, *SSX*, *NY-ESO-1*, and *LAGE-1*, and similarly for all remaining CTA encoding genes except *SCP-1* (data not shown), CTAs that were not expressed at significant levels before treatment became detectable. In the case of *SCP-1*, expression was not modulated by the treatment. Together with the observation that the expression of this gene is not clustered with that of other CTAs (Table 1), this indicates that the expression of *SCP-1* is regulated through different mechanisms. It is of note that, unlike the other members of the group, *SCP-1* is not located on chromosome X (14). The increased, or induced, CTA expression resulting from treatment of tumor cells with demethylating agents should, in principle, lead to increased tumor recognition by CTA-specific CTLs. Figure 1B illustrates the effect of these agents on the recognition of SW 872 cells by HLA-A2 restricted, tumor-reactive CTL clones specific for CTA-derived peptides. In the case of high CTA expression levels prior to any treatment (that is, *MAGE-A10* and *SSX-2*), recognition of sarcoma cells by specific CTLs was similar in the presence or in the absence of peptide. In contrast, when antigen expression was not detectable by PCR prior to treatment (that is, *NY-ESO-1* and *LAGE-1*), no tumor recognition by CTLs was detected in the absence of exogenously added peptide. Under these experimental conditions, no significant difference in antigen recognition was detected after treatment with IFN-gamma. In contrast, recognition of antigens whose expression was undetectable in untreated tumor cells became evident after treatment of tumor cells with 5-AZA-CdR.

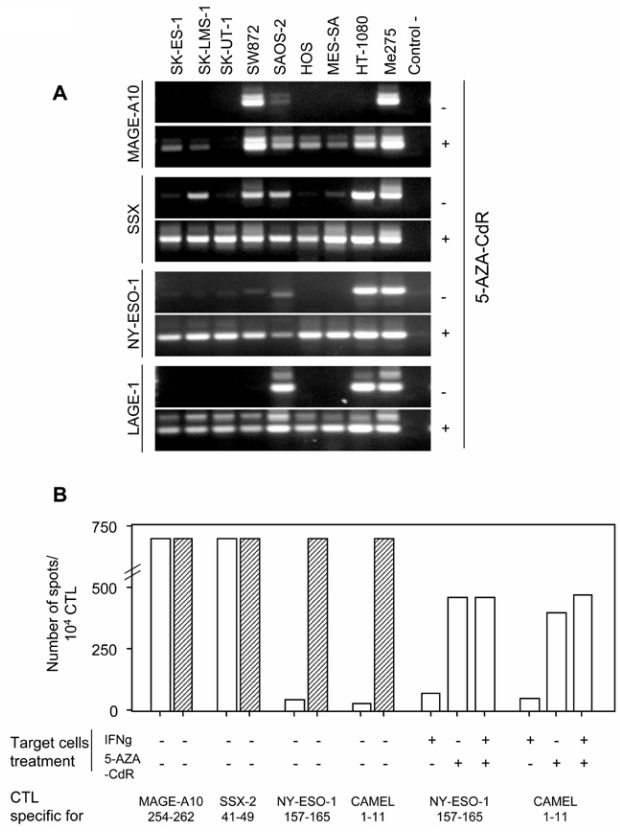

Figure 1. Impact of 5-AZA-CdR on CTA expression in sarcoma cells and recognition by CTLs. (A) RT-PCR analysis of untreated tumor cells or cells treated with 5-AZA-CdR. (B) Antigen recognition of SW 872 sarcoma cells by CTA-specific CTLs in the absence (white bars) or presence (dashed bars) of 1 µg/ml of the corresponding peptide.

Assessment of spontaneous immune responses to CTAs in sarcoma patients

Spontaneous humoral and T cell responses to CTAs have been documented in patients with different tumor types. Among the patients listed in Table 1, sera were available in the case of 10 patients. We assessed the presence of antibodies against NY-ESO-1 or MAGE-A4 in these sera by using the corresponding recombinant proteins in an ELISA assay, as described previously (15). Four of the sera tested were from patients with detectable *MAGE-A4* expression in the corresponding tumor lesions [uterine leiomyosarcoma case 1, synovial sarcoma (SS) case 2, angiosarcoma case 1, gastrointestinal stromal tumor (GIST) case 1] and four were from *NY-ESO-1*-expressing patients (SS case 2, angiosarcoma case 1, GIST case 1 and case 3). Only in the case of SS case 2, which expressed the highest levels of NY-ESO-1 (see Figure 2 for IHC), were specific antibodies clearly detectable in the serum (Figure 3A). Antibodies against MAGE-A4, however, were not detectable in any of the tested sera, although two of the patients (SS case 2 and angiosarcoma case 1) expressed high antigen levels (Table 1). PBLs were available in the cases of four patients who expressed HLA-A2 antigens (SS case 2, liposarcoma case 1, GIST case 1 and case 3). For these patients, CD8+ T cell responses to known HLA-A2-restricted peptides derived from CTAs expressed in the corresponding tumors (NY-ESO-$1_{157-165}$, SSX-2_{41-49}, CAMEL$_{1-11}$, MAGE-A$4_{230-239}$, and MAGE-A$10_{254-262}$) were assessed by *in vitro* stimulation with the corresponding peptide, followed by staining with fluorescent HLA-A2/peptide multimers, as described (16). Consistent with his serological response to NY-ESO-1, a clear CD8+ T cell response to NY-ESO-$1_{157-165}$ was detected for SS case 2 (Figure 3B). No specific CTL responses to the other CTA-derived peptides were detected in this patient or in the other patients (data not shown). It is of note that SS case 2 was a patient with advanced disease. Homogeneous expression of NY-ESO-1 has been reported in more than 80% of SS cases analyzed (17). In agreement with these data, the two SS cases analyzed in this study expressed high levels of NY-ESO-1. Detection of strong humoral and cellular responses to NY-ESO-1 in SS case 2 is consistent with previous findings in patients with advanced NY-ESO-1-expressing cancers (18). It is likely that such spontaneous responses develop so late in the disease because the high load of tumor antigen present in progressing patients is required for optimal cross-priming by professional APCs (19). We hope that by vaccinating patients bearing tumor antigen-expressing lesions with tumor antigen-derived immunogens at earlier stages of the disease we can stimulate an earlier response and have a favorable impact on their immune system's ability to control the disease.

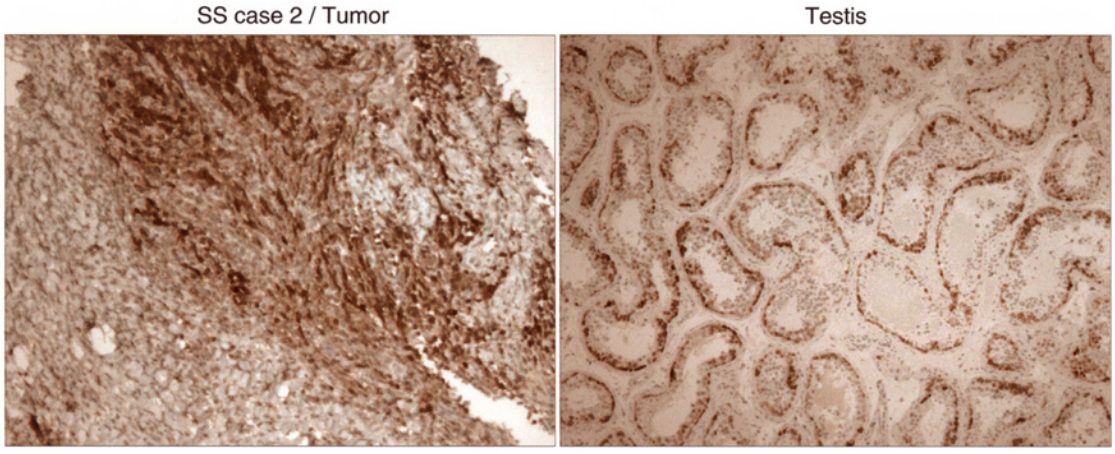

Figure 2. Expression of NY-ESO-1 antigen in a patient with synovial sarcoma. The expression of NY-ESO-1 in a tumor specimen from patient SS case 2 was assessed using monoclonal antibody ES121. For comparison, ES121-specific staining is shown for spermatogonia/primary spermatocytes in the periphery of seminiferous tubles.

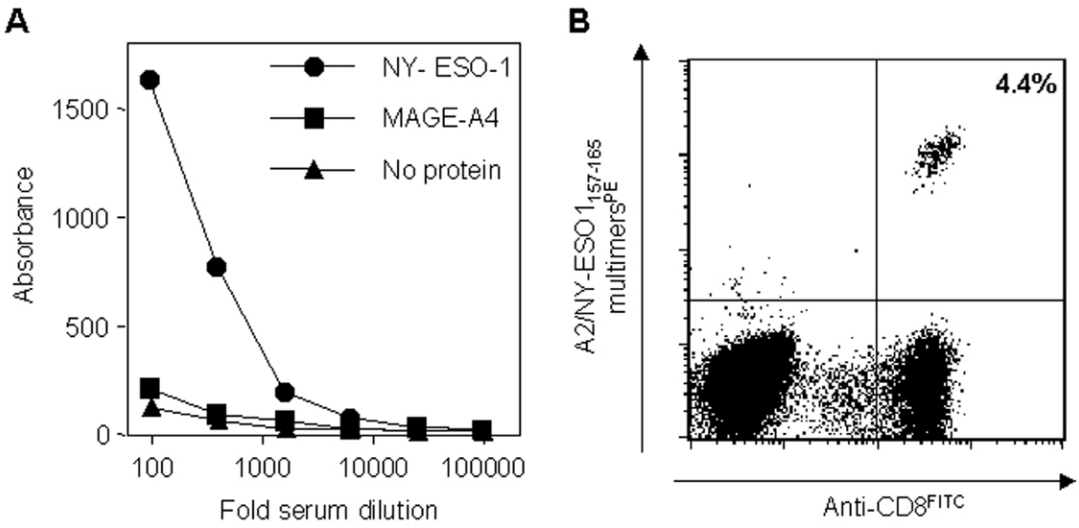

Figure 3. Spontaneous immune response to NY-ESO-1 antigen in SS case 2. (A) The presence of NY-ESO-1-specific circulating antibodies was assessed by ELISA using serial serum dilutions. (B) NY-ESO-1-specific CTLs were assessed among circulating CD8+ T lymphocytes, 1 wk after *in vitro* stimulation with peptide NY-ESO-$1_{157-165}$, using fluorescent soluble HLA-A2/NY-ESO-1 peptide multimers and anti-CD8 mAb.

Discussion

Over a century ago, after observing the absence of tumor recurrence in a patient with small cell sarcoma after severe infection with erysipelas, Dr. W. B. Coley (20) investigated the beneficial effects of administering microbial injections into metastatic cancer patients, thereby launching the concept that immunotherapy could be an option in the treatment of cancer. During the last decade, the identification of a growing number of tumor-specific antigens has allowed the implementation of the first clinical trials involving tumor antigen-specific vaccination of cancer patients (2). Paradoxically, immunotherapeutic approaches for the treatment of sarcoma, the tumor type that first inspired cancer immunotherapy, have been envisaged only very recently (21, 22). The aim of this study is to address the relevance of CTA as vaccine targets for immunotherapy of sarcoma.

Our results show that the expression of CTAs is very frequent in sarcoma, with frequencies comparable to those of other malignancies, such as melanoma or lung cancer, for which there are ongoing clinical trials involving immunotargeting (3). Similar to the results obtained in other malignancies (3), CTA expression in sarcoma samples and tumor cell lines was often clustered, with multiple CTAs being simultaneously expressed in the same tumor. The clustered expression of CTA genes favors a common mechanism leading to their expression. A shared feature of CTA gene expression is induction by the DNA demethylating agent 5-aza-2'-deoxycytidine (5-AZA-CdR) (3, 13). We observed that expression of all CTA genes tested, except *SCP-1*, could be significantly augmented or induced *de novo* upon treatment of sarcoma cell lines with 5-AZA-CdR. *SCP-1* expression in tumor samples was indeed not clustered with that of other CTAs (Table 1), indicating that the expression of *SCP-1* is regulated through different mechanisms.

Assessment of MHC class I expression in sarcoma cell lines and tumor samples showed, in about two-thirds of the cases, expression levels that would allow antigen recognition by tumor-specific CTLs. MHC class I expression was strongly upregulated by *in vitro* treatment of sarcoma cell lines with IFN-gamma. A spontaneous humoral and cellular response to one of the test antigens (NY-ESO-1) was detected in one antigen-expressing sarcoma patient with advanced disease. In a previous study, antibody responses to NY-ESO-1 were detected in

sera from 2 of 54 sarcoma patients, suggesting that the spontaneous immune responses against this antigen in sarcoma patients could be less frequent than in other malignancies (15, 23). A more rigorous comparison of the spontaneous immunogenicity of CTAs in sarcoma and in other malignancies, however, would require assessment of CTA-specific humoral and cellular immune responses in a large panel of antigen-expressing patients.

Together, our data strongly encourage the introduction of clinical trials involving the vaccination of sarcoma patients with CTA-derived immunogens. To include a large number of sarcoma patients, the vaccine should contain multiple T cell epitopes from the most commonly expressed CTAs. Vaccination could be given as an adjuvant therapy, after standard therapy is completed. Longitudinal molecular monitoring of the elicited immune responses would provide information regarding magnitude, quality, and persistency of the induced CTA-specific immune responses. Due to the generally rapid progression of the disease, statistically significant information on the impact of vaccination on the patients' survival should be obtained within a time period of 1 to 2 yr. In the case of patients with advanced disease, additional therapeutic options such as adoptive transfer of CTA-specific T cells, possibly in combination with administration of DNA demethylating agents, should also be contemplated.

Abbreviations

5-AZA-CdR, 5-aza-2'-deoxycytidine; CTA, cancer/testis antigens; IHC, immunohistochemistry

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Materials and methods

Patients, tumor samples, and cell lines

Patients gave their written informed consent for the use of blood and tissue for research purposes. The local Institutional Review Board approved the study (IRB#9853). Tumor tissues were obtained during routine surgery at the Presbyterian Hospital, Columbia University, New York, USA. Tissues were preserved in RNAlater (Sigma, St. Louis, MO, USA) and stored at -20°C. Human sarcoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA).

RT-PCR

Aliquots of tissue samples were processed using an FP 120 Fast Prep Cell Disrupter (Savant Instruments). Total cellular RNA was prepared from frozen tumor tissue specimens or cell lines using a Nucleospin® RNA II extraction kit (Macherey-Nagel, Düren, Germany). Complementary DNA synthesis was performed using Promega Reverse Transcription System A3500 (Madison, WI, USA). The integrity of the cDNA was tested by amplifying beta-actin in a 35-cycle PCR reaction. The panel of CTAs analyzed included SSX, NY-ESO-1, LAGE-1, MAGE-A1, -A3, -A4, -A10, BAGE, GAGE, CT7/MAGE-C1, CT10, and SCP-1. Messenger RNA expression of the CTAs in tumor tissue samples or in tumor cell lines was assessed by using previously described oligonucleotide primers and conditions: SSX (13), NY-ESO-1 (24), LAGE-1 (25), MAGE-A1, MAGE-A3, MAGE-A4 (26), MAGE-A10 (27), BAGE (28), GAGE (29), CT7/MAGE-C1 (26), CT10 (30), and SCP-1 (14).

Antigen recognition assay

Tumor recognition by CTA-specific CTLs was assessed by IFN-gamma ELISPOT assay using nitrocellulose-lined 96-well microplates (MAHA S45, Millipore, Bedford, MA, USA) and an IFN-gamma ELISPOT kit (DIACLONE, Besancon, France). Sarcoma cell lines expressing the HLA-A2 antigen or the melanoma cell line Me 275 (HLA-A2+ SSX-2+) were used as APCs. APCs (5 x 10^4/well) pulsed or not with the relevant peptide (1 µM) during 1 h at 37°C were added together with the T cell clone indicated (10^4/well). The assay was carried out as described previously (31). Spots were counted using a stereomicroscope with a magnification of 15×.

Analysis of MHC class I and NY-ESO-1 antigen expression

Expression of total MHC class I or HLA-A2 antigens on tumor cell lines was assessed by FACS® analysis (FACScan, Becton Dickinson, San José, CA, USA) using anti-HLA-ABC^{FITC} (W6/32, eBioscience, San Diego, CA, USA) or anti-HLA-A2^{FITC} (BB7.2, BD Pharmingen, San Diego, CA, USA) mAbs, respectively. Samples were stained with antibodies in PBS containing 5% FCS for 20 min at 4°C, washed, and immediately analyzed. Data analysis was performed using Cell Quest™ software. For IHC analysis of MHC class I antigens expression, tissue sections were fixed with cold acetone. Blocking serum was added for 30 min, followed by washing and incubation with anti-HLA-ABC antibody (W6/32, eBioscience, San Diego, CA, USA) for 1 h. Immunostaining was performed on a Dako autostainer, using a Vector biotinylated secondary antimouse antibody during 30 min, followed by Avidin Biotin Peroxidase (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) and signal detection with diaminobenzidine (DAB). Sections were counterstained with methyl green (ethyl green) (Sigma, St. Louis, MO, USA). MHC class I staining of samples was evaluated by a semiquantitative scoring system. The system assesses cytoplasmic staining intensity as a 4-level ordered categorical variable (0 = none, 1 = mild, 2 = moderate, 3 = strong), and the percentage of positive cells as a 3-level ordered categorical (A: <25%, B: 25 to 75%, C: >75%). Cases were considered negative for MHC class I expression if the cytoplasmic IHC staining had an intensity score of none to mild in 25% or fewer of tumor cells (A0 or A1). All other cases were considered positive. For IHC analysis of NY-ESO-1 expression, 5-µm cuts of standard formalin-fixed, paraffin-embedded tissue specimens were applied to slides for IHC (Fisherbrand Superfrost/Plus Slides) and heated at 60°C for 2 h to ensure adherence. The slides were then deparaffinized in xylene and rehydrated in a series of graded alcohols. IHC detection was carried out with monoclonal antibody ES121 to NY-ESO-1. Heating the slides in 1mM EDTA ph 8.0 in a microwave oven before IHC staining retrieved the antigens. ES121 was applied at room temperature for 45 min and then detected with the Envision Plus system with DAB (Dako, Carpinteria, CA, USA) as the chromogen. Counterstaining was performed with a hematoxylin solution. Staining was interpreted as follows: 0 = no positive cells, negative sample; 1 = rare and scattered positive cells, negative sample; 2 = up to 50% positive cells, positive sample; 3 = more than 50% positive cells, positive sample.

Assessment of CTA-specific antibodies

The presence of CTA-specific antibodies in the sera of sarcoma patients was assessed by ELISA as previously described (15). Briefly, microwell plates (Nunc, Roskilde, Denmark) were incubated with recombinant protein (1 µg/ml) in coating buffer. Plates were washed with PBS and saturated with PBS containing 2% BSA. After washing, serial dilution of patients' and control sera were added. Goat antihuman IgG-AP (Southern Biotechnology, Birmingham, AL, USA) was used as secondary antibody, and Attophose substrate (JBL Scientific, San Louis Obispo, CA, USA) was added for 25 min at room temperature. Plates were read immediately using a CytoFluor 4000 (Millipore, Bedford, MA, USA).

Peptide stimulation and MHC class I/peptide multimer staining

Patients' PBMCs were cultured *in vitro* for 1 wk in CTL medium (16) containing recombinant human IL-2 (10 IU/ml, Roche, Basel, Switzerland) and recombinant human IL-7 (10 ng/ml, R&D, Minneapolis, MN, USA) in the presence of each of the peptides used (1 µM). PE-conjugated multimeric HLA-A2/peptide complexes containing

peptides NY-ESO-$1_{157-165}$, SSX-2_{41-49}, $CAMEL_{1-11}$, MAGE-$A4_{230-239}$, and MAGE-$A10_{254-262}$ were synthesized as described previously (9, 16, 31, 32). Peptide-stimulated samples were stained with HLA-A2/peptide multimers (4.5 µg/ml) in PBS containing 5% FCS for 1 h at room temperature, washed once in the same buffer, stained with anti-CD8 mAb (Becton Dickinson, San José, CA, USA) for 20 min at 4°C, washed again, and analyzed by flow cytometry (FACScan, Becton Dickinson, San José, CA, USA). Data analysis was performed using Cell Quest™ software.

Contact

Address correspondence to:

Danila Valmori
Ludwig Institute Clinical Trial Center, Division of Medical Oncology
Department of Medicine, Columbia University College of Physicians and Surgeons
650 West 168th Street, Black Building Room 20-09
New York, NY 10032
USA
Tel.: + 1 212 305 1962
Fax: + 1 212 305 7348
E-mail: dv2117@columbia.edu