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Accumulation of the p53 Protein Allows Recognition by Human CTL of a Wild-Type p53 Epitope Presented by Breast Carcinomas and Melanomas

Sacha Gnjatic,* Zhenzi Cai,† Mireille Viguier,* Salem Chouaib,† Jean-Gérard Guillet,* and Jeannine Choppin²*

The p53 protein is accumulated in tumor cells of many human cancers and can elicit in vivo humoral and proliferative responses. Rare reports about p53-mediated tumor recognition by CTLs have remained questioned. We therefore studied a panel of breast tumor and melanoma cell lines that we assayed for the presence of accumulated p53 and surface HLA-A2 and for the presentation of p53 epitopes. From PBMC of a healthy donor, we have generated a CTL line, D5/L9V, directed against HLA-A2-restricted peptide 264–272 from wild-type p53. It efficiently lysed breast adenocarcinomas MCF-7, MCF7/ADR, and MDA-MB-231, and melanoma M8, which all accumulate the p53 protein. Using competition assays, we made sure that tumor lysis by D5/L9V was due to recognition of endogenously produced p53 peptide 264–272 associated with the HLA-A2.1 molecule on the surface of these tumor cells. Cells with undetectable levels of wild-type p53, such as lymphoblastoid cells and melanoma M74, were not recognized by D5/L9V. Neither were breast tumor cell line MCF7/ADR nor melanoma line M44 because of HLA loss. This study therefore shows that it is possible to obtain in vitro CTL lines that specifically recognize a p53 epitope spontaneously presented by a variety of HLA-A2* transformed cell lines provided they display abnormal patterns of p53 expression. This work points out that breast tumors and melanomas share a p53 epitope, and raises hopes for future immunotherapeutic approaches.


Cytotoxic T lymphocytes are able to control or even abolish tumor progression by recognizing tumor-specific Ags (1, 2). The panel of such tumor-associated proteins has expanded rapidly in the last few years (3–5). The p53 protein is a particularly good candidate since many malignant cells display abnormal amounts of p53 that elicit in vivo immune reactions. Abs directed against the p53 tumor suppressor have been found in sera of patients with many different cancers (6, 7). We have also shown that p53 stimulates the proliferation of specific T cells from patients with breast cancer (8). These immune responses are generally linked to mutations of the p53 gene that result in the accumulation of dysfunctional p53 product in the nucleus or cytoplasm of tumor cells (9). The spectrum of p53 mutations is very wide and variable (10). When considering CTL recognition of p53, it is thus difficult to conceive targeting a specific mutant p53 epitope for each tumor. Fortunately, as shown with Ab responses and T cell proliferation, it is possible to obtain specific in vitro immune responses to wild-type (WT) p53 protein from patients with a p53 gene mutation (7, 8). This suggests that WT p53 is normally ignored by the immune system due to insufficient amounts and a too short half-life. Mutated p53 would become immunogenic due to stabilization and accumulation in tumor cells to unusually high concentrations. This should allow specific targeting of p53 in tumors via WT sequences undetectable under normal conditions.

Extensive immunization studies using a mutant p53 epitope have been conducted in mice (11, 12), but WT p53 epitopes on murine tumor cells have been shown only recently to be recognized by CTLs (13). Mice can also be protected against challenge with tumors presenting the immunizing p53 epitope (14). Human p53 epitopes presented by various tumor cells were shown to be recognized by CTLs obtained from HLA-A2 transgenic mice (15). Recently, Röpke et al. showed killing of human squamous cell carcinomas of head and neck by a human p53 peptide-induced CTL clone (16).

To confirm the notion of p53 as a general tumor Ag, it was necessary to establish the capacity of killing tumor cells from different tissue origin with human CTLs directed against p53. We focused on the recognition of two different cancers: breast carcinomas, for which we had reported previously the presence of Ab and Th responses in patients (8), and melanomas. Mutations of p53 are frequent in breast cancer (40% of cases), while their occurrence is uncommon in melanoma, except during late stages or metastases (17). Our work shows that it is possible to easily obtain CTL lines in vitro from the PBMC of healthy human donors, which recognize an epitope from endogenously processed p53 protein presented by a variety of breast carcinomas and melanomas, provided they show accumulation of the p53 protein.

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2 Address correspondence and reprint requests to Dr. Sacha Gnjatic, INSERM U445, ICGM, Hôpital Cochin, 27 rue du Faubourg Saint-Jacques, 75014 Paris, France. E-mail address: gnjatic@cochin.inserm.fr

Abbreviation used in this paper: WT, wild-type.
Materials and Methods

Peptides

Peptide p53 264–272, LLGRNSFEV, derived from the WT p53 sequence, and peptide M58–66, GILGFVFTL, derived from the matrix of the influenza virus, were both synthesized by Neosystem (Strasbourg, France).

Cell lines

All of the cell lines used displayed HLA-A2 expression (except K562, MCF7/ADR, and M44), as assessed by immunohistochemical labeling with mAb BB7.2 (10 μg/ml) specific for HLA-A2 (Table I). MDA-MB-231 (American Type Culture Collection [ATCC], Rockville, MD; HTB26) is a breast cancer cell line displaying high p53 concentrations in the nucleus due to a p53 gene mutation in codon 280. MCF-7 (ATCC; HTB22) is a breast cancer cell line with WT p53 accumulated in the cytoplasm (18). MCF7/RA1 and MCF7/ADR (19) are variants of MCF-7 selected in culture for resistance to TNF-α and Adriamycin, respectively, and both have acquired a p53 mutation, unlike the parental line. In addition, MCF7/ADR no longer expresses any HLA (Table I). Melanoma cell lines M8, M44, and M74 were kindly given by Dr. F. Jotereau (INSERM U211, Nantes, France). M8 and M44 display p53 accumulation in their nuclei, whereas M74 has an undetectable p53 level. Moreover, M44 has lost its HLA expression (Table I). JHE-LCL is an EBV-immortalized B lymphoblastoid cell line. The T2 cell line is a processing-deficient lymphoma (TAP-1 mutation). K562 is a HLA class I-negative erythroleukemia cell line that is sensitive to NK lysis. All cells were grown in DMEM or RPMI with Glutamax-I (Life Technologies, Cergy Pontoise, France) supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM HEPES, MEM, and antibiotics. Adherent cells were detached with trypsin-EDTA for passage or assaying.

CTL generation

Antipeptide CTLs were induced in vitro using a modification of the protocol described by Cerny et al. (20). Briefly, four wells with 4 × 10^6 PBMC from a healthy HLA-A2-positive human donor (DS) were incubated with 4000 U/ml purified protein derivative (Pasteur, Paris, France) for helper effect, and 1 μM p53 peptide 264–272. IL-7 (20 U/ml; Tebu, Le Perray-en-Yvelines, France) was added on day 3. Cell lines were then restimulated weekly with irradiated autologous PBMC pulsed with 2 μM peptide. IL-2 (10 U/ml; Boehringer Mannheim Corp., Mannheim, Germany) and IL-7 (20 U/ml) were added the day after each stimulation and 3 days later. Long-term specific cytotoxic activity was detected in two of four wells, with similar reactivity, from the fifth round of stimulation onward. The lytic activity of CTL lines remained stable for more than 3 mo in culture, and phenotyping indicated they were 85% CD8-positive cells (not shown). This method proved efficient for generating different antipeptide CTL lines in more than five healthy donors, with 10 to 60% of the wells positive (unpublished data).

Measurement of cytotoxic activity and competition assays

Cytolysis was measured with a standard 4-h chromium release test. CTL effectors and targets labeled with 51Cr, pulsed or not with 5 μM peptide, were incubated together at different ratios. The radioactivity released into supernatants was a measure of target cell lysis. For peptide dilution assays, labeled target cells were pulsed with peptide in concentrations ranging from 10 μM to 0.1 nM. For competition assays, unlabeled T2 target cells, pulsed with 2 μM peptide (L9V or M58–66) or not pulsed, were added at a competitor:target ratio of 60:1. To block target cell lysis by Abs, ascites of mAbs W6/32 (ATCC; HB95) and L243 (ATCC; HB55) recognizing HLA class I and class II molecules, respectively, were diluted to 1/400 and preincubated on targets at 4°C for 15 min before adding the effectors to the chromium release test.

Results

Characterization of human CTL lines specific for p53 peptide 264–272

Peptide 264–272 from WT p53 may be a potential T epitope because of its strong affinity for HLA-A2 (21). It has also been shown to be immunogenic (22–24), and to be presented by tumor cells (15). Recently, it was shown that a human CTL clone recognizes this epitope processed from endogenous p53 of squamous cell carcinomas (16).

We have generated two HLA-A2-restricted CTL lines from a healthy donor that recognize p53 peptide 264–272. The results shown in this work for one of them, D5/L9V, describe their common features. D5/L9V efficiently lysed T2 cells pulsed with 5 μM p53 peptide 264–272, while unpulsed T2 cells were unaffected (Fig. 1A). D5/L9V was restricted to HLA-A2.1, since no lysis was detected on non-HLA-A2.1 lymphoblastoid targets pulsed with p53 peptide 264–272 (not shown). Most (85%) activated D5/L9V lymphocytes were CD8+, as assessed by flow cytometry of labeled cells (not shown). The minimal peptide concentration required for sensitizing targets to lysis by D5/L9V may be a critical factor for tumor cell recognition. D5/L9V lysed targets pulsed with amounts of p53 264–272 as low as 1 nM (Fig. 1B). Notably, this concentration is similar to that of the efficient antiviral effectors routinely studied in our laboratory.

Ag-presenting capacity of tumor cell lines

After immunohistochemical labeling, flow cytometry analysis showed that all of the carcinomas studied express HLA-A2, except MCF7/ADR and M44 (Table I). We have also verified the presence of the HLA-A2.1 subtype at the surface of the tumor cells by testing their capacity to present exogenous peptides to HLA-A2.1-restricted CTLs. The HLA-A2-positive breast tumor cell lines MCF-7, MCF7/RA1, and MDA-MB-231, incubated with 5 μM synthetic p53 peptide 264–272, were very efficiently recognized by D5/L9V, with lysis levels comparable with that of peptide-pulsed lymphoblastoid cell line JHE-LCL (Fig. 2). Recognition by D5/L9V of HLA-A2-positive melanoma cell lines M8 and M74 was also efficient in the presence of exogenous peptide. Conversely, melanoma M44 and breast tumor cell line MCF7/ADR, which is a variant of the MCF-7 line selected for its resistance to Adriamycin, were devoid of any detectable HLA molecule expression (Table I), and therefore could not present exogenous peptide 264–272. These lines had a comparable or lesser sensitivity to lysis to that of K562 cells, which do not present HLA class I molecules and are classically used to detect NK activity (Fig. 2).

Tumor lysis dependent on presentation of endogenous p53 peptide 264–272

Control cells T2 and JHE-LCL, without accumulated p53, were not lysed in the absence of exogenous peptide (Fig. 2). Similarly, melanoma M74, which displayed undetectable levels of p53 by immunostaining (Fig. 3G, control, Fig. 3H), was not susceptible to D5/L9V lysis either (Fig. 4C), even though it was capable of presenting exogenous peptide (Fig. 2). This indicated that these cells that do not accumulate p53 could not present peptide 264–272.

Table I. Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Cell Type</th>
<th>% HLA-A2 Expression</th>
<th>p53 Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>JHE-LCL</td>
<td>EBV-immortalized line</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>M74</td>
<td>Melanoma</td>
<td>68</td>
<td>–</td>
</tr>
<tr>
<td>M44</td>
<td>Melanoma</td>
<td>1</td>
<td>+ (nucleus)</td>
</tr>
<tr>
<td>M8</td>
<td>Melanoma</td>
<td>94</td>
<td>+ (nucleus)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast carcinoma</td>
<td>98</td>
<td>+ (nucleus)</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast carcinoma</td>
<td>89</td>
<td>+ (cytoplasm)</td>
</tr>
<tr>
<td>MCF7/RA1</td>
<td>Breast carcinoma</td>
<td>95</td>
<td>+ (nucleus)</td>
</tr>
<tr>
<td>MCF7/ADR</td>
<td>Breast carcinoma</td>
<td>2</td>
<td>+ (nucleus)</td>
</tr>
</tbody>
</table>

* As assessed by flow cytometry after immunohistochemical labeling of cells with 10 μg/ml mAb BB7.2 specific for HLA-A2. Revelation with FITC-conjugated anti-murine IgG (Coulter).

* Refer to Figure 3.
from endogenous WT p53. By contrast, melanoma M8 accumulated p53 (Fig. 3C) and was killed spontaneously by D5/L9V with high efficiency in absence of exogenous peptide (Fig. 4B, black circles). Similarly, breast tumor cells MDA-MB-231 and MCF7/RA1, which have both undergone a p53 mutation and highly accumulate mutant p53 in their nuclei (Fig. 3A and D), were lysed by D5/L9V (Fig. 4A). D5/L9V also extensively lysed MCF-7 cells (Fig. 4A), in which p53 was WT, but stabilized in the cytoplasm (Fig. 3B). We made sure that the observed killing of targets was HLA class I restricted since it was only blocked by anti-class I Abs, W6/32, while anti-class II Abs, L243, had no effect. Figure 5 shows this Ab-mediated inhibition of lysis for MDA-MB-231 and M8 lines. Tumor cell line MCF7/ADR, although accumulating mutant p53 (Fig. 3F), was not recognized significantly by D5/L9V since it has lost HLA-A2 expression (Fig. 4C). Conversely, cold T2 cells pulsed with p53 264–272 greatly reduced the lysis by D5/L9V of breast tumor lines MCF-7, MCF7/RA1, and MDA-MB-231, and of melanoma M8, thus showing that HLA-A21+ malignant cells that accumulate mutant or cytoplasmic WT p53 are lysed naturally and specifically by presenting the WT p53 264–272 epitope (Fig. 4, A and B).

**Discussion**

Peptides from p53 can elicit human CTL responses in vitro (22, 23), and the existence of CTL precursors directed against WT p53

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**FIGURE 2.** Lysis by D5/L9V of different cells pulsed with 5 μM p53 peptide 264–272 or not pulsed at an E:T ratio of 10:1. Results are representative of at least three independent and reproducible assays.
epitopes in the PBMC of healthy individuals has also been reported (24). The presentation of p53 epitopes by tumor cells has been demonstrated in mouse models (11–13). We have shown that mouse CTLs recognize an endogenous p53 epitope presented by mouse hepatocarcinomas (13). CTLs generated against human p53 epitopes in HLA-A2-transgenic mice have also been shown to recognize human tumors (15). It has been suggested that species differences in p53 sequence allow mouse CTLs to respond to epitopes that, in humans, may cause efficient effectors to be tolerized (25). Nevertheless, a human CTL clone recognizing p53 epitope 264–272 has recently been shown to kill squamous cell carcinomas by recognizing endogenous p53 (16). In the present study, we have generated in vitro a CTL line, D5/L9V, that recognizes endogenous peptide 264–272 from naturally processed p53 on breast carcinoma and melanoma cells displaying accumulated p53 protein. These results point out that recognition of a human anti-p53 CTL line is not restricted to a tumor tissue type, which is the main advantage of the p53 tumor Ag.

Our competition experiments show that D5/L9V can specifically lyse tumor cells through presentation of p53 264–272/HLA-A2 complexes. These antigenic complexes are only recognized at the surface of tumors that accumulate p53, provided they have adequate HLA-A2. D5/L9V is incapable of specifically lysing cells with normal levels of nuclear WT p53, even when these are transformed cells. Several mechanisms may be involved in selective recognition of tumor cells with abnormal p53. Mutations in p53 induce changes in conformation that can lead to new patterns of proteolysis, when compared with WT p53 degradation (26); there might thus be a specific delivery of antigenic peptides from altered p53 processing. Abnormal cell localization and sequestration by various intracellular components may also redirect p53 toward new degradation pathways (27, 28). Finally, accumulation of abnormal p53 in tumor cells may be one of the criteria involved in specific recognition by CTLs. Along with Th (8) and B cell responses (7), our findings support the concept that p53 is expressed insufficiently in normal cells of an individual to cause central tolerance by deletion of autoreactive clones. Existence of precursors and attainment of CTLs sensitive enough to detect and endogenous p53 epitopes argue in favor of a peripheral ignorance rather than deletional tolerance of the immune system for these p53 epitopes. Nevertheless, our in vitro conditions used to generate CTLs may have broken a state of in vivo peripheral tolerance of nonresponsive precursors, but the outcoming effectors do not trigger recognition of normal cells with ubiquitary WT p53.

Although accumulation and/or delocalization of p53 seems necessary for epitope presentation, it is not always a sufficient requirement. Numerous steps in Ag presentation, including modulation of HLA and cofactor expression or cytokine secretion by the target, may impair epitope recognition by CTLs. Despite the natural heterogeneity of our tumor cell lines, four of them were killed very efficiently by D5/L9V at high E:T ratios. They displayed a strong HLA-A2 expression similar to that of reference cells when exogenous peptide was added, and were thus not limited for the spontaneous presentation of p53 peptide, as shown with the MART-1 Ag in melanomas (29). Moreover, p53 peptide 264–272 shows a stable association with HLA-A2 in vitro (not shown), which could favor CTL recognition. Notably, we have obtained sensitive T effectors that kill tumor cell lines without the need for prior cytokine treatment of target cells. This contrasts with CTLs obtained in HLA-A2 transgenic mice that required breast adenocarcinomas to be incubated with IFN-γ and/or TNF-α to be recognized (15). Our results may reflect a more efficient recognition of tumors due to adequate cofactor molecules. Lack of cytokine requirement is a crucial factor since IFN-γ has been shown to enhance the metastatic capacity of certain tumors (30). Effector cell line D5/L9V probably arose from a limited number of precursors, since it required multiple stimulations that were shown to select for a small number of high avidity clones in mouse models in which anti-mdm2 CTLs were obtained in vitro (31).

The ready in vitro response of anti-p53 CTLs obtained from PBMC of healthy donors raises questions about the status of human p53 recognition in vivo. Further studies with primed anti-p53 CTLs from cancer patients, should they exist, may show whether p53 from in vivo tumors also causes immunization, as in mouse models, and whether the kinetics of this potential antitumor activity allows an efficient response. Anti-p53 human CTLs generated in vitro are compatible with the expansion of autologous cytotoxic effectors from cancer patients in the development of immunotherapies. Another tumor-associated Ag shared between several distinct types of epithelial tumors, a HER2/neu-derived HLA-A2-restricted peptide, has been previously identified (32). Tumor-associated lymphocytes directed to this peptide have been isolated from breast cancer patients, and authors have suggested that they might be important for the development of broadly applicable vaccine therapies. In the same prospect, anti-p53 264–272 CTLs would not only hit original tumors of various types, but may also prove useful against metastases.
FIGURE 4. Spontaneous lysis by D5/L9V of A, ⁵¹Cr-labeled breast tumor cell lines MCF-7, MCF7/RA1, and MDA-MB-231; B, ⁵¹Cr-labeled melanoma line M8; and C, ⁵¹Cr-labeled melanomas M74 and M44 and breast carcinoma MCF7/ADR in the presence of unlabeled competitor cells (competitor:target ratio of 60:1). Results are representative of at least three independent and reproducible assays.
ACKNOWLEDGMENTS

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6. Crawford, L. V., D. C. Pim, and R. D. Bulbrook. 1982. Detection of antibodies to HLA class I mAbs, but not affected by anti-class II mAbs. Control JHE-LCL cells were pulsed with 1 μM p53 peptide 264–272, whereas breast carcinoma MDA-MB-231 and melanoma M8 were not pulsed. The E:T ratio was 10:1.