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CD4+ T helper responses in squamous cell carcinoma of the head and neck

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Summary

Anti-tumor immunity plays an important role in the development of and protection from malignancy. However, there is a lack of information regarding induction of CD4+ T helper responses in patients with squamous cell carcinoma (SCCHN). To explore anti-tumor immune responses against SCCHN, a permanent cell line, Gun-1 was established from a squamous cell carcinoma of the hypopharynx. In addition to its characterization, we performed mixed lymphocyte-tumor cell cultures (MLTC) using peripheral blood lymphocytes and autologous tumor cells. Furthermore, T cell responses to wild type (wt) p53-derived peptides were assessed. Gun-1 cells overexpressed p53 and were negative for HLA-A2 expression. No tumor-specific or wt p53-specific CD8+ CTL lines could be established from peripheral blood mononuclear cells (PBMCs) of this patient. Autologous tumor-specific HLA-DR-restricted CD4+ T helper clone was obtained by limiting dilutions using bulk populations from MLTC. This clone produced IFN- γ but not IL-5 in response to autologous tumor cells. In addition, CD4+ T cells were generated from the patient's PBMCs which responded to two HLA-DP5-restricted wt p53-derived peptides. Our results suggest that the immune cells specific for autologous tumor as well as wt p53-derived epitopes are present in the peripheral circulation of this cancer patient. However, helper-type CD4+ T lymphocytes represent the predominant anti-tumor response.

Keywords

CD4+ T cells; Autologous tumor cells; Squamous cell carcinoma of the head and neck; p53

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Conflict of Interest Statement

We have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications, opinions stated.

Introduction

It has been suggested that anti-tumor immunity plays an important role in the development of and protection from malignancy. T cells serve as effector cells responsible for specific long-lasting immunity against the tumor. CD8+ cyto-toxic T lymphocytes (CTLs) can recognize and kill tumor cells expressing peptides that are presented by MHC class I molecules, while CD4+ T helper cells are activated by recognition of peptides presented by MHC class II molecules. To date, functions and interactions of these effector cells have been a focus of attention, and this contributed to a better understanding of the mechanisms necessary for the development of an effective immunotherapy. It has been determined that while CTLs play a major role in tumor eradication, the participation of CD4+ T helper cells is also needed for the development of optimal anti-tumor responses. Increasingly, evidence has accumulated indicating that CD4+ T cells have a pivotal role in generating, and maintaining anti-tumor immune responses through their interactions with CTLs, B cells, macrophages, and NK cells.^{1,2}

In general, patients with cancer, including squamous cell carcinoma of the head and neck (SCCHN), are known to be immunologically compromised.^{3,4} Considerable evidence for the presence of functional defects of peripheral blood lymphocytes (PBLs) and tumor-infiltrating lymphocytes (TILs) in patients with SCCHN has been accumulated.⁵ It is also apparent that SCCHN not only actively corrupt the host anti-tumor response via various well-recognized mechanisms but also manage to effectively escape from the host immune system. For example, the production of immunosuppressive factors such as transforming growth factor- β (TGF- β), interleukin (IL)-10, or prostaglandin E-2 (PGE-2) and low levels or lack of expression of tumor antigens, major histocompatibility complex (MHC) molecules, or costimulatory molecules are some of the mechanisms facilitating tumor escape.⁶ Therefore, it is not surprising that strong activation signals are necessary to induce and detect autologous tumor-specific T-cell responses in PBLs and TILs of patients with SCCHN.

In SCCHN, similar to other solid tumors, we and other groups have demonstrated that autologous tumor-specific CD8+ CTL can be generated from PBL or TIL.⁷⁻⁹ On the other hand, tumor-specific CD4+ T cells restricted by MHC class II molecules have been studied in patients with lymphoma, sarcoma, colon cancer, and breast cancer,^{10,11} however, there is little information regarding induction of tumor-specific CD4+ T helper cells in patients with SCCHN. In this study, we have established a permanent cell line from a squamous cell carcinoma of the hypopharynx. In addition to characterization of this cell line, we performed mixed lymphocyte-tumor cell (MLTC) cultures by using mitomycin C (MMC)-treated tumor cells and autologous peripheral blood lymphocytes. Furthermore, we examined T-cell responses to wild type (wt) p53-derived peptides in PBMCs obtained from the patient. We report here the analysis of CD4+ T helper responses in a patient with SCCHN.

Materials and methods

Patient

An 80-year-old man who was human leukocyte antigens (HLA)-A2+, A24+, B13+, B40+, Cw3+, Cw8+, DR9+, DR12+, DQ3+, DP2+, DP5+ presented in December 2004 with a squamous cell carcinoma of the hypopharynx in stage IVA (T3N2bM0) (Fig. 1A). The patient received systemic chemotherapy consisting of cisplatin and 5-fluorouracil as neoadjuvant chemotherapy, and then surgery was performed. Subsequently, the patient was treated with radiotherapy (60Gy). He has had no evidence of disease since then.

Cell lines

Tumor cell line Gun-1 was derived from the primary tumor obtained at surgery. These cells grew in a complete medium (CM) consisting of RPMI-1640 (Sigma–Aldrich, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all reagents were from Invitrogen, Grand Island, NY). The cultures were maintained at 37 °C in 5% CO₂ in air. The T2-A24 cell line, HLA-A*2402-transfected T2 cell line¹² and H0301 cell line, HLA-DPB1*0501 homozygous Epstein-Barr virus-transformed B lymphocytes (EBV-B cells) were kindly provided by Dr. Kuzushima (Aichi Cancer Center, Nagoya, Japan) and Dr. Nishimura (Kumamoto University, Kumamoto, Japan), respectively. These two cell lines were used as the peptide-presenting cell in ELISPOT assays.

p53 mutation analysis, immunohistochemistry, and detection of p53 antibody

p53 mutation analysis of the Gun-1 tumor was performed using PCR-SSCP analysis, followed by direct DNA sequencing. DNA was isolated from Gun-1 cells and the sets of amplification primers flanking exon 5 through 8 of the p53 gene were used in four separate PCR. SSCP analysis of the PCR product was carried out on 6% non-denaturing polyacrylamide gels. Purified PCR products of the samples showing mobility shift on SSCP analysis were analyzed with an automated DNA sequencer ABI prism 310.

For p53 immunohistochemistry in tumor specimens, formalin-fixed, paraffin-embedded tissue blocks were sectioned (2 µm thick) and deparaffinized. The slides were autoclaved in 0.01 M citrate buffer for 15 min (w/v) for antigen retrieval. After washing, sections were incubated with 1% (w/v) bovine serum albumin (Sigma–Aldrich, St. Louis, MO) and 5% (w/v) normal horse serum (IBL, Gunma, Japan) for 30 min and subsequently treated overnight with anti-p53 monoclonal antibody (mAb) (DO-7; DAKO Cytomation, Inc., Carpinteria, CA). Next, sections were incubated with biotinylated goat anti-mouse antibodies (DAKO, Copenhagen, Denmark). The avidin–biotin complex technique (Vectastain Elite Kit, Vector Inc., Burlingame, CA) was performed according to the manufacturer's instructions, and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Wako, Osaka, Japan) was used for color development. Sections were counterstained with hematoxylin.

Ab to p53 in the patient's sera was detected by an ELISA purchased from PharmaCell Immunotech Coulter (Miami, FL) using microtiter plates coated with recombinant human wt p53 protein.

Flow cytometry analysis

Expression of HLA-A2, A24, and HLA class I on Gun-1 cells and PBMCs obtained from the patient were examined by flow cytometry. HLA-A2-specific antibodies (MA2.1 and BB7.2) and an HLA class I antibody (w6/32) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and an HLA-A24-specific antibody (anti-HLA-A23, A24 mAb, IgG2b) were from One Lambda, Inc. (Canoga Park, CA). In some experiments, Gun-1 cells were treated with interferon (IFN)-γ (100 u/ml) for 48 h, and then stained with the antibody.

MLTC and the establishment of CD4+ T cell clone

The patient's PBMCs were isolated by density-gradient centrifugation over Ficoll-Hypaque gradients (Amersham Biosciences, Uppsala, Sweden), washed, counted in the presence of a trypan blue dye, and cryopreserved in liquid nitrogen. Gun-1 cells were incubated with IFN-γ (100 IU/ml) for 48 h before being used as stimulators. Autologous MLTC was performed as follows: 2×10^6 PBMCs were stimulated with 2×10^5 MMC (kindly provided by Kyowa

Hakko, Tokyo, Japan)-treated Gun-1 cells (100 µg/ml for 30 min at 37 °C). The cells were suspended in 2 ml AIM-V medium supplemented with 10% (v/v) heat-inactivated human AB serum, 20 IU/ml of IL-2 (kindly provided by Shionogi & Co., Ltd. Osaka, Japan), and 25 IU/ml of IL-4 (kindly provided by Ono Pharmaceutical Co., Ltd. Osaka, Japan). On days 7 and 14, 1×10^6 the responder lymphocytes were restimulated with 10^5 MMC-treated Gun-1 cells. On day 21, the responder cells were harvested and the T-cell reactivity against autologous tumor (Gun-1) cells was evaluated. On day 28, the responder cells were cloned by limiting dilution. Each microwell contained 10^3 MMC-treated Gun-1 cells, 10^4 MMC-treated EBV-B cells, and 10^5 MMC-treated PBMCs mixture obtained from healthy donors as feeder cells, in 200 µl of AIM-V medium supplemented with 10% (v/v) heat-inactivated human AB serum, 100 IU/ml of IL-2, and 50 IU/ml of IL-4. The T cell clones were maintained by weekly passages of 1×10^6 T cells with 2×10^4 MMC-treated Gun-1 cells, 5×10^5 MMC-treated EBV-B cells, and 2×10^6 MMC-treated PBMCs mixture in 2 ml culture medium as described above.

In vitro stimulation (IVS) of PBMCs using wild type p53-derived peptides

PBMCs were cultured in the presence of wt p53-derived peptide (10 µg/ml) in wells of 24-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in a final volume of 2 ml AIM-V medium (Invitrogen) containing 10% (v/v) human AB serum. Following 7 days of incubation at 37 °C, PBMCs were harvested, washed and tested in ELISPOT assays. Four HLA-A2-restricted wt p53 peptides (p53₆₅₋₇₃: RMPEAAPPV, p53₁₄₉₋₁₅₇: STPPPGTRV, p53₂₁₇₋₂₂₅: VVPYEP-PEV, p53₂₆₄₋₂₇₂: LLGRNSFEV), three HLA-A24-restricted peptides (p53₁₂₅₋₁₃₄: TYSPALNKMF, p53₁₆₁₋₁₆₉: AIYKQSQHM, p53₂₀₄₋₂₁₂: EYLDDRNTF), and two HLA-DP5-restricted peptides (p53₁₀₈₋₁₂₂: GFRLGFLHSGTAKSV, p53₁₅₃₋₁₆₆: PGTRVRAMAIYKQS) were synthesized using standard N-(9-fluorenyl) methoxycarbonyl methodology. These peptides were stored as lyophilized preparations. The amino acid sequence was confirmed by mass spectrometric analysis.

IFN-γ and IL-5 ELISPOT assays

ELISPOT assays were performed in 96-well flat-bottomed plates with nitrocellulose membrane inserts (Millipore, Bedford, MA) as previously described.¹³ Briefly, the plates were coated overnight at 4 °C with 10 µg/ml of anti-human IFN-γ mAb (1-D1K: Mabtech, Nacka Strand, Sweden), or 5 µg/ml of anti-human IL-5 mAb (TRFK5: BD Pharmingen, San Diego, CA) in PBS. The IFN-γ-treated Gun-1 cells (1×10^4 cells/well) or T2-A24 cells (2×10^4 cells/well) or H0301 cells (2×10^4 cells/well) pulsed with a wt p53-derived peptide (10 µg/ml), were plated in triplicate wells. The responder cells (1×10^5 cells/well or 1×10^3 cells/well in case of CD4+ cloned T cells) were added to AIM-V medium at a final volume of 200 µl. PBMCs stimulated with 12.5 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin were used as a positive control. The plates were incubated at 37 °C for 20 h for IFN-γ assessment, and 40 h for IL-5 assessment. After incubation, the plates were washed with PBS/ 0.05% Tween 20, and supplemented with biotinylated anti-IFN-γ mAb (7-B6-1: Mabtech) or biotinylated anti-IL-5 mAb (JES1-5A10: BD Pharmingen). After 2 h incubation, plates were washed with PBS/0.05% Tween 20, and developed with the avidin-peroxidase complex (Vectastain Elite kit; Vector) for 1 h. 3-Amino-9-ethyl-carbozole (Sigma, St. Louis, MO) was added and incubated for 5 min for the IFN-γ ELISPOT assay and the TMB substrate for peroxidase (3,3',5,5'-Tetramethylbenzidine; Vector Laboratories) was added and incubated for 10 min for the IL-5 ELISPOT assay. The mean number of spots in control wells (no peptide) was subtracted from the mean number of spots in experimental wells. A T-cell response to a given wt p53 peptide was considered to be positive if at least 10 cells per 1×10^5 responder cells secreted IFN-γ or IL-5, as described

by Nagorsen et al.¹⁴ In some experiments, IFN- γ -treated Gun-1 cells were used as stimulators.

Results

Establishment and characteristics of the Gun-1 cell line

Gun-1 cell line was established from the fresh SCCHN tumor biopsy specimen. Once established, the tumor cell line grew rapidly. In culture, the Gun-1 cell line grew as sharply demarcated, compact islands of cells with a distinct epithelial morphology (Fig. 2). The tumor tissue stained positive with anti-p53 mAb (Fig. 1B), and, therefore, p53 mutation analysis of Gun-1 cell line was performed. The p53 gene in Gun-1 cell line had a missense mutation at codon 273 and 280 (data not shown).

Next, the expression of HLA class I molecules on Gun-1 cells was analyzed using flow cytometry. Interestingly, the Gun-1 cell line was HLA-A2 negative, although HLA-A24 as well as HLA class I were detected as shown in Fig. 3A. As expected, PBMCs obtained from the patient showed HLA-A2, A24, and class I molecules expression (Fig. 3B). When treated with IFN- γ , Gun-1 cell line up-regulated expression of HLA-DR and DP molecules; however HLA-A2 expression on Gun-1 cell line was not restored (data not shown).

Induction of autologous tumor-specific T cell responses

We generated autologous tumor-specific T cells from PBMCs obtained from the patient by MLTC. After 3xIVS, the outgrowing T cells were tested in ELISPOT assays for cytokine production in response to autologous tumor. The bulk population of effectors, which consisted of 52% CD4⁺ and 48% CD8⁺ T cells, produced both IFN- γ and IL-5 in response to autologous Gun-1 tumor cells (Fig. 4). The production of IFN- γ to autologous tumor cells was blocked by anti-HLA class I mAb and anti-HLA-DR mAb, but not anti-HLA DP mAb. On the other hand, the production of IL-5 to autologous tumor cells was blocked by anti-HLA-DR mAb and anti-HLA-DP mAb, but not anti-HLA class I mAb. However, it was not possible to establish autologous tumor-specific CD8⁺ CTL lines or clones despite various attempts to facilitate CTL generation. For example, we used a protocol previously established for generation of autologous tumor-specific CD8⁺ CTLs in our laboratory consisting of purified CD8⁺ T cells stimulated with autologous dendritic cells (DCs) pulsed with apoptotic Gun-1 cells. The cross-presentation of tumor antigens by DCs in this protocol providing adequate HLA class I presentation and co-stimulation considered to be necessary for generation tumor-specific CD8⁺ CTLs.¹⁵ Unfortunately, autologous tumor-specific CD8⁺ CTL lines were not induced even using this culture system (data not shown). Instead, we isolated several CD4⁺ T cell clones from the bulk population in MLTC by limiting dilution and selected CD4⁺ T cell clone 5D1. Clone 5D1 produced IFN- γ in response to autologous tumor cells, but not IL-5. This reactivity was inhibited by anti-HLA-DR mAb, but not anti-HLA-DP mAb (Fig. 5). These results suggested that clone 5D1 was an HLA-DR-restricted T helper type 1 (Th1) clone.

T-cell responses to wt p53 peptides of PBMCs obtained from the patient following IVS

Since tumor tissue overexpressed p53 protein associated with a p53 mutation, wt p53-derived epitopes may be recognized by the patient's immune system. To investigate this possibility, we assessed T-cell responses to wt p53 peptides using IFN- γ and IL-5 ELISPOT assays. Based on the established profile of HLA alleles in the patient, a total of 9 wt p53-derived peptides (4 HLA-A2-restricted, 3 A24-restricted, and 2 DP5-restricted) was selected, synthesized, and tested. PBMCs obtained from the patient were co-cultured in the presence of each peptide added to the cultures. Following 7-day IVS (1xIVS), T cells were evaluated for peptide-specific reactivity (Table 1). Unfortunately, the patient's PBMCs did

not respond to HLA A2 or A24-restricted wt p53 peptides. However, PBMCs responded to two HLA DP5-restricted wt p53 peptides. Interestingly, patient's PBMCs showed T helper type 2 (Th2) response to p53_{108–122} peptide and T helper type 0 (Th0) response to p53_{153–166} peptide, respectively.

Discussion

Advances in the field of tumor immunology have allowed us to identify and classify tumor associated antigens recognized by the immune system and to monitor the *in vivo* distribution and function of T cells recognizing those antigens. However, not all antigens on human solid tumors are immunogenic. Thus, although autologous tumor-specific T cells can be obtained from peripheral blood or TILs in some patients with solid tumors, including SCCHN,^{7–9} in most cases, it has not been possible to document the presence of such T cells. Several mechanisms exist that contribute to the *in vivo* paucity of T cells with specificity for autologous tumor. Patients with cancer, especially those with advanced disease, are known to be immunologically suppressed. Data from our and other laboratories have demonstrated various abnormalities in immune cells of patients with cancer, including DCs and T cells isolated from the tumor site or from the peripheral circulation.^{5,16,17} On the other hand, tumors escape from immune surveillance by mechanisms such as down-modulation of HLA expression, mutation of these and other tumor antigens, the lack of costimulatory factors, production of immunosuppressive cytokines and apoptosis of effector T cells.⁶

To generate tumor-specific T cells from PBMCs or TILs of patients with cancer, permanent tumor cell lines are usually established from tumor specimens. In general, tumor specimens from head and neck regions are difficult to propagate, and they are more often contaminated by bacteria, viruses, and mycoplasma due to their location, compared with the tumors from other locations. Mandruzzato et al. have reported a 19% success rate for establishment of tumor cell line from head and neck carcinoma.⁹ But even if permanent tumor cell lines are established, it may still be difficult to obtain tumor-specific T cell lines by performing MLTC with tumor cells and autologous PBMCs or TILs. This may be due to immunoinhibitory properties of tumor cells or to absence on tumor cells of key molecules necessary for immune recognition. For example, allele-specific deletion of HLA-A2 molecules was observed in Gun-1 cells by staining with anti-HLA-A2 mAbs. Takenoyama et al. have also reported the loss of HLA-A2 antigens on a lung adenocarcinoma cell line.¹⁸ Using immunohistochemistry, Meissner et al. have reported that HLA class I antigens were not detectable in 12% of the lesions from SCCHN.¹⁹ It is generally accepted that IFN- γ transcriptionally modulates the expression of HLA class I antigens and of antigen processing machinery components, including the proteasome subunits LMP2, LMP7, and LMP10.²⁰ However, when Gun-1 cells were treated with IFN- γ and examined for HLA-A2 expression by flow cytometry, we failed to up-regulate HLA-A2 expression on these cells. This suggested that mechanisms not involving the IFN- γ pathway may be involved in the loss of HLA-A2 antigens in Gun-1 cells.

The failure to establish a tumor-specific CD8⁺ T-cell line from this patient was compensated by the isolation of tumor-specific CD4⁺ T cell clone from the bulk MLTC population by limiting dilution. This clone, 5D1, produced IFN- γ but not IL-5 upon challenge with Gun-1 cells, and this reactivity was inhibited by anti-HLA-DR mAb, suggesting that autologous tumor-specific HLA-DR restricted Th1 cells may be present in the peripheral blood of this patient. The essential role of CD4⁺ T cells in anti-tumor immunity has become evident in murine models^{21,22} as well as humans.^{23,24} CD4⁺ T cells initiate and control anti-tumor immune responses by providing help in the form of secreted cytokines and cell surface ligands. Our results also suggest that CD4⁺ T cells may be crucial participants in the development of CTL responses against autologous tumors in this patient. Furthermore, when

we examined T-cell responses to wt p53 derived peptides using PBMCs, CD4+ T helper responses to HLA-DP5 restricted wt p53 peptides were detected. PBMCs from a patient produced IL-5 upon stimulation with wt p53_{108–122}, while PBMCs stimulated with wt p53_{153–166} produced both IFN- γ and IL-5. These results suggest that anti-wt p53 CD4+ T helper cells are also present in peripheral blood of this patient. The p53 gene was mutated and expressed in the patient's tumor, which is a common event in SCCHN.²⁵ A number of studies relating identification of tumor-associated antigens (TAAs) have demonstrated that wild-type peptides derived from p53 molecules can act as TAAs, and anti-wt p53 peptide-specific CTLs and CD4+ T helper cells have been previously established as reactive SCCHN cell lines.^{26–28} Similar to MLTC results with Gun-1 cells, we were unable to generate CD8+ CTL responses to HLA-A2 or A24-restricted wt p53 peptides. Previously, Hoffmann et al have demonstrated that CD8+ T cells in the circulation of patients with SCCHN are significantly more sensitive to, and preferentially targeted for, apoptosis than CD4+ T cells.²⁹ Moreover, Tsukishiro et al. have shown that the CD8+CD28-effector subset contained the highest proportion of Annexin-binding (i.e., early apoptotic) cells as compared with naïve and memory CD8+ T cells,³⁰ suggesting a high turnover rate of the CD8+CD28-effector cells in patients with SCCHN. Other data suggest that T cells with the V β -restricted repertoire or tetramer+ tumor peptide-specific CD8+ T cells may be particularly sensitive to spontaneous apoptosis in SCCHN patients.³¹ Given sensitivity of CD8+ T cells to spontaneous apoptosis in PBMCs of patients with SCCHN, generation of anti-tumor CD8+ CTLs is likely to be especially difficult. In our study, autologous tumor antigen-specific or wt p53-specific CD8+ T cell precursors may be present in peripheral blood; however, activation and maintenance of such precursors represents a considerable challenge. In contrast, CD4+ effector T cells are less sensitive to apoptosis²⁹ and their isolation, culture and long-term maintenance are feasible.

Taken together, our results showed that CD4+ effector/ helper cells specific for autologous tumor as well as wt p53-derived peptides has been elicited in this patient. The problems associated with the isolation and culture of CD8+ or CD4+ anti-tumor effector cells may be related not to the absence or paucity of these precursor cells but rather to *in vivo* operative mechanisms of tumor escape. The development of new strategies able to overcome functional impairments induced *in vivo* in immune cells is necessary to elicit strong CD8+ CTL responses as well as CD4+ T cell helper responses targeting autologous tumor.

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Abbreviations

SCCHN	squamous cell carcinoma of the head and neck
MLTC	mixed lymphocyte-tumor cell cultures
wt	wild type
PBMCs	peripheral blood mononuclear cells
CTLs	cytotoxic T lymphocytes
PBLs	peripheral blood lymphocytes
TILs	tumor-infiltrating lymphocytes
TGF	transforming growth factor

IL	interleukin
PGE-2	prostaglandin E-2
MHC	major histocompatibility complex
MMC	mitomycin C
HLA	human leukocyte antigens
CM	complete medium
FBS	fetal bovine serum
EBV-B	Epstein-Barr virus-transformed B
mAb	monoclonal antibody
IVS	in vitro stimulation
IFN	interferon
PMA	phorbol 12-myristate 13-acetate
DCs	dendritic cells
Th1	T helper type 1
Th2	T helper type 2
Th0	T helper type 0
TAAAs	tumor-associated antigens

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A

B**Figure 1.**

A Histological sections of the original tumor obtained from biopsy. H&E (original magnification, $\times 100$).

B Immunohistochemical staining of p53 in the original tumor tissue. p53 MoAb stained nuclei of the tumor cells (original magnification, $\times 100$).

Figure 2.
Microscopic characteristics of the Gun-1 cell line, which grow as a compact monolayer
(original magnification, $\times 400$).

A

B

Figure 3.

A Expression of the HLA-A2, A24, and class I antigens on Gun-1 cells. Allele-specific deletion of HLA-A2 antigen was observed, whereas HLA-A24 and class I antigens were detected.

B Expression of the HLA-A2, A24, and class I antigens on PBMCs obtained from a patient. The HLA haplotype of a patient is HLA-A2/A24.

Figure 4. Induction of autologous tumor-specific T cell responses. After 3 $\times$ IVS with autologous tumor cells, the outgrowing T cells were tested for their cytokine production against autologous tumor cells. Asterisk (*), significant mAb blocking of cytokine production against autologous tumor cells. Data are expressed as the mean number of spots $\pm$ SD of triplicate cultures.

Figure 5.

Clone 5D1 is an HLA-DR-restricted Th1 typed CD4⁺ T cell clone. *Asterisk* (*), significant mAb blocking of cytokine production against autologous tumor cells. Data are expressed as the mean number of spots $\pm$ SD of triplicate cultures.

Table 1Analysis of T-cell responses to wild-type p53 derived peptides in a patients with hypopharyngeal cancer^a

HLA restriction	Peptides	Number of spots	
		IFN- γ	IL-5
A2	p65–73	2	ND ^b
	p149–157	0	ND
	p217–225	0	ND
	p264–272	0	ND
A24	p125–134	3	ND
	p161–169	0	ND
	p204–212	6	ND
DP5	p108–122	0	20
	p153–166	17	14

^aData are the mean numbers of spots/105 T cells/well in ELISPOT assays.^bND, not done.