Generation of Renal Cell Carcinoma-specific $CD4^+/CD8^+$ T Cells Restricted by an HLA-39 from a RCC Patient Vaccinated with GM-CSF Gene-Transduced Tumor Cells

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ABSTRACT

Background: Granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transduced tumor cell vaccines induce very potent systemic anti-tumor immunity in preclinical and clinical models. Our previous phase I clinical trial in patients with metastatic renal cell carcinoma (RCC) has demonstrated both immune cell infiltration at vaccine sites and T cell-mediated delayed-type hypersensitivity (DTH) response to whole tumor cell vaccines. **Methods:** To investigate the immune responses to autologous genetically- modified tumor cell vaccines, tumor-specific $CD8^+$ T cell lines were generated from peripheral blood lymphocytes (PBL) of a RCC patient 1.24 by repeated *in vitro* stimulation with either B7.1-transduced autologous RCC tumor cells or B7.1-transduced autologous tumor cells treated with interferon gamma (IFNγ), and cloned by limiting dilution. **Results:** Among several RCC-specific cytotoxic T lymphocytes (CTLs), a $CD4^+/CD8^+$ double positive T cell clone (17/A2) appeared to recognize IFNγ-treated autologous RCC restricted by HLA-B39. The 17/A2 also recognized other HLA-B39 positive RCC tumor cells after IFNγ treatment. **Conclusion:** These results demonstrate that autologous RCC vaccination successfully generates the tumor-specific CTL 17/A2, and suggest that the presentation and recognition of the tumor antigen by the 17/A2 might be upregulated by IFNγ. **(Immune Network 2003;3(2):96-102)**

Key Words: RCC, GM-CSF, tumor-specific cytotoxic T cells, IFNγ, cancer vaccine

Introduction

Current treatment modalities to human cancer, which is the most complicate and persistent disease to human, include the surgical resection, chemotherapy, radiation therapy, and immunotherapy. In the past decades, the convergence of information from basic studies of cellular and molecular immunology and the application of advanced recombinant DNA technologies have continually developed views concerning the immune response to human cancer. Immunotherapy with cancer vaccine is a potential therapeutic approach to the treatment of various human cancer. The immunologic killing of tumor cells is different from the mechanical killing of tumor cells by chemotherapy and radiation therapy, in that it depends on tumor-specific cytotoxic T cell response and thus causes no side effects damaging normal cells. Recently, it has been reported that the granulocyte-macrophage colony stimulation factor (GM-CSF) gene-transduced tumor cell vaccines can induce the most potent, specific and long-lasting anti-tumor immunity in poorly immunogenic models (1). Subsequently, the efficacy of GM-CSF-secreting vaccines has been shown in large number of preclinical tumor models and several human tumors including RCC (2,3), melanoma (4), prostate cancer (5), and pancreas cancer (6,7). The therapeutic activity of GM-CSF involves the paracrine action of this cytokine at the vaccine site in recruiting antigen-presenting cells, such as dendritic cells (DC) to take up antigens from dying tumor cells and then present to $CD4^+$ and $CD8^+$ T cells. Moreover, GM-CSF-secreting vaccines also elicit multiple immune responses exerted by various immune cells including Th1/Th2, B cells, macrophages, and eosinophils (8-10).

Renal cell carcinoma has long been considered an

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immunogenic tumor. This contention is based on clinical reports of late relapses after nephrectomy, prolonged stabilization of disease in the absence of systemic treatment, and spontaneous regresions as well as reproducible albeit infrequent responses with IFNγ and IL-2 against this chemotherapy-refractory malignancy (11). In our previous phase I clinical trial, patients with advanced RCC was vaccinated with irradiated autologous, GM-CSF-transduced tumor cells (3). Although assessment of efficacy was not a primary objective of the study, one patient had regression of multiple pulmonary metastasis following treatment. To further investigate a potential contribution of $CD8^+$ T cells in the systemic anti-tumor immune response elicited by the vaccine, peripheral blood lymphocytes (PBL) from RCC patient 1.24 were stimulated in vitro with B7.1-transduced, autologous whole tumor cells, and several specific $CD8^+$ T cells against RCC tumor antigens were generated in vitro. In the present study, we have investigated RCC-specific cytotoxicity of 17/A2 clone that is one of the CTL clones generated from PBL of RCC patient 1.24. The results demonstrate that the 17/A2 is a $CD4^+/CD8^+$ double positive T cell clone, and its recognition of tumor antigen appears to depend on cell density and prolonged interferon gamma (IFNγ) treatment. In addition, the results indicate that the 17/A2 recognizes autologous RCC tumor cells as well as other allogeneic RCC, but does not recognize autologous B cells and NK-sensitive K562 cells.

Materials and Methods

Patients and cell lines. TheRCC tumor cell lines were established from the RCC patients enrolled in a Phase I Clinical Trial (3) as previously described (12). All cells were maintained in RPMI 1640 supplemented with20% fetal bovine serum (FBS), 10% tryptose phosphate broth, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100μg/ml streptomycin. This RPMI 1640 medium was referred to as the complete medium. For the Epstein-Barr virus immortalized B cell lines (EBV 1.24) and K562 were grown in RPMI 1640 containing 10% FBS, 1% NEAA, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100μg/ml streptomycin.

Generation of $CD8^+$ T cell lines and clones. The cytotoxic $CD8^+$ T cell line against autologous RCC tumor cells were generated from PBL of patient 1.24, and obtained 92 days post vaccination by leukopheresis. After Ficoll purification, macrophage/monocytes were removed by plastic adherence. The $CD4^+$ T cells among the non-adherent cells were depleted by plastic adherence cells by using anti-CD4 coated AIS CELLector flasks (Applied Immune Science, Menlo park, CA, USA). For the generation of RCC-specific T cell lines, two different stimulator cells were used; B7.1-transduced autologous RCC tumor cells or B7.1-transduced autologous RCC tumor cells treated with IFN-γ. After irradiation (20 Gy), 2×10^5 tumor cells were co-cultivated with 1×10^6 T cells in one well of a 24-well plate in 2 ml of the complete medium. Two days after T cell stimulation, 60 U/ml of IL-2 (Proleukin; Centry, Emeryville, CA, USA) were added, three stimulations were performed at an interval of between 9～14 days.

RCC tumor-specific T cell clones were established from T cell lines by limiting dilution method. Briefly, the cloning was done by incubating 0.5～1.0 T cell per well of 96-well plate containing irradiated feeder cells and B7.1-transduced RCC tumor cells in presence of 60 U/ml of IL-2. Outgrowing clones were transferred to additional wells and ultimately further expanded in 24-well plate. RCC-specific cytotoxic activity of outgrowing clone was tested with smallquotes against K562, (EBV)-tansformed autologous B cells, and B7.1-transduced autologous RCC tumor cells.

Assay for cytolytic activity. Measurement of cytotoxicity was performed by chromium release assay as described (11). Target cells were labeled with 200μCi/ml of $Na^{51}CrO_4$ (Dupont, Dreieich, Germany) at a concentration of 5×10^4 cells, then washedthree times, and resuspended in the complete medium. The ^{51}Cr labeled target cells and effector lymphocyte suspension were mixed in 96-well plates, and incubated for 4 hr. The plates were centrifuged at 200 rpm for 5 min, and the radioactivity of supernatant was measured in a γ-counter. The percentage of specific chromium release was calculated as follow:

$$\% \text{ specific chromium release} = \frac{(\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})}{(\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})} \times 100$$

Maximum ^{51}Cr release was obtained by adding 100μl of 1% NP-40 to labeled target cells. Spontaneous ^{51}Cr release ranged from 5% and 20% of total counts incorporated into cells.

GM-CSF assay. T cells (1×10^5) were incubated with 1×10^3 target cells in 96-well flat form plate in a final volume of 200μl of the media for 24 hr. Cells were removed and medium tested for GM-CSF with Quantikine kit (R&D Systems, Minneapolis, MN, USA).

Determination of HLA restriction of 17/A2 clone. Specificity of the T cells was tested 10 days after

stimulation. T cells (1×10^5) were incubated with 1×10^3 of different target cells in 96-well flat bottom plate in a final volume of 200μl of the complete medium. For the identification of MHC restriction, monoclonal antibodies directed against HLA determinants in W6/32 (anti-HLA-A, B, C), BB7.2 (anti-HLA-A2), CRII-351 (anti-HLA-A2), B1.23.7 (anti-HLA-B, C), L243 (anti-HLA class II) were incubated at 50μg/ml for 30 min at room temperature before addition of the effector T cells. The ELISA plate was incubated at 37°C for 20～24 hr. After incubation, the supernatants were assayed for cytokine production by ELISA plates (R&D Systems, Minneapolis, MN, USA).

HLA typing. HLA serotypes and DNA genotypes of cell lines were determined by the Johns Hopkins University Immunogenetic Facility. The HLA class I genotype for patient 1.24 was found to be: HLA-A1, A2, B39, B62, C1, Cw7.

Flow cytometry analysis. Approximately 1×10^5 cells were incubated with monoclonal antibodies at saturation concentrations for 30 min on ice, washed with PBS containing 2% FBS, and then stained with polyclonal goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) for another 30 min on ice and washed again. The cells were analyzed in a FACSAN (Becton Dickinson, Mountain View, CA, USA).

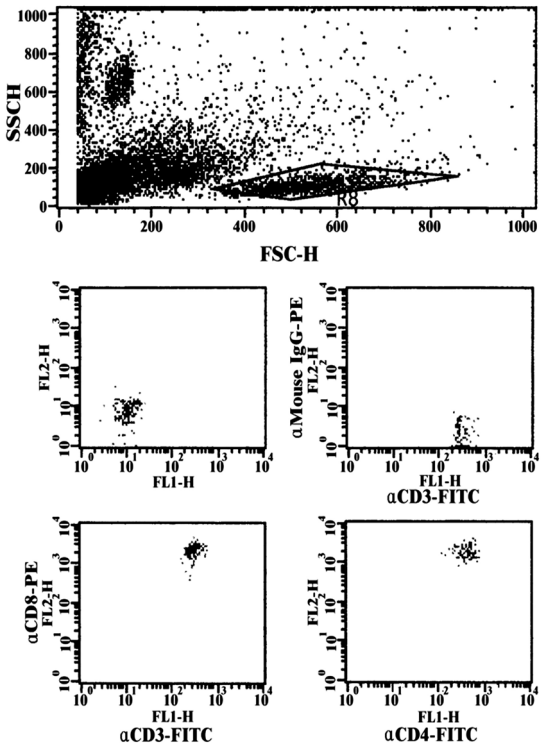

Figure 1. Phenotypic analysis of $CD4^+/CD8^+$ double positive T cell clone 17/A2 which is generated by limiting dilution method from the CTL line of RCC1.24 patient.

Results

Generation of $CD4^+/CD8^+$ double positive T cells specified to a RCC patient 1.24. Vaccination of RCC patient 1.24 with irradiated autologous GM-CSF-secreting tumorcells induced an intense infiltration of immune cells at the vaccine site along with a strong DTH reaction toward both tumor cells and normal kidney cells. Notably, the 1.24 patient had a remarkable regression of multiple pulmonary metastases 28 days after the first vaccination, indicating that the GM-CSF-secreting tumor cells might elicit anti-tumor immunity in vivo. To directly verify T-cell-mediated anti-tumor immunity in vitro, we attempted to identify RCC tumor-specific $CD8^+$ T cells produced inthe 1.24 patient. When the PBL of RCC patient 1.24 was repeatedly stimulated with autologous RCC tumor cells transfected by B7.1 that is known as the costimulatory molecule for T-cell activation, MHC class I-restricted and RCC-specific CTL lines were established. To generate these CTLs, we used two differently prepared autologous RCC tumor cells. One was used after treatment of 30 units/ml of IFNγ for 3 days, and the other was not treated. For each stimulation, IL-2 at a final concentration of 60 U/ml was added along with PBL from 3 different donors as feeder, and autologous RCC tumor cells were used as stimulator. Once the $CD8^+$ CTL lines against RCC 1.24 tumor cells were established, then the $CD8^+$ clones were obtained by limiting dilution method as described

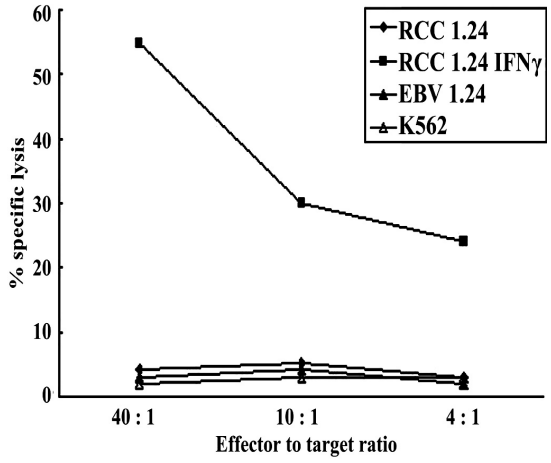

Figure 2. Specific cytotoxic activity of the autologous tumor cells by the 17/A2 clone. Autologous tumor cells (◆), autologous tumor cells treated with 30 units/ml of IFNγ (■), EBV-transformed B cell line (▲), and K562 (△) were used as different target cells. The specific lytic activity of the 17/A2 clone against target cells was tested by ^{51}Cr release assay at three different effector: target ratio.

in Materials and Methods. The 17/A2 clone was isolated from the CTL lines that were generated using IFNγ-treated tumor cells as the stimulator. FACS analysis revealed that 17/A2 T cells are $CD3^+$ TCR positive, and also $CD4^+/CD8^+$ double positive being stained by both anti-CD4-FITC and anti-CD8-PE antibodies (Fig. 1).

Cytolytic activity of CTL17/A2 clone toward autologous RCC tumor cells. The specificity of cytolytic effect of the 17/A2 clone on RCC tumor cells was tested by chromium- release assay method as described in Material and Methods.

As shown in Fig. 2, the 17/A2 cloneappeared to possess cytolytic activity against IFNγ-treated B7.1-transduced autologous RCC tumor cells at three different ratio of effector versus target (40 : 1～4 : 1) with a maximum activity at 40:1. However, the 17/A2 clone failed to show cytotoxicity against autologous RCC tumor that were not treated with IFN γ. Under these conditions, neither EBV-transformed B-cell line nor K562 cells were affected by cytotoxic activity of the 17/A2 clone. These results indicate that the 17/A2 clone can specifically recognize a tumor antigen presented in autologous RCC tumor cells, and suggest that the presentation of tumor antigen may be upregulated by the treatment of IFNγ.

Characterization of 17/A2CTL clone. Since the 17/A2 clone was able to target only the RCC tumor antigen which appeared to be more efficiently presented by IFNγ treatment, we investigated the effect of time-period of IFNγ treatment using two different densities of the RCC tumor cells on antigen recognition of the 17/A2 to find the optimum condition. The recognition of the RCC tumor antigen by the 17/A2 clone was evaluated by quantitating the level of GM-CSF produced by the 17/A2 clone, which reflects its activation following recognition of the RCC tumor antigen. Either 1×10^5 or 5×10^5 of B7.1-transduced autologous RCC tumor cells were treated with 30 units/ml of IFNγ in a T75 culture flask for upto 7 days. As shown in Fig. 3, the RCC tumor cells treated with IFNγ (30 units/ml) at a concentrations of 5×10^5 cells/T75 flask were well recognized by the 17/A2 clone, and the recognition began to be detectable at 3 days and reached a maximum 4 days after IFNγ treatment. However, when the RCC tumor cells were treated with IFNγ (30 units/ml) at a concentration of 1×10^5 cells/T75 flask, these cells were not recognized by the 17/A2 clone regardless of time period of IFNγ treatment. Again, under these conditions, both the RCC tumor cells untreated with IFNγ and EBV-transformed autologous B cells were refractory to recognition by the 17/A2 clone, resulting in production of nearly detectable level of GM-CSF.

To confirm whether the tumor antigen of B7.1-transduced autologous RCC tumor cells, recognized by the 17/A2 clone, is present in the primary RCC tumor cells from RCC 1.24 patient and can be induced following IFNγ treatment, we investigated the CTL activation activity of the 17/A2 clone using RCC 1.24 primary tumor cells as the target cells. Since

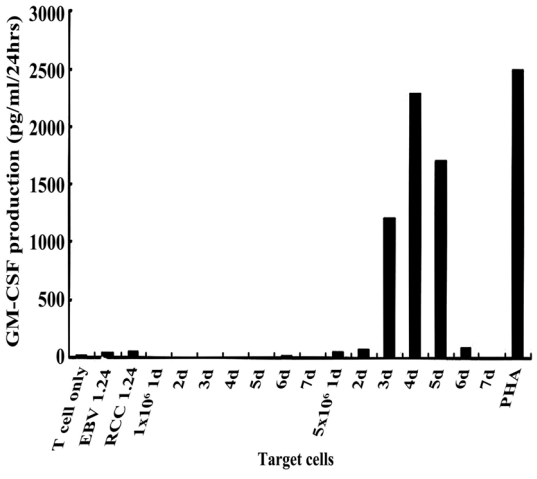

Figure 3. Antigen recognition of the 17/A2 clone is dependent on cell density and prolonged IFNγ treatment. Different number of tumor cells (1×10^5 or 5×10^5 cells per T75 culture flask) were treated with IFNγ (30 units/ml) for various periods. After treatment of IFNγ, cells were harvested and plated out into 96-well plate with 1×10^3 cells per well. Then all target cells were incubated with 1×10^4 cells of clone 17/A2 for 24 hr at 37°C. Supernatant were assayed for GM-CSF cytokine with ELISA kit.

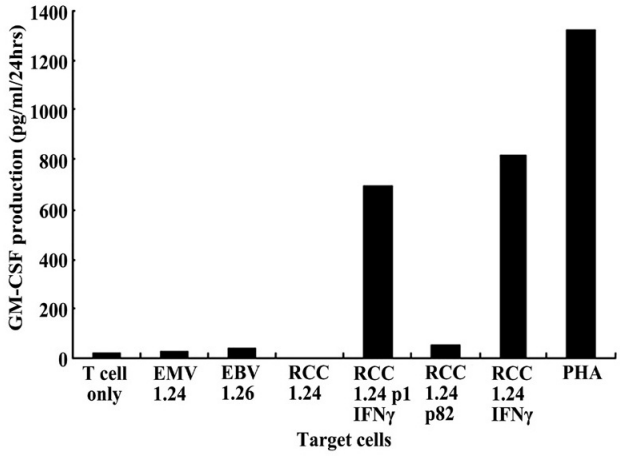

Figure 4. Recognition analysis of the 17/A2 clone on autologous primary tumor cells. Uncultured primary RCC1.24 tumor cells were thawed and used as a target cells (1×10^3 cells). Clone 17/A2 recognized the uncultured primary tumor cells as well as in vitro cultured tumor cells only after treatment of IFNγ.

the growth rate of the primary tumor cells were very slow as compared to that of B7.1-transduced RCC tumor cells, the primary RCC tumor cells (5×10^5/T75 flask) were treated with IFNγ for 8 days at a concentration of 20 units/ml. During the treatment of IFNγ, the medium was changed with fresh culture medium every 2 day incubation. As shown in Fig. 4, the 17/A2 clone was able to specifically recognize IFNγ-treated primary RCC tumor cells, whereas the 17/A2 clone failed to react with IFNγ-untreated tumor cells. These results demonstrated that the 17/A2 clone established from PBL ofRCC 1.24 patient can recognize the tumor antigen intrinsically present in the primary RCC tumor cells although its expression appears to be dependent on IFNγ treatment.

MHC restriction analysis. To understand molecular mechanisms underlying the specific recognition of the tumor antigen by the 17/A2 clone, we decided to identify the MHC restriction molecule involved in the antigen recognition. Cytokine release assay was done by MHC class I and MHC class II specific monoclonal antibodies. Monoclonal anti-HLA class I (anti-A, B, C=W6/32; anti-A2=BB7.2 or CR11-351; anti-B, C=B1.23.7) and monoclonal anti-HLA class II (anti-DR, -DP, -DQ=L-243) molecules were used to determine the role and importance of MHC restriction for 17/A2 T cell activation. As shown in Fig. 5, the 17/A2 clone was able to produce GM-CSF upon stimulation with IFNγ-treated autologous RCC 1.24 tumor cells, and this GM-CSF production was abrogated by the presence of either mAb W6/32 that can bind to all HLA class I molecules or mAb B 1.23.7 that reacts with HLA class I B and C molecules. However, the GM-CSF production was not blocked by addition of anti-HLA-A2 (BB7.2 or CR11-351) and anti-HLA class II (L-243). These results indicate that the CTL reactivity of the 17/A2 restricted by HLA class I B or C molecule. Since the HLA type of RCC 1.24 patient was HLA-A1, A2, B39, B62, C1, Cw7, these results suggest a possible involvement of B39, B62, C1, or Cw7 in the RCC tumor antigen presentation. In order to further identify the MHC restriction molecule for the 17/A2 clone, we sequentially performed cytokine release assay by employing various allogeneic RCC tumor cells from NIH patients who had been fully HLA-typed. These RCC tumor cells included the RCC-NIH-B (HLA-A2, A3, B7, B39, C0, Cw7), the RCC-NIH-F (HLA-A1, A25, B8, B51, C1, Cw7), and the RCC-NIH-S (HLA-A1, A2, B57, B62, C0, Cw7). All RCC tumor cells were treated with 30 units/ml of IFNγ for 4 days before using as targets for the 17/A2 clone. As expected, the GM-CSF production of the 17/A2 clone upon stimulation with RCC 1.24 tumor cells was significantly reduced in the presence of either W6/32 mAb that reacts with HLA class I A, B, and C molecules, or B1.23.7 mAb that reacts with HLA class I B and C molecules (Fig. 6). Under the same condition, the GM-CSF production of the 17/A2 upon stimulation with RCC-NIH-B tumor cells, which appeared to be comparable to the production level upon stimulation with RCC 1.24, was also significantly blocked. However, both RCC-NIH-F and RCC-NIH-S tumor cells were poorly recognized by the clone 17/A2, and thus induced a

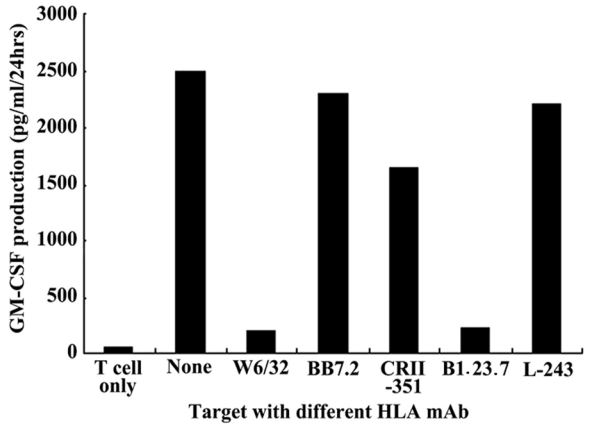

Figure 5. Recognition of interferon gamma treated RCC1.24 tumor cells by the 17/A2 clone is inhibited by anti HLA-B and C mAb. Autologous tumor cells (1×10^3 cells) were incubated with 1×10^4 of the 17/A2 clone for 4 hr either in the absence or presence of mAb W6/32 (anti-HLA-class I A, B, and C), BB7.2 (anti-HLA-A2), CR11-351 (anti-HLA-A2), B1.23.7 (anti-HLA-B and C) or L-243 (anti-pan HLA class II). The production of GM-CSF in the supernatant was assayed by ELISA kit.

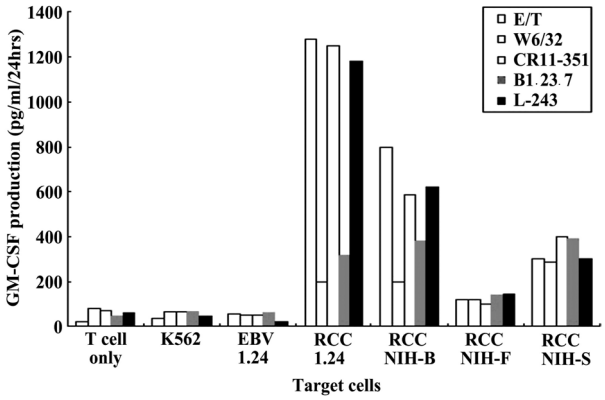

Figure 6. Identification of the HLA-restriction molecule of the 17/A2 clone using a panel of cultured autologous and allogeneic cells. Clone 17/A2 cross reacts with HLA-B39-matched allogeneic RCC cells. All target cells were treated with IFNγ. The HLA phenotype for patients are: RCC1.24: A1, A2, B39, B62, C1, Cw7, RCC-NIH-B: A2, A3, B7, B39, C0, Cw7, RCC-NIH-F: A1, A25, B8, B51, C1, Cw7, RCC-NIH-S: A1, A2, B57, B62, C0, Cw7.

very low level of GM-CSF. In addition, this low level of GM-CSF production was not affected by the addition of mAb W6/32, CR11-351, B1.23.7, or L-243, suggesting that the recognition of RCC-NIH-F and RCC-NIH-S tumor cells by the 17/A2 may be due to nonspecific reactivity of the 17/A2. These results indicate that HLA-B39 molecule that is present in both RCC 1.24 and RCC-NIH-B patients in common is involved in the antigen presentation. These data also suggest that the HLA-39-restricted RCC tumor antigen targeted by the 17/A2 clone may be a shared tumor antigen between RCC tumor cells.

Discussion

Tumor models have identified T cells as the main immunological effector cells in the immune response against tumors. Analyses of T cell responses against human tumors have focused on CD 8^+ cytotoxic T cells as exemplified by an increasing list of tumor antigens identified by tumor-reactive $CD8^+$ CTLs (14). Elucidation of regulation and specificity of T cell responses against tumors is the critical first step in understanding the relationship between the immune system and the endogenously arising cancers and provides the basis for the development of antigen-specific vaccines as well as other forms of T cell-mediated cancer immunotherapy. Recently, there are many reports describing about the tumor-antigen based cancer vaccine strategies (15-17). Antigen identification requires methods for generating T cell lines and clones, and for isolating either the gene coding the tumor antigen or the antigen itself.

In general, renal cell carcinoma has been considered an immunogenic human tumor type along with melanoma because its spontaneous regressions and late relapses after nephrectomy have been observed. Although a large number of tumor antigens have been identified in melanoma, there are only four RCC antigens have been reported so far. The first RCC antigen was cloned by Gauler et al. (18) and encoded by RAGE. This antigen is silent in most normal tissues but expressed in one out of 57 RCC cell lines. The second and third ones belong to the unique antigens resulting from a point mutation in the HLA-A2 (19) or in the hsp 70 gene (20). A fourth RCC antigen was reported recently and this antigen was produced by a rare mechanism of anti-sense strand translation (21).

This study examines the cytotoxic T cell clone responses against the autologous tumor in a patient with advanced renal cell carcinoma (RCC). In our previous clinical trial, following vaccination with autologous, GM-CSF gene transduced, irradiated tumor cells, the patient 1.24 showed regression of multiple pulmonary metastases, implying that a systemic antitumor immunity had been induced by the vaccine (3). The isolation of tumor-reactive $CD8^+$ T cells is of critical importance for elucidating antitumor cytotoxic T cell responses, and can be applicable to development of tumor antigen-based cancer vaccine against RCC.

The RCC-specific CTL cells were generated from peripheral blood lymphocytes (PBL) of the RCC patient 1.24 using B7.1-transduced autologous RCC tumor cells, that were pretreated with IFNγ, as stimulator. The 17/A2 clone was one of the CTL clones obtained after three-time stimulation. The 17/A2 clone appears to be a $CD4^+/CD8^+$ double positive T cell clone that possesses cytolytic activity toward autologous RCC tumor cells via MHC-class I restriction. The CTL 17/A2 clone was able to specifically target autologous RCC tumor cells treated with IFNγ and its CTL reactivity appeared to be restricted by the HLA-B39 molecule. The HLA-39 restriction was determined based on two different observations: specific blocking of the CTL reactivity of the 17/A2 clone by an anti-HLA-B, C mAb, and cross-reactivity with allogeneic HLA-B39-positive RCC cells. One interesting observation is that the RCC tumor antigen recognized by the 17/A2 clone is induced after IFNγ treatment in a cell density-dependent fashion. The IFNγ is a Type II interferon which is known to be secreted by T cells under certain conditions of activation and by natural killer (NK) cells. According to the World Wild Web documents, there are over 200 genes to be regulated by IFNγ. Particularly, it has been reported that IFNγ is a critical cytokine for the induction of efficient antigen processing in MHC-mediated antigen presentation, which plays clearly defined roles in pathogen resistance (22,23).

Taken together these results indicate that although the 17/A2 clone is not a fully differentiated functional cytotoxic T cells, it can specifically recognize HLA-B39-restricted RCC tumor antigen. The 17/A2 clone will be useful to identify the RCC-specific tumor antigen that can be applicable to elucidate the development or arising mechanism of tumor in normal cells.

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