HER2/neu-derived Peptides Are Shared Antigens among Human Non-Small Cell Lung Cancer and Ovarian Cancer

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Abstract

Previously, we have reported a correlation between the expression of HER2/neu and sensitivity to HLA-A2-restricted cytotoxic T-cells (CTL) in ovarian cancer. To investigate the role of HER2/neu in human ovarian and breast cancers, we established autologous tumor-specific CTL from tumor-infiltrating lymphocytes of HLA-A2+ HER2/neu+ ovarian cancer patients. These CTL lines specifically recognized HLA-A2+ HER2/neu+ autologous and allogeneic NSCLC cell lines as well as HLA-A2+ HER2/neu+ heterologous ovarian cancer cell lines. Furthermore, these CTL recognized an overexpressed, HER2/neu-derived peptide. From these results, we conclude that HLA-A2 serves as a restriction element in NSCLC. More importantly, at least one HER2/neu-derived peptide is a tumor-associated antigen in NSCLC and ovarian cancer.

Introduction

Tumor-specific CTL have been detected in several human cancers and could provide a therapeutic arm against malignant diseases in the form of a response to a tumor vaccine or through sensitization for subsequent adoptive immunotherapy. However, identification and characterization of TAA of human clinical tumors is required for optimal manipulation of CTL for clinical use. It would be even more beneficial for this purpose if we were to identify TAA which are shared by different tumors and not expressed by normal cells. In this sense, peptides derived from oncogene products may be potentially good targets for CTL. Recently, peptides derived from oncogene products such as p53 (3) and Ras (4, 5) have been shown to induce T-cell responses. We have previously demonstrated the relationship between HER2/neu expression and sensitivity to CTL in ovarian cancer (6). Peptide analysis revealed that a HER2/neu-derived peptide is one of the crucial T-cell epitopes recognized by HLA-A2-restricted, tumor-specific CTL, and this peptide is recognized in both ovarian and breast cancer. These findings have encouraged us to establish new therapeutic approaches to these malignancies. Lung cancer is one of the leading causes of death because of its frequency and its resistance to various therapeutic modalities (7), and therefore, poses a challenge for the development of novel therapeutic approaches to improve the prognosis of patients with this disease. Immunotherapy utilizing lymphokine-activated killer cells or TIL have been shown to be ineffective thus far (8, 9). In this study, we established CTL against NSCLC cells, and based on our previous results from ovarian and breast cancer, we examined if HER2/neu serves as a source of TAA in NSCLC since HER2/neu is also frequently expressed in NSCLC (10, 11).

Materials and Methods

CM. CM consisted of RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% heat inactivated fetal calf serum (BioWhittaker), 2 mM L-glutamine (BioWhittaker), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (BioWhittaker), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (BioWhittaker).

Preparation of TIL and Autologous Tumor Cells. TIL and autologous tumor cells were prepared as described previously (12). Briefly, fresh tumor tissues were excised from surgical specimens and minced into small pieces with scissors. This minced tissue was placed in a flask with a mixture of 0.1 mg/ml DNase type I, 1 mg/ml collagenase type IV, and 0.5 mg/ml hyaluronidase type V (all from Sigma, St. Louis, MO) in RPMI 1640 and stirred at room temperature for 2 to 4 h. The resultant cell suspension was washed in Hank’s balanced salt solution and subjected to Ficoll-Hypaque gradient (LSM, Organon Teknika, Durham, NC) centrifugation (1000 X g, 30 min). The interface was collected, suspended in CM, and subsequently subjected to two-layered (75 and 100%) Ficoll-Hypaque discontinuous density gradient centrifugation at 1000 X g for 20 min. Cells from 100% interface and 75% interface were used as TIL and tumor cells, respectively. TIL and tumor cells were also obtained from malignant pleural effusion or ascites with the use of the above discontinuous gradient centrifugation method.

Tumor Cell Lines. Non-small cell lung cancer cell lines (NCI-H460, NCI-H-647, NCI-H820, NCI-H1155, and NCI-H1355) were established and characterized in the NCI-Navy Medical Oncology Branch (Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) (11). NCI-H460/NCI-H1155, NCI-H647, and NCI-H820/NCI-H1355 originate in large cell carcinoma, adenocarcinoma, and adenoscarcinoma, respectively. These lung cancer cell lines were cultured in the specially conditioned medium, ACL-4 (13). Ovarian epithelial tumor lines (Ov1, Ov2, and Ov3) were established in the Division of Surgical Oncology, Brigham and Women’s Hospital (Boston, MA) from tumor specimens as described above. The expression of HER2/neu by these tumor cell lines was determined by Northern blotting analysis (11) and/or flow cytometry analysis, and the HER2/A2 expression of the tumor cell lines was determined by flow cytometry analysis by using BB7.2 (anti-HLA-A2 mAb). HER2/neu expression detected by flow cytometry was matched with the results of Northern blot. Characteristics of each tumor cell line are summarized in Fig. 1. T2 is an HLA-A2+ human melanoma cell line which is defective in endogenous antigen presentation secondary to the deletion of the gene encoding TAP-1 and TAP-2 (14), and was used as a peptide-pulsed target in a chromium release assay.

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3 The abbreviations used are: CTL, cytotoxic T-cells; CM, culture medium; NSCLC, non-small-cell lung cancer; mAb, monoclonal antibody; TAA, tumor-associated antigen; TIL, tumor-infiltrating lymphocytes; FITC, fluorescein isothiocyanate.


CTL. HLA-A2\(^+\) TIL obtained as described above were stimulated with solid phase anti-CD3 mAb (Ortho Pharmaceutical Corp., Raritan, NJ) for 48 h and expanded in CM containing 50 IU/ml recombinant interleukin 2 (Amgen, Thousand Oaks, CA) for 6 to 7 weeks. For the last 3 or 4 weeks, TIL were stimulated with irradiated autologous or allogeneic tumor cells weekly. We established CTL1/CTL2, CTL3/CTL4, and CTL5/CTL6 with stimulation of autologous non-small cell lung cancer cells, HLA-A2\(^+\) allogeneic non-small cell lung cancer cells, and HLA-A2\(^+\) allogeneic ovarian cancer cells, respectively. CTL1/CTL3, CTL5, and CTL2, and CTL4/CTL6 were derived from three patients with adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma, respectively.

mAb. Anti-CD4 mAb (phycoerythrin-conjugated) and anti-CD8 mAb (FITC-conjugated) were purchased from Becton Dickinson (Mountain View, CA). Anti-HLA-A2 mAb (BB-7.2) and anti-HLA-A2 (BB7.2), respectively.

Flow Cytometry. For direct single or two-color analyses, cells (5 \(\times\) 10\(^6\)) were incubated for 30 min at 4\(^\circ\)C with phycoerythrin- and/or FITC-conjugated mAb or murine control antibodies. In indirect staining, cells were incubated with the first mAb, or murine IgG (control) in staining medium (Hanks' balanced salt solution with 5% fetal calf serum) for 30 min at 4\(^\circ\)C. After washing, cells were stained with FITC-conjugated anti-mouse IgG. The stained cells were fixed with 1% paraformaldehyde and kept at 4\(^\circ\)C until analysis. Fluorescence analysis was performed by using a flow cytometer (Coulter, Hialeah, FL).

Cytotoxicity Assay. Cytotoxicity of CTL against tumor cells was examined by a standard \(^{51}\)Cr-release cytotoxicity assay as described previously (12). Briefly, tumor targets were labeled for 1 h at 37\(^\circ\)C with \(^{51}\)Cr and washed. The target cells (2.5 \(\times\) 10\(^6\)) were incubated with 2.5 \(\times\) 10\(^5\) to 1 \(\times\) 10\(^5\) of effector cells (effector:target ratio = 10:1 to 40:1) in 200 \(\mu\)l of CM in a 96-well round bottom microtitr plate for 4 h at 37\(^\circ\)C. The supernatant (100 \(\mu\)l) was collected and the samples were counted in a gamma counter (Gamma, Tm Analytic, Elk Grove, IL). Percentage of specific lysis was evaluated by measuring the experimental minus the spontaneous divided by the maximum minus the spontaneous \(^{51}\)Cr release.

Blocking of CTL Activity by mAb. Target cells were incubated with 1:10-diluted supernatant of L227-producing hybridoma or BB7.2-producing hybridoma for 30 min at 4\(^\circ\)C after labeling with chromium. The mAb were added to the effector cells and target cells in 1:4-dilution, and a 4-h \(^{51}\)Cr-release assay was performed as described above.

Synthesis of HER2/neu-derived Peptides. We synthesized the HER2/neu-derived peptide (654–662) with the sequence LSAVVGIL (GP2) and peptide (650–658) with the sequence PLTSI!SAV (GP1) at the Biopolymer Synthesis Laboratory at Brigham and Women's Hospital with an Applied Biosystems 430 peptide synthesizer. Both peptides have a point mutation at position 655 (valine to isoleucine) and contain a binding motif for the HLA-A2 molecule.

Statistical Analysis. Differences between cytotoxicities of CTL were examined by paired \(t\) test, and were considered significant when the \(P\) value was less than 0.05.

Results and Discussion

We successfully established autologous NSCLC-specific CTL (CTL1 and CTL2) by repetitive stimulation of TIL with irradiated autologous tumor cells following initial stimulation with immobilized anti-CD3 mAb and expansion in low-dose recombinant interleukin 2 (Fig. 1). Subsequently, we examined the cross-reactivity of the CTL against allogeneic HER2/neu-expressing tumor cells.

Both CTL1 and CTL2 showed cytotoxicity against the allogeneic HER2/neu\(^+\) HLA-A2\(^+\) NSCLC cell line, NCI-H1355, as well as autologous tumor cells, but there was no substantial killing against either HER2/neu\(^+\) HLA-A2\(^-\) NSCLC lines (NCI-H820) or HER2/neu\(^-\) HLA-A2\(^-\) lung large cell carcinoma cell lines (NCI-H460 and NCI-H1155) (Fig. 1A). CTL1 also killed HER2/neu\(^+\) HLA-A2\(^-\) lung adenosquamous carcinoma cell line (NCI-H647) (Fig. 1A); however, this cytolytic activity was not blocked by anti-HLA-A2 mAb (data not shown), indicating that this cytotoxicity is non-HLA-A2 restricted, and potentially an allogeneic response. Interestingly, CTL1 and CTL2 also killed allogeneic HER2/neu\(^+\) HLA-A2\(^+\) ovarian cancer cells (Ov1 and Ov3), while HER2/neu\(^-\) HLA-A2\(^+\) Ov2 was not killed (Fig. 1B). The cytolytic activity of CTL1 and CTL2 against autologous tumor cells, NCI-H1355 and Ov1, was blocked by anti-HLA-A2 mAb (Fig. 2). These data indicate that NSCLC-specific CTL recognize TAA in the context of HLA-A2 similar to ovarian cancer-specific CTL as previously reported (16). Furthermore, NSCLC appear to share common TAA with ovarian cancer, and our findings implicate HER2/neu.
HER2/neu-DERIVED TUMOR ANTIGEN IN NSCLC

To confirm that NSCLC TIL recognize a HER2/neu-derived antigen, we tested cytotoxicity of these CTLs against the T2 cell line pulsed with two HER2/neu-derived peptides (GP1 and GP2), both of which share a binding motif for HLA-A2. CTL1, CTL2, CTL5, and CTL6 killed GP2-pulsed T2 at a significantly higher level as compared with the killing of unpulsed T2 (P < 0.05), although the cytotoxicity of CTL3 and CTL4 against peptide-pulsed and unpulsed T2 was not significantly different (Fig. 4A). The cytolytic activities against T2 pulsed with GP2 were significantly blocked by anti-HLA-A2 (P < 0.05) (Fig. 4B). There was no substantial cytotoxicity against GP1-pulsed T2 among these CTL (Fig. 4B). From these results, we conclude that at least one HER2/neu-derived peptide (GP2) is a tumor antigen presented by HLA-A2 and recognized by NSCLC-specific CTL. Moreover, this tumor antigen is shared with ovarian carcinoma.

In this study, we used TIL obtained from clinical cases and succeeded in generating HER2/neu tumor-specific CTL restricted by HLA-A2 by stimulation with irradiated tumor cells 3–4 times after activation with anti-CD3 mAb and expansion in low-dose recombinant interleukin 2. Disis et al. (17) reported that 10 stimulations were required to induce effective CTL directed against a HER2/neu-HLA-A2 complex when peripheral blood lymphocytes from normal donors and peptide-loaded antigen-presenting cells are used. These findings suggest that there are differences in the frequency of precursor CTL between TIL and lymphocytes from normal blood, and indicate the existence of circulating T-cells recognizing HER2/neu-derived peptides and their accumulation in tumor sites.

For therapeutic purposes, peptides recognized by CTL may be derived from p53 (3), Ras (4, 5), and HER2/neu (17, 18), and thus have been investigated extensively in human and murine in vitro systems. HER2/neu is widely expressed and the overexpression of this gene accelerates tumor growth resulting in a poor prognosis in these patients (19, 20). We have demonstrated a HER2/neu-derived immunogenic peptide presented by HLA-A2 and recognized in ovarian cancer, breast cancer, and non-small cell lung cancer, suggesting that this antigen is widespread among human tumors. This antigen system may be theoretically useful in a peptide-based anti-tumor vaccine, as well as in the generation of anti-tumor CTL for use in...
adjuvant immunotherapy. In the present study, however, there was a discrepancy between the cytotoxicity against autologous tumor cells (Fig. 1) and that against GP2-pulsed T2 (Fig. 4) in both CTL1 and CTL2, and between that against HER2/neu tumor cells (Fig. 3) and that against GP2-pulsed T2 (Fig. 4) in both CTL3 and CTL4, suggesting that HER2/neu peptides presented to CTL in the context of HLA-A2 are diverse in NSCLC and ovarian cancer potentially related to differences in the level of expression of HLA-A2, HER2/neu, or other possible TAA among these tumors. Further investigation is required to clarify which peptides may be involved in different HER2/neu-expressing tumors.

Previously, immunotherapy has not been shown to be effective in lung cancer; however, the results presented in this study form the basis for further investigation into novel therapeutic modalities for NSCLC such as peptide vaccines and adoptive immunotherapy utilizing the HER2/neu-derived peptide-driven immune reaction.

References

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