Her-2/neu-derived Peptides Are Tumor-associated Antigens Expressed by Human Renal Cell and Colon Carcinoma Lines and Are Recognized by in Vitro Induced Specific Cytotoxic T Lymphocytes

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ABSTRACT

The Her-2/neu oncogene encodes a M, 185,000 transmembrane protein with homology to the epidermal growth factor receptor. It is overexpressed in 30–40% of breast and ovarian cancers, and this overexpression was shown to correlate with aggressiveness of malignancy and poor prognosis. Using tumor-associated lymphocytes isolated from patients with ovarian or breast cancer, several HLA-A2-restricted, Her-2/neu-derived peptides were identified. Further studies revealed that these tumor-associated CTLs can also lyse other tumors, including non-small cell lung and pancreatic cancer cells, suggesting that Her-2/neu epitopes are shared between several distinct types of epithelial tumors. To analyze whether Her-2/neu epitopes are tumor-associated antigens for renal cell carcinoma (RCC) and colon carcinoma, we induced Her-2/neu peptide-specific CTL responses by primary in vitro immunization and used these CTLs to determine the presentation of Her-2/neu epitopes on human tumor lines. Autologous dendritic cells (DCs) generated from peripheral blood monocytes were pulsed with Her-2/neu-derived peptides E75 and GP2 and used as antigen-presenting cells for CTL priming. High CTL activity toward peptide-pulsed targets was observed after two weekly restimulations. CTLs induced with DCs generated in the presence of TNF-α elicited a higher cytotoxic activity when they were stimulated with the cognate peptide than did CTLs induced with DCs grown in granulocyte macrophage colony-stimulating factor and interleukin 4 alone. The cytotoxicity of induced CTLs was antigen specific and HLA-A2 restricted. Furthermore, these CTLs lysed, in a MHC- and antigen-restricted fashion, not only breast cancer cells but also colon carcinoma and RCC cell lines expressing Her-2/neu. The cytotoxic activity against tumor cells was blocked by cold HLA-A2-positive targets pulsed with the cognate peptide in cold target inhibition assay and by anti-HLA-A2 monoclonal Ab. These results suggest that epitopes derived from Her-2/neu protein might be attractive candidates for broadly applicable vaccine therapies and may prove useful for adoptive immunotherapies designed for colon carcinoma or RCC.

INTRODUCTION

Peripheral CD8+ T cells recognize peptide antigens that are presented in the groove of MHC class I molecules on APCs. Recently, the definition of MHC class I allele-specific motifs allowed the definition of epitopes contained within a given antigen and provided new opportunities for the development of vaccine therapies (1–3). However, to date, with a few exceptions (melanoma-associated antigens), there is only limited information about the identity of CTL epitopes presented by human malignant cells (4).

The Her-2/neu oncogene is a M, 185,000 transmembrane protein with tyrosine kinase activity and extensive homology to the epidermal growth factor receptor (5, 6). It is overexpressed in 30–40% of breast and ovarian cancers, and this overexpression was shown to correlate with aggressiveness of malignancy and poor prognosis (7, 8).

Using tumor-associated lymphocytes isolated from patients with ovarian or breast cancers, two HLA-A2 restricted Her-2/neu-derived peptides were identified (E75 and GP2; Refs. 9 and 10). Further studies revealed that these tumor-associated lymphocytes can also lyse other tumors, including non-small cell lung and pancreatic cancer cells, suggesting that Her-2/neu epitopes are shared between several distinct types of epithelial tumors and can be an appropriate candidate for broadly applicable vaccine therapies (11–13).

In vitro immunization methods using epitopes derived from self-antigens have often proved unsatisfactory because of the low affinity of the elicted CTLs and, consequently, the lack of a sufficient recognition of naturally processed antigens by these CTLs (14, 15). Presentation of antigens by professional APCs may be critical for the effectiveness of an induced immune response, and the nature of the APCs can determine the outcome, ranging from immunity to tolerance (16).

DCs are key regulators in immune responses, being capable of priming naive resting T cells and initiating primary T cell responses when they are pulsed with antigenic peptides or proteins (17–26).

Here, we demonstrate that DCs pulsed with Her-2/neu-derived peptides E75 and GP2 elicited a peptide-specific CTL response by primary in vitro immunization in a culture system using peripheral blood from a normal individual. These CTLs showed an antigen-specific and HLA-A2-restricted lysis of target cells coated with the antigenic peptide and established tumor cell lines expressing Her-2/neu, including colon carcinoma and RCC. These results suggest that epitopes derived from Her-2/neu protein may prove useful for adoptive immunotherapies designed for colon carcinoma or RCC.

MATERIALS AND METHODS

Tumor Cell Lines. Tumor cell lines used in these experiments were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 50 µM 2-mercaptoethanol, and antibiotics. HLA-A2 expressing tumors were as follows: MB-MDA-231 (breast cancer), MCF7 (breast cancer), A-498 (RCC), HCT116 (colon carcinoma), MKR (malignant melanoma, kindly provided by U. Keilholz, University of Heidelberg, Heidelberg, Germany), and T2 (174×CEM.T2 hybridoma, TAP1 and TAP2 deficient). Croft (HLA-A2) is an EBV-immortalized B-cell line that was kindly donated by O. J. Finn (University of Pittsburgh School of Medicine, Pittsburgh, PA). TW-33 (RCC) was kindly provided by G. Müller (University of Göttingen, Göttingen, Germany). SK-OV-3 (HLA-A3) is an ovarian cell line that overexpresses HER-2/neu.

Cell Isolation and Cultures. Generation of DCs from peripheral blood monocytes was performed as described previously (26, 27). In brief, PBMCs were isolated by Ficoll/Paque (Life Technologies, Inc., Grand Island, NY)
density gradient centrifugation of heparinized blood obtained from a healthy male HLA-A2-positive volunteer. Cells were seeded (1 X 10^7 cells/well; 3 ml/well) into six-well plates (Costar, Cambridge, MA) in RP10 medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mm l-glutamine, 50 µM 2-mercaptoethanol, and antibiotics). After 2 h of incubation at 37°C, nonadherent cells were removed, and the adherent cells were cultured in RP10 medium supplemented with cytokines. The following cytokines were used: 100 ng/ml human recombinant GM-CSF (Leukomax; Sandoz), 1000 units/ml IL-4 (Genzyme), and 10 ng/ml TNF-α (Genzyme). The phenotypes of the DCs were analyzed by flow cytometry after 7 days of culture.

**Immunostaining.** Cell staining was performed using FITC- or phycoerythrin-conjugated mouse mAbs against the following: CD86; CD40 (Pharmingen, Hamburg, Germany); CD80; HLA DR; CD34; CD14 (Becton Dickinson, Heidelberg, Germany); HLA A, B, and C (W6/32; DAKO A/S, Glostrup, Denmark); CD83 (Coulter-Immunotech, Hamburg, Germany); CD1a (OKT6; Ortho Diagnostic Systems); and mouse IgG isotype controls (Becton Dickinson). The level of HLA-A2 expression was analyzed using a purified mAb that was specific for HLA-A2 (BB7.2). Her-2/neu expression was determined using unlabelled antibodies c-neu (Ab-5; Calbiochem, Cambridge, MA) and c-erbB-2/Her-2/neu (Upstate Biotechnology, Inc., Lake Placid, NY), followed by FITC-conjugated goat antimouse antibody (Becton Dickinson). The samples were analyzed on a FACScan Calibur (Becton Dickinson).

**Induction of Antigen-specific CTL Response Using HLA-A2-restricted Synthetic Peptides.** The IMP peptide (amino acids 58–66; GILGFVFTL) and the Her-2/neu-derived peptides E75 (amino acids 369–377; KIFGSLAFL) and GP2 (amino acids 654–662; IISAVVGIL) were synthesized using standard N-(9-fluorenylmethoxycarbonyl) chemistry on a peptide synthesizer (model 432A; Applied Biosystems, Weiterstadt, Germany) and analyzed by reverse-phase high-performance liquid chromatography and mass spectrometry. The HLA-A2 binding of synthetic peptides was confirmed by T2 stabilization assay. For CTL induction, 5 X 10^6 DCs were pulsed with 50 µg/ml synthetic peptide for 2 h, washed, and incubated with 2.5 X 10^6 autologous PBMCs in RP10 medium. After 7 days of culture, cells were restimulated with autologous peptide-pulsed PBMCs, and human recombinant IL-2 (Genzyme) was added at 5 units/ml on days 1, 3, and 5. The cytolytic activity of induced CTLs was analyzed on day 5 after the last restimulation in a standard Cr-release assay.

**CTL Assay.** The standard 51Cr-release assay was performed, with some modifications, as described (14). Target cells were pulsed with 50 µg/ml peptide for 2 h and labeled with [51Cr]sodium chromate in RP10 for 1 h at 37°C. Cells (10^6) were transferred to a well of a round-bottomed 96-well plate. Varying numbers of CTLs were added to give a final volume of 200 µl, and the mixtures were incubated for 4 h at 37°C. At the end of the assay, supernatants (50 µl/well) were harvested and counted in a β-counter. To increase the lysis of human tumor cells, target cells were incubated for 24 h with 100 units/ml IFN-γ (Genzyme). Preincubation of target cells with IFN-γ increased their lysis by CTLs up to 10–20%. However, the CTLs also lysed target cells in an antigen- and HLA-specific manner without IFN-γ treatment (data not shown).

Antigen specificity of tumor cell lysis was further determined in a cold target inhibition assay by analysis of the capacity of peptide-pulsed unlabeled T2 cells to block lysis of tumor cells at a ratio of 20:1 (inhibitor:target ratio). For antibody blocking, target cells were incubated after 51Cr labeling and washing for 30 min with BB7.2 or isotype antibody before being seeded in 96-well plates.

**RESULTS**

**Generation of DCs.** Adherent PBMCs were grown for 7 days in RP10 medium supplemented with GM-CSF and IL-4, with or without the addition of TNF-α. Analysis of surface markers showed that the addition of TNF-α to the culture medium resulted in increased levels of MHC class I and II molecules, CD80, CD86, CD40, and CD54. In line with high expression of adhesion and costimulatory molecules and corresponding to phenotypic characteristics of mature DCs, GM-CSF-, IL-4-, and TNF-α-induced DCs expressed high levels of CD83, as compared to DCs generated with GM-CSF and IL-4 alone (data not shown). In a typical experiment, after 7 days of culture, about 70–90% of the cells appear as loosely adherent cells with typical DC morphology and phenotype, as assessed by light microscopy and flow cytometry, in line with recently reported results (26, 27).

**Induction of a Peptide-specific CTL Response Using DCs Generated from Adherent PBMCs.** DCs pulsed with the synthetic peptides derived from IMP or Her-2/neu oncogene (GP2 and E75) were used to induce a CTL response in vitro. As shown in Fig. 1, CTL lines obtained after two weekly restimulations demonstrated peptide-specific killing. It was observed that T cells only exhibited a cytotoxic response against targets coated with the cognate peptide. CTLs induced with DCs generated in the presence of TNF-α elicited a higher cytotoxic activity when they were stimulated with the cognate peptide than did CTLs induced with DCs grown in GM-CSF and IL-4 alone (data not shown). Incubation of PBMCs with unpulsed DCs did not result in CTL induction. Repeated attempts to obtain a Her-2/neu-specific T-cell response by using peptide-pulsed PBMCs as APCs did not elicit a measurable response (data not shown).

Cultures containing CTL (referred to as CTL.IMP, CTL.E75, and CTL.GP2) were examined for their antigen-specific proliferation. Triplicate wells containing 10^5 T cells were cultured in the presence of autologous irradiated PBMCs (2 X 10^5/well) coated with the cognate or irrelevant peptide. Forty-eight h later, cultures were pulsed with [3H]thymidine, and then an additional 16 h, cultures were harvested. As shown in Fig. 2, the induced CTL.E75 and CTL.GP2 elicited antigen-specific proliferative responses upon stimulation with the cognate peptide, although the responses were weaker than those elicited by CTL.IMP.

**Fig. 1.** Induction of CTL responses by peptide-pulsed DCs. Adherent PBMCs were grown for 7 days in RP10 medium supplemented with GM-CSF and IL-4, without (O and □) or with (■ and △) the addition of TNF-α. DCs pulsed with the synthetic peptides derived from IMP (A) or Her-2/neu oncogene (GP2 (B) and E75 (C)) were used to induce a CTL response in vitro. Cytotoxic activity of induced CTLs was determined in a standard Cr-release assay using T2 cells as targets pulsed for 2 h with 50 µg of the cognate peptide (■ and △) or left unpulsed (O and □).
Fig. 2. Antigen-specific proliferative response of induced CTLs after stimulation with the cognate peptide. Ten days after their last restimulation, triplicate wells containing 10^5 T cells were cultured in the presence of autologous irradiated PBMNCs (2 x 10^5/well) coated with the cognate or irrelevant peptide. Forty-eight h later, cultures were pulsed with [3H]thymidine, and they were harvested after an additional 16 h. The assay was performed after four restimulations. Columns, means of triplicate cultures; bars, SD.

Lysis of Allogeneic Breast Cancer Cells by CTLs Specific for Her-2/neu Is Antigen Specific and HLA-A2 Restricted. After a number of weekly restimulations, the induced CTL lines were analyzed for their ability to lyse tumor cells naturally expressing the Her-2/neu protein. The expression of Her-2/neu and HLA-A2 molecules on tumor cells was determined by flow cytometry, and the results are shown in Fig. 3. Interestingly, expression of Her-2/neu was also detected on tumor cell lines derived from colon carcinoma (HCT116) and RCC (A-498 and TW-33).

To analyze the ability of CTL.E75 and CTL.GP2 to lyse tumor cells, primary Her-2/neu and MB-MDA-231 breast cancer cells were used as targets in a standard Cr-release assay. As demonstrated in Fig. 4, A and B, CTL.E75 and CTL.GP2 lysed breast cancer cells, and their cytotoxic activities could be blocked in a cold target inhibition assay with unlabeled T2 cells pulsed with the cognate peptide. No inhibition was observed when T2 cells were used with an irrelevant peptide.

Significant cytolytic activity toward MCF7 tumor cells was also obtained, whereas CTL.GP2 and CTL.E75 did not lyse K562 cells, indicating that they did not express natural killer activity (Table 1). The CTL lines did not recognize HLA-A2-positive, Her-2/neu-negative tumors (Croft and MKR cell lines), and they did not show detectable cytotoxicity against the HLA-A2-negative SK-OV-3 line (HLA-A3), which overexpresses Her-2/neu protein. These findings suggested MHC restriction and antigen specificity of the cytolytic activity mediated by CTL.GP2 and CTL.E75.

Lysis of RCC and Colon Carcinoma Cells by Her-2/neu-specific CTLs. Flow cytometric analysis of Her-2/neu expression of different tumor cell types using specific mAbs revealed Her-2/neu expression in the HCT116 colon carcinoma line and two RCC cell lines (A-498 and TW-33). We, therefore, analyzed the presentation of Her-2/neu-derived peptides by these cell lines and used them as targets in a standard Cr-release assay. As shown in Fig. 4, C and D, and Table 1, CTL.E75 and CTL.GP2 did lyse the HCT116 and A-498 cells, suggesting that the E75 and GP2 peptides are presented by these tumors. The lysis of A-498 cells could be blocked by cold target inhibition and presence of HLA-A2-specific mAb BB7.2 in the assay, suggesting antigen specificity and MHC restriction of the cytotoxic activity (data not shown). No detectable recognition was found against the TW-33 line, which is Her-2/neu positive but showed no expression of HLA-A2 when stained with BB7.2 (Fig. 3).

DISCUSSION

DCs are recognized as the most efficient professional APCs for induction of primary immune responses. Several previous studies have demonstrated that DCs can develop from CD14+ blood monocytes cultured with GM-CSF and IL-4. These cells have the phenotypical and functional characteristics of immature DCs and can be further induced to mature by activation with TNF-α, lipopolysaccharide, IL-1, or monocyte-conditioned medium (19, 26-29). Here, DCs generated with GM-CSF and IL-4 demonstrated, after stimulation with TNF-α, a greater ability to initiate a primary-antigen specific CTL response.

Recently, it was demonstrated that CTLs specific for the Her-2/neu-derived GP2 peptide lysed not only breast and ovarian cancer cells but also non-small lung and pancreatic cancer cells, confirming that this epitope is a shared antigen between many epithelial tumors and is a suitable candidate for broadly applicable vaccine therapies (11-13). Our results extend the list of epithelial tumors that present Her-2/neu-derived T cell epitopes, which increases the possible clinical use of these peptides.

![Fig. 3. Flow cytometric analysis of HLA-A2 and Her-2/neu expression on the human tumor cell lines.](image-url)
Melanoma and RCC are generally considered to be immunogenic tumors, and in the case of melanoma, MHC class I- and II-restricted T-cell epitopes can be identified (4, 30). In contrast, there are only a few reports about tumor-specific CTLs recognizing RCC, and only one gene coding for an antigen recognized by cytolytic T lymphocytes on human renal carcinoma has been identified thus far (31–35). A recent report on Her-2/neu expression in RCC showed overexpression of Her-2/neu in a high percentage of RCCs and found a close correlation of high protein levels with an unfavorable tumor grade and the occurrence of distant metastasis (35). Our findings indicate that Her-2/neu epitopes might be tumor-associated antigens in RCC that are recognized by CTLs.

Because protocols for maintenance and expansion of human DCs generated from bone marrow-derived progenitors or peripheral blood monocytes have recently been established (19, 26–29, 36, 37), it is possible now to generate sufficient numbers of DCs from patients and apply them in vaccination therapies.

The use of DCs pulsed with antigenic peptides could provide an alternative or additional approach to established therapies of tumors, and Her-2/neu peptides could be attractive candidates for designing immunotherapy protocols for the treatment of epithelial tumors, such as RCC and colon cancer, in addition to breast and ovary cancers.

Table 1  Lysis of human tumor cell lines by Her-2/neu-specific CTLs

<table>
<thead>
<tr>
<th>Tumor cell lines</th>
<th>CTL.GP2 (% specific lysis) at E:T</th>
<th>CTL.E75 (% specific lysis) at E:T</th>
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<tr>
<td>MCF7</td>
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<td>37</td>
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<td>HC7116</td>
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</tr>
<tr>
<td>K562</td>
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<td>2</td>
</tr>
</tbody>
</table>

*The cytotoxic activities of CTL.E75 and CTL.GP2 against human tumor cell lines were analyzed in a standard 51Cr-release assay.*

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