Expression of Her-2/neu on Acute Lymphoblastic Leukemias: Implications for the Development of Immunotherapeutic Approaches

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ABSTRACT

Her-2/neu is a tumor-associated antigen that is expressed on several adenocarcinomas and correlates with poor prognosis. In a previous study (H. J. Bühring et al., Blood, 86: 1916–1923, 1995), it has been demonstrated that Her-2/neu expression can be detected on blast cells from patients with hematological malignancies including acute lymphoblastic leukemia (ALL). Here, we show that Her-2/neu-specific CTLs induced in vitro using peptide-pulsed dendritic cells efficiently lyse primary ALL blasts constitutively expressing both Her-2/neu and human leukocyte antigen A2 in an antigen-specific and MHC-restricted manner. Furthermore, we analyzed the feasibility of this approach in an autologous setting and induced Her-2/neu-specific CTLs using dendritic cells generated from peripheral blood mononuclear cells from an ALL patient that were pulsed with peptides or transfected with in vitro-transcribed Her-2/neu mRNA. Our data demonstrate that Her-2/neu could be used as a potential target for the application of Her-2/neu-directed treatment strategies in ALL including vaccination approaches.

INTRODUCTION

Her-2/neu is a Mₜ, 185,000 transmembrane protein with tyrosine kinase activity that is a member of the epidermal growth factor receptor family (1). Overexpression of Her-2/neu has important biological and therapeutic implications. In preclinical studies, overexpression of Her-2/neu was found to contribute to the oncogenic transformation and metastatic potential of tumor cells (2, 3). In breast cancer, Her-2/neu overexpression correlates with poor prognosis and is associated with more aggressive tumors (2). Her-2/neu is overexpressed in 20–30% of human breast cancers and can be found on several adenocarcinomas such as ovarian, colorectal, or RCCs (2–4). Her-2/neu protein currently represents a viable therapeutic target for the development of novel treatment strategies using mAbs or tyrosine kinase inhibitors that have been shown to be effective in the treatment of malignant diseases (3, 5–7). Furthermore, in recent vaccination studies, DCs presenting T-cell epitopes derived from the Her-2/neu oncogene were demonstrated to induce antigen-specific CTLs in cancer patients that are able to recognize tumor cells expressing Her-2/neu (8). In a previous report (9), it was found that Her-2/neu is expressed in some hematological malignancies including ALL. To further extend the possible use of Her-2/neu-derived T-cell epitopes in immunotherapeutic approaches, we analyzed the presentation of antigenic peptides by these malignant cells using in vitro-induced Her-2/neu-specific CTLs. CTLs generated from healthy donors by primary in vitro immunization recognized malignant cells from patients with ALL in an antigen-specific manner. Furthermore, we show that Her-2/neu-specific CTLs able to lyse autologous malignant cells can be induced in patients with ALL using peptide-pulsed or RNA-transfected DCs, indicating that functional DCs and CTLs can be generated in these patients. Our results demonstrate that Her-2/neu is a tumor-associated antigen expressed in ALL cells and contribute to the development of novel therapeutic strategies in ALL.

MATERIALS AND METHODS

Tumor Cell Lines. Tumor cell lines used in the experiments were grown in RPMI medium (4). The following tumor cells were used in the experiments: A498 (RCC; Her-2/neu+; HLA-A2+); Croft [EBV-immortalized B-cell line kindly donated by O. J. Finn (Pittsburgh, PA); Her-2/neu−; HLA-A2+]; SK-OV-3 (ovarian carcinoma cell line; Her-2/neu+; HLA-A3+; kindly provided by O. J. Finn); and K562 (chronic myelogenous leukemia cell line).

Blasts from ALL Patients. Blasts from seven ALL patients were used for the experiments: (a) patient 1, HLA-A2+, Her-2/neu+; (b) patient 2, HLA-A2+, Her-2/neu+; (c) patient 3, HLA-A2−, Her-2/neu+; (d) patient 4, HLA-A2+, Her-2/
(e) patient 5, HLA-A2+, Her-2/neu+; (f) patient 6, HLA-A2−, Her-2/neu−; and (g) patient 7, HLA-A2+, Her-2/neu−. Blasts from patients with ALL were grown in RP10 medium for 24 h before they were used as target cells in a standard 51Cr release assay.

**Generation of DCs.** Generation of DCs from adherent PBMCs was performed as described previously (4, 10).

**Cell Isolation.** B cells and CD34+ peripheral blood progenitor cells were isolated using the B Cell Isolation Kit (human) and the CD34+ Progenitor Cell Isolation Kit [human (Miltenyi Biotec, Bergisch Gladbach, Germany)] according to the manual provided by the manufacturer. Isolated cell populations were characterized by flow cytometry.

**Immunostaining.** Cell immunostaining and detection of Her-2/neu expression were performed as described elsewhere (4, 8, 10, 11).

**Induction of an Antigen-specific CTL Response using HLA-A2-restricted Synthetic Peptides and CTL Assay.** CTL induction with the Her-2/neu-derived peptides [E75, KIFGSLAFL (12); GP2, IISAVVGIL (13)] and the 51Cr release assay for CTL activity were performed as described previously (4, 10, 14–16). Briefly, DCs were pulsed with 50 μg/ml synthetic peptide for 2 h, washed, and incubated with 3 × 10⁶ autologous PBMCs in RP10 medium. After 7 days and 14 days of culture, cells were restimulated with autologous peptide-pulsed PBMCs, and 1 ng/ml human recombinant IL-2 (R&D Systems, Wiesbaden, Germany) was added every second day after the first restimulation. The cytolytic activity of induced CTLs was analyzed on day 19 (day 5 after the last restimulation) in a standard 51Cr release assay. Antigen specificity of tumor cell lysis was further determined in a cold target inhibition assay (4) by analyzing the capacity of peptide-pulsed unlabeled Croft cells to block the lysis of tumor cells at a ratio of 20:1 (inhibitor: target ratio) and by blocking studies. For antibody blocking, target cells were incubated for 30 min with 20 μg/ml mAb BB7.2 recognizing the HLA-A2 molecule or isotype control antibody. For preincubation with IFN-γ, blasts were cultured for 24 h in RP10 medium with 100 units/ml IFN-γ (Genzyme, Neu-Isenburg, Germany) before the assay.

**Generation of Her-2/neu mRNA by in Vitro Transcription.** For generation of Her-2/neu mRNA, plasmid pSPJC1 with an insert coding for Her-2/neu (kindly provided by Prof. A. Ulrich, Martinsried, Germany) which allows in vitro transcription under the control of a Sp6 promoter, was used. The plasmid was linearized using NdeI (MBI Fermentas, St. Leon-Rot, Germany) and in vitro transcribed with Sp6 Cap Scribe (Roche, Mannheim, Germany) according to the protocol provided by the manufacturer.

### Table 1 Mean fluorescence intensity of Her-2/neu + ALL blasts

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Her-2/neu expression MFIa</th>
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<tbody>
<tr>
<td>1</td>
<td>43.71</td>
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<tr>
<td>2</td>
<td>38.21</td>
</tr>
<tr>
<td>3</td>
<td>58.37</td>
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<tr>
<td>4</td>
<td>10.22</td>
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<tr>
<td>5</td>
<td>45.29</td>
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a MFI, mean fluorescence intensity. The range of Her-2/neu protein expression in all five Her-2/neu + patients used in the experiments was analyzed by flow cytometry. The level of surface expression is indicated as the mean fluorescence intensity of the Her-2/neu + cell population.
manufacturer. Purification of in vitro transcripts was performed with RNeasy Mini anion-exchange spin columns (Qiagen, Hilden, Germany) according to the RNA cleanup protocol provided by the manufacturer. In vitro transcripts were routinely checked by formaldehyde/agarose gel electrophoresis for size and integrity and stored at −80°C in small aliquots.

Electroporation of DCs and CTL Induction. Before electroporation on day 6, immature DCs were washed twice with serum-free X-VIVO 20 medium (BioWhittaker, Walkersville, MD) and resuspended to a final concentration of 2 × 10^7 cells/ml. Subsequently, 200 µl of the cell suspension were mixed with 5 µg of Her-2/neu mRNA and electroporated in a 4-mm cuvette using an Easyject Plus unit (Peqlab, Erlangen, Germany; Ref. 17). The physical parameters were voltage of 300 V, capacitance of 150 µF, resistance of 1540 Ω, and pulse time of 231 ms. After electroporation, the cells were immediately transferred into RP10 medium and returned to the incubator. After transfection, DCs were incubated for 24 h in RP10 medium containing 10 ng/ml TNF-α for maturation of DCs. For CTL induction, 5 × 10^5 DCs (transfected with Her-2/neu RNA) were cocultured with 2.5 × 10^6 autologous PBMNCs in RP10 medium. Human recombinant IL-2 (1 ng/ml; Genzyme) was added on days 7, 9, and 11. The cytolytic activity of induced CTLs was analyzed on day 11 after CTL induction in a standard 51Cr release assay.

RESULTS AND DISCUSSION

Her-2/neu Expression on Primary ALL Blasts. We analyzed the expression of Her-2/neu on primary blasts from patients with ALL using a Her-2/neu-specific monoclonal antibody. In 5 of 33 tested patients with ALL, expression of Her-2/neu was detected. Interestingly, all five patients had a poor outcome with early relapse or resistance to standard treatment. As demonstrated in Fig. 1A, ALL blasts were characterized by high levels of CD10, CD19, CD34, and HLA-DR expression. In Fig. 1B, the level of Her-2/neu expression on ALL blasts was further tested against all blasts from patient 5 (Her-2/neu+, HLA-A2+) as well as against nonmalignant B cells, DCs, bone marrow and CD34+ peripheral hematopoietic progenitor cells (all HLA-A2+). In Fig. 1C, Her-2/neu Epitopes Recognized by CTLs on ALL Cells.

Fig. 2 Cytotoxic specificity of CTLs induced with Her-2/neu peptide-pulsed DCs from healthy donors. DCs were generated from adherent PBMNCs grown for 7 days in RP10 medium supplemented with GM-CSF, IL-4, and TNF-α and pulsed with the synthetic Her-2/neu-derived peptides E75 (left column) or GP2 (right column) and used as antigen-presenting cells to induce a CTL response in vitro. The cytotoxic activity of induced Her-2/neu-specific CTLs (CTL E75 and CTL GP2) was determined in a standard 51Cr release assay after two weekly restimulations. Targets used were the EBV-immortalized B-cell line Croft (Her-2/neu+, HLA-A2+), pulsed with the Her-2/neu-derived peptides E75 and GP2, the RCC cell line A498 (Her-2/neu+, HLA-A2+), and the ovarian carcinoma cell line SK-OV-3 [Her-2/neu+, HLA-A2+ (A)]. To rule out NK-mediated cytotoxicity, K562 cells were also included as target cells. A498 cells were incubated with a HLA-A2-specific mAb (BB7.2) before the assay and used as target cells to show the HLA-A2 restriction of the CTLs. A498 cells incubated with mouse IgG were included as an isotype control (B). The cytotoxic activity of the two CTL lines was further tested against ALL blasts from patient 5 (Her-2/neu+, HLA-A2+) as well as against nonmalignant B cells, DCs, bone marrow and CD34+ peripheral hematopoietic progenitor cells (all HLA-A2+).
healthy donors as antigen-presenting cells (4, 10). The two recently described HLA-A2-binding peptides E75 (12) and GP2 (13) were used for CTL induction in vitro (4). Croft cells (HLA-A2+/H11001/Her-2/neu/H11002) were lysed when they were pulsed with the cognate peptide used for CTL induction but were spared when they were pulsed with an irrelevant peptide or left untreated. As shown in Fig. 2A, CTL lines obtained after two weekly restimulations demonstrated peptide-specific killing. Both CTL lines were able to efficiently lyse A498 cells (HLA-A2+/H11001/Her-2/neu/H11001), whereas there was no lysis of the ovarian cancer SK-OV-3 cells (Her-2/neu/H11001/HLA-A3+/H11001). There was no killing of K562 cells (Fig. 2B), demonstrating that the cytotoxic activity was not mediated by NK cells. The cytolytic activity against RCC line A498 could be blocked entirely by using a monoclonal antibody against HLA-A2. These results demonstrate that the presentation of Her-2/neu peptides in the context of HLA-A2 molecules on the target cells is necessary for the efficient lysis of tumor cells and confirm the antigen specificity and MHC restriction of the induced CTLs. As shown in Fig. 2C, the Her-2/neu-specific CTLs did not recognize autologous non-malignant B cells, DCs, bone marrow, and mobilized CD34+ hematopoietic progenitor cells from a healthy HLA-A2+ donor, whereas there was a significant lysis of leukemic blasts from a HLA-A2+ and Her-2/neu+ ALL patient. These results demonstrate that the induced cytolytic activity is directed specifically against the Her-2/neu-expressing ALL blasts.

In the next set of experiments, we analyzed the ability of the in vitro-induced CTLs to lyse primary leukemic cells from patients with ALL. Flow cytometric analysis revealed Her-2/neu expression on blasts obtained from five patients with ALL (Fig. 1 and Table 1). As shown in Fig. 3A, Her-2/neu peptide-specific CTLs recognized primary Her-2/neu-expressing ALL blasts obtained from three HLA-A2+ patients (patients 1, 2, and 4), suggesting that Her-2/neu peptides are presented by these leukemic cells. In contrast, there was no lysis of cells lacking the corresponding HLA haplotype (HLA-A2−, patient 3). The antigen specificity mediated by the in vitro-induced CTL lines was further confirmed in a cold target inhibition assay (Fig. 3B). The lysis of the ALL cells (patient 1) could be blocked by the addition of unlabeled “cold” Croft cells pulsed with the cognate peptide, whereas cells pulsed with an irrelevant peptide showed no effect. In our experiments, we observed a diminished lysis of primary ALL blasts from patient 2 that was not due to a decreased expression of Her-2/neu protein on the ALL blasts (Fig. 4A). We therefore analyzed the expression of HLA class I molecules on the surface of these cells, and it was reduced as compared with the blasts from the other patients and could be
Her-2/neu Epitopes Recognized by CTLs on ALL Cells

Fig. 5 Induction of Her-2/neu-specific CTLs with RNA-transfected or peptide-pulsed DCs from ALL patients. DCs were generated using adherent PBMNCs from a patient with a newly diagnosed ALL. Autologous DCs were cultured in RPMI medium supplemented with GM-CSF and IL-4 and electroporated with Her-2/neu mRNA using an EasyJet Plus unit on day 6 after induction. After an additional 24 h in RPMI medium supplemented with GM-CSF, IL-4, and TNF-α, DCs were used for CTL induction. Cytolytic activity was determined by a standard 51Cr release assay on day 11 after CTL induction (A). DCs used for peptide pulsing (B) were prepared as described in the previous experiments. Cytolytic activity was analyzed by a standard 51Cr release assay after two weekly restimulations with peptide-pulsed autologous PBMNCs. Target cells used in the assays were autologous ALL blasts (Her-2/neu+, HLA-A2+), the EBV-immortalized B-cell line Croft (Her-2/neu+, HLA-A2+) pulsed with the Her-2/neu-derived peptides E75 or GP2 or with the irrelevant HIV peptide, the RCC cell line A498 (Her-2/neu+, HLA-A2+), and the ovarian cancer carcinoma line SK-OV-3 (Her-2/neu+, HLA-A2−). To assess for potential NK cell-mediated cytotoxicity, the K562 cell line was included.

up-regulated by incubation with IFN-γ. In line with these results, the lysis of the ALL blasts of patient 2 by the CTLs could be increased upon treatment of the target cells with IFN-γ (Fig. 4B).

Her-2/neu-specific CTLs Can Be Induced using PBMNCs from ALL Patients. Next we analyzed the induction of Her-2/neu-specific CTLs in an autologous setting using PBMNCs from a patient (patient 5) with a newly diagnosed Her-2/neu+ ALL. DCs were generated from peripheral blood mononuclear cells of this patient and pulsed with the GP2 and E75 peptides. In addition, these DCs were transfected with an in vitro-transcribed Her-2/neu mRNA and used as antigen-presenting cells for CTL induction. The cytotoxic activity of the T cells generated with RNA-transfected DCs was determined on day 11, whereas the peptide-induced CTLs were analyzed on day 19 after additional weekly restimulation with peptide-pulsed autologous PBMNCs.

As demonstrated in Fig. 5A, CTLs generated with Her-2/neu mRNA-transfected DCs lysed HLA-A2-matched allogeneic tumor cells and autologous malignant ALL cells. There was no significant cytolytic activity directed against K562 cells, demonstrating that the induced response was not mediated by NK cells. Interestingly, the CTLs induced with Her-2/neu RNA-transfected DCs also recognized targets pulsed with the E75 or GP2 peptides, demonstrating that these two peptides are presented upon transfection of DCs with Her-2/neu RNA and contribute to the observed cytolytic activity.

Lysis of autologous malignant cells was also detected when CTLs generated with peptide-pulsed DCs were applied in the assay (Fig. 5B). However, these CTLs required additional restimulation, indicating that transfection of DCs with RNA derived from Her-2/neu is a more potent approach to elicit tumorspecific CTLs.

In our study, we demonstrate that the tumor-associated antigen Her-2/neu is expressed in ALL and that primary blasts from patients with ALL can be lysed by CTLs specific for Her-2/neu-derived peptides. In a previous report, it was shown that Her-2/neu is expressed in a subset of hematological malignancies, especially in CD10+/CD19- common-ALL (in 9 of 19 patients; Ref. 9) indicating that treatment strategies targeting Her-2/neu could be evaluated in this subgroup of patients. Recent developments in the treatment of malignant diseases using mAbs, tyrosine kinase inhibitors, or cellular immunotherapies have revealed Her-2/neu as an important therapeutic target (3, 5–8).

In contrast to chronic myelogenous leukemia, where remissions of the disease can be induced in 60–80% of patients after donor lymphocyte infusions, such clinical and immunological effects are rare in ALL patients due to the induction of a graft-versus-leukemia effect (18, 19). This might be due to a reduced induction of ALL-specific T cells after donor lymphocyte infusions or might result from inefficient recognition of ALL blasts by the CTLs. However, our data indicate that ALL-specific T cells can be generated in the peripheral blood of patients with ALL in vitro and that these CTLs are able to efficiently lyse primary autologous leukemia cells. Thus, enhanced antigen presentation by a powerful antigen-presenting cell (such as DCs) might induce a strong T-cell response capable of mediating the lysis of ALL blasts, which could result in clinical remission of the leukemic disease.

In a Phase I study using DCs pulsed with HLA-A2-binding peptides derived from Her-2/neu or MUC1 tumor antigens (8), we were recently able to induce peptide-specific CTLs in patients with metastatic breast and ovarian cancers in vivo without any side effects or autoimmune reactions, demonstrating that these peptides can be safely and efficiently used in clinical studies.

In conclusion, our results suggest that therapies targeting Her-2/neu, including vaccination studies with peptide-pulsed DCs, could be applied in ALL.

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