The Epithelial Tumor Antigen MUC1 Is Expressed in Hematological Malignancies and Is Recognized by MUC1-specific Cytotoxic T-Lymphocytes

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ABSTRACT

The epithelial mucin MUC1 is overexpressed on the cell surface of many epithelial malignancies as well as on some B-cell lymphomas and multiple myelomas. Recently, we identified two HLA-A2-restricted T-cell epitopes derived from the MUC1 protein. To further extend the potential application of these peptides, we analyzed the expression of MUC1 on blast cells from patients with acute myelogenous leukemia (AML; n = 43) and several other hematological malignancies including acute lymphoblastic leukemia (n = 24), chronic lymphocytic leukemia (n = 36), hairy cell leukemia (n = 9), follicular lymphoma (n = 7), and multiple myeloma (n = 12). Using reverse transcription-PCR and MUC1-specific monoclonal antibodies, MUC1 expression was found in 67% of AML samples and 92% of myeloma samples. To analyze the presentation of MUC1 peptides by primary AML blasts, we induced MUC1-specific CTLs in vitro using peptide-pulsed dendritic cells from HLA-A2+ healthy donors as antigen-presenting cells. These CTLs efficiently lysed in an antigen-specific and HLA-A2-restricted manner not only target cells pulsed with the antigenic peptide but also tumor cell lines including multiple myeloma cells and primary AML blasts that constitutively expressed both MUC1 and HLA-A2. The specificity of the CTLs was confirmed in a cold target inhibition assay. Our data demonstrate that MUC1-derived peptides are tumor antigens in AML and several other hematological malignancies that could potentially be used for immunotherapeutic approaches.

INTRODUCTION

MUC1 is a highly glycosylated type I transmembrane glycoprotein that is abundantly overexpressed on the cell surface of many human adenocarcinomas like breast and ovarian cancers. Moreover, MUC1 expression has been demonstrated in multiple myeloma and some B-cell Non-Hodgkin lymphomas making MUC1 an attractive and broadly applicable target for immunotherapeutic strategies (1–9). Several recent reports (8–12) demonstrated that cytotoxic MHC-unrestricted T cells from ovarian, breast, pancreatic, and multiple myeloma tumors can recognize epitopes of the MUC1 protein core localized in the tandem repeat.

Recently (4), we identified two HLA-A2-binding peptides derived from the MUC1 protein. One of the peptides is derived from the tandem repeat region of the MUC1 protein, referred to as M1.1. The second peptide (referred to as M1.2) is localized within the signal sequence of MUC1. Using MUC1-peptide-pulsed DCs as antigen-presenting cells, CTLs were generated that lysed tumors endogenously expressing MUC1 in an antigen-specific and HLA-A2-restricted fashion. More recently (13), we have shown that MUC1-specific CTLs could also be induced in vivo after vaccination of breast and ovarian cancer patients with peptide-pulsed DCs.

To extend the possible use of MUC1-derived T-cell epitopes in immunotherapeutic approaches, we screened the expression of MUC1 on normal hematopoietic cells (14) as well as on various hematological malignancies using monoclonal antibodies specific for the MUC1 tumor antigen. To prove the presentation of T-cell epitopes by the malignant cells, we induced MUC1-specific CTLs in vitro using peptide-pulsed DCs as antigen-presenting cells. We show here that the CTLs generated from several healthy donors by primary in vitro immunization elicited an antigen-specific and HLA-A2-restricted cytolytic activity against target cells endogenously expressing MUC1 including primary AML blasts and multiple myeloma cell lines, thus extending the number of malignancies expressing the MUC1 tumor-rejection antigen.

MATERIALS AND METHODS

Tumor Cell Lines. Tumor cell lines used in the experiments were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics. The following tumor lines were used in experiments: MCF7 (MUC1+, HLA-A2+; purchased from the American Type Culture Collection), A498 (renal cell carcinoma, MUC1+, HLA-A2+), U266 (multiple myeloma, MUC1+, HLA-A2+), IM9 (multiple myeloma, MUC1+, HLA-A2+), Croft [EBV-immortalized B-cell line, kindly donated by O. J. Finn (Pittsburgh, PA), MUC1-, HLA-A2+], SK-OV-3 (ovarian cell line, MUC1+, HLA-A3; kindly provided by Dr. O. J. Finn, University of Pittsburgh School of Medicine). Blasts from patients with AML were grown in RPMI 10 medium containing GM-CSF (Leukomax; Novartis, Basel, Switzerland; 100 ng/ml) for 2 days before they were used as target cells in a standard 3Cr-labeled release assay.

Cell Isolation and Generation of DCs from Adherent PBMCs. Generation of DCs from peripheral blood monocytes was performed as described previously (4, 15, 16). In brief, PBMCs were isolated from buffy coat preparations of healthy volunteers from the blood bank of the University of Tübingen. Cells were seeded (1 × 107 cells/ml) into 6-well plates (Costar, Cambridge, MA) in 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 blood monocytes were cultured in RPMI 1640 medium supplemented with the following cytokines: human recombinant GM-CSF (Leukomax; Novartis; 100 ng/ml), IL-4 (Genzyme; 1000 IU/ml), and TNF-α (Genzyme; 10 ng/ml). The phenotype of DCs was analyzed by flow cytometry after 7 days of culture. Isolation of CD14+ monocytes, CD15+ granulocytes, and CD34+ peripheral blood progenitor cells was performed using MACS technology, as recommended by the manufacturer. The purity of the cells was >90%.

Immunostaining. Cell staining was performed using FITC- or PE-conjugated mouse mononclonal antibodies against CD86, CD40 (PharMingen, Hamburg, Germany), CD80, HLA-DR, CD54, CD14 (Becton Dickinson, Heidelberg, Germany), CD83 (Coulter-Immunotech, Hamburg, Germany), and CD1a (OKT6; Ortho Diagnostic Systems). Appropriate mouse IgG isotypes were used as controls (Becton Dickinson). The level of HLA-A2 expression was analyzed using a purified mononclonal antibody specific for HLA-A2 (BB7.2; data not shown). The MUC1 expression was determined using the monoclonal antibodies BM-2, BM-7 (Ref. 17; kindly provided by Dr. Sepp Kaul, University of Heidelberg, Heidelberg, Germany), and HMFG-1 (Ref. 4; IgG1; No-
intron-spanning primer pair for the H9252

ard F-moc chemistry on a peptide synthesizer (432A; Applied Biosystems, 20, LLLLTVLTV from the leader sequence) were synthesized using stand–

Synthetic Peptides.

with ethidium bromide for visualization under UV light.

RT-PCR reactions were electrophoresed through a 3% agarose gel and stained

analyzed on a FACScan Calibur (Becton Dickinson).

jugated goat antimouse antibody (Becton Dickinson). The samples were

vocastra Laboratories, Newcastle, United Kingdom), followed by FITC-con-

uated goat antimouse antibody (Becton Dickinson). The samples were

alyzed on a FACScan Calibur (Becton Dickinson).

RT-PCR. RT-PCR was performed with some modifications as described

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expression of MUC1 tumor antigen on AML blasts. The expression of MUC1 on malignant hematopoietic cells was determined using the MUC1-specific monoclonal antibody BM-2 (14, 17). As demonstrated in Table 1, MUC1 expression could be detected in 92% of the samples from multiple myeloma patients and in 67% of the blast samples from patients with AML. Interestingly, the frequency and the level of MUC1 expression were higher on AML FAB M4 and M5 blasts as compared with the FAB M1 and M2 subtypes (Table 1). An example of MUC1 expression on AML blasts from three patients is presented in Fig. 1 where three different MUC1-specific antibodies were used. The protein expression results obtained by flow cytometry were further confirmed using RT-PCR and MUC1-specific primers (Fig. 2A). Interestingly, as demonstrated in Fig. 2B, we could not detect any MUC1 transcripts in other myeloid cells purified from peripheral blood like CD14+ (monocytes) and CD15+ (granulo-

Table 1 Expression of MUC-1 (BM-2) on primary malignant cells from patients with various hematological malignancies

<table>
<thead>
<tr>
<th>Percentage of positive samples</th>
<th>MFI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-CLL</td>
<td>19%</td>
</tr>
<tr>
<td>T-CLL</td>
<td>25%</td>
</tr>
<tr>
<td>HCL</td>
<td>22%</td>
</tr>
<tr>
<td>FL</td>
<td>43%</td>
</tr>
<tr>
<td>MCL</td>
<td>8%</td>
</tr>
<tr>
<td>MZL</td>
<td>25%</td>
</tr>
<tr>
<td>Myeloma</td>
<td>92%</td>
</tr>
<tr>
<td>T-ALL</td>
<td>0%</td>
</tr>
<tr>
<td>Common-ALL</td>
<td>33%</td>
</tr>
<tr>
<td>AML</td>
<td>67%</td>
</tr>
<tr>
<td>AMM-M1</td>
<td>50%</td>
</tr>
<tr>
<td>AMM-M2</td>
<td>50%</td>
</tr>
<tr>
<td>AMM-M4</td>
<td>64%</td>
</tr>
<tr>
<td>AMM-M5</td>
<td>87%</td>
</tr>
<tr>
<td>AML unclassified</td>
<td>57%</td>
</tr>
<tr>
<td>CML-BCmy</td>
<td>56%</td>
</tr>
<tr>
<td>CML-BCly</td>
<td>2%</td>
</tr>
</tbody>
</table>

*a MFI, median fluorescence intensity. MFI levels <3 were considered negative, levels >3 were considered to be positive; HCL, hairy cell leukemia; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal cell lymphoma; c-ALL, common acute lymphoblastic leukemia; CML-BCly, chronic myelogenous leukemia in myeloid blast crisis; CML-BCmy, chronic myelogenous leukemia in lymphoid blast crisis.

Expression of MUC-1 (BM-2) on primary malignant cells from patients with various hematological malignancies.

Fig. 1. Flow cytometric analysis of MUC1 expression on AML blasts obtained from three different patients. The MUC1 expression was determined using unlabeled antibodies BM2 (bold line), BM2 (thick solid line) and HMPG1 (dotted line) by staining with FITC-conjugated goat antimouse antibody. Filled histograms represent isotype-matched controls.

Induction of Antigen-specific CTL Response Using HLA-A2-restricted Synthetic Peptides. The MUC1-derived peptides M1.1 (amino acids 950–958, STAPPVHNV from the tandem repeat domain) and M1.2 (amino acids 12–20, LLLLLTVLTV from the leader sequence) were synthesized using standard F-moc chemistry on a peptide synthesizer (432A; Applied Biosystems, Weiterstadt, Germany) and analyzed by reversed-phase high-performance liquid chromatography and mass spectrometry (4). For CTL induction, 5 × 10^5 DCs were pulsed with 50 μg/ml synthetic peptide for 2 h, washed, and incubated with 2.5 × 10^6 autologous PBMCs in RP10 medium. After 7 days of culture, cells were restimulated with autologous peptide-pulsed PBMCs, and 1 ng/ml human recombinant IL-2 (Genzyme) was added on days 1, 3, and 5. The cytolytic activity of induced CTL was analyzed on day 5 after the last restimulation in a standard ^51 Cr-labeled release assay (4, 16).

**C**TL **Assay.** The standard ^51 Cr-labeled release assay was performed as described (4, 16). Target cells were pulsed with 50 μg/ml peptide for 2 h and labeled with ^51 Cr-sodium chromate in RP10 for 1 h at 37°C. Cells (10^5) 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 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 plate. The percentage of specific lysis was calculated as: 100 × (experimental release – spontaneous release)/spontaneous release. Spontaneous and maximal releases were determined in the presence of either medium or 1% Triton X-100, respectively.

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 lysis of tumor cells at a ratio of 20:1 (inhibitor/target ratio).

RESULTS

Expression of MUC1 Tumor Antigen on AML Blasts. The expression of MUC1 on malignant hematopoietic cells was determined using the MUC1-specific monoclonal antibody BM-2 (14, 17). As demonstrated in Table 1, MUC1 expression could be detected in 92% of the samples from multiple myeloma patients and in 67% of the blast samples from patients with AML. Interestingly, the frequency and the level of MUC1 expression were higher on AML FAB M4 and M5 blasts as compared with the FAB M1 and M2 subtypes (Table 1). An example of MUC1 expression on AML blasts from three patients is presented in Fig. 1 where three different MUC1-specific antibodies were used. The protein expression results obtained by flow cytometry were further confirmed using RT-PCR and MUC1-specific primers (Fig. 2A). Interestingly, as demonstrated in Fig. 2B, we could not detect any MUC1 transcripts in other myeloid cells purified from peripheral blood like CD14+ (monocytes) and CD15+ (granulo-
Fig. 3. Induction of MUC1-specific CTL responses by peptide-pulsed mature DCs. Adherent PBMCs were grown for 7 days in RPMI medium supplemented with GM-CSF, IL-4, and TNF-α. DCs pulsed with the synthetic peptides derived from the MUC1 protein (M1.1 and M1.2) were used to induce a CTL response in vitro. Cytotoxic activity of induced CTL (CTL.M1.1 and CTL.M1.2) was determined in a standard 51Cr-labeled release assay using the multiple myeloma cell lines U266 (MUC1+, HLA-A2+) and IM9 (MUC1−, HLA-A2+), and the EBV-immortalized B-cell line Croft (MUC1−, HLA-A2+) as targets. Croft cells were pulsed for 2 h with 50 μg of the M1.1 (●) or M1.2 peptide (△).

Table 2. Lysis of human tumor cell lines by MUC1-specific CTL.

<table>
<thead>
<tr>
<th>Tumor cell lines</th>
<th>HLA-A2 expression</th>
<th>MUC1 expression</th>
<th>CTL.M1.1 Percentage of specific lysis at E:T</th>
<th>CTL.M1.2 Percentage of specific lysis at E:T</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-498</td>
<td>+</td>
<td>-</td>
<td>28 22 14</td>
<td>22 14 4</td>
</tr>
<tr>
<td>U266</td>
<td>+</td>
<td>-</td>
<td>19 15 7</td>
<td>16 26 3</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>-</td>
<td>+</td>
<td>1 2 -2</td>
<td>2 -3 -2</td>
</tr>
<tr>
<td>IM-9</td>
<td>+</td>
<td>-</td>
<td>15 -4 -2</td>
<td>14 -13 -12</td>
</tr>
</tbody>
</table>

The cytotoxic activity of CTL.M1.1 and CTL.M1.2 against human tumor cell lines was analyzed in a standard 51Cr-labeled release assay. As shown in Fig. 3, both CTL.M1.1 and CTL.M1.2 were able to efficiently lyse U266 and A498 cells (both HLA-A2+/MUC1+). There was no lysis of the ovarian cancer cells SK-OV-3 (MUC1+/HLA-A3+), IM9 cells (multiple myeloma; HLA-A2+, MUC1−), or Croft cells (HLA-A2+, MUC1−). These results demonstrate that the presentation of MUC1 epitopes in context of HLA-A2 molecules on the target cells is necessary for the efficient lysis of target cells and confirm the antigen specificity and MHC restriction of the CTL. Furthermore, these data show that the MUC1 peptides can be presented in an HLA-restricted manner by multiple myeloma cells on the cell surface.

Lysis of Primary AML Blasts by MUC1-specific CTLs. Flow cytometric analysis of different tumor cell types revealed MUC1 expression on several malignant hematopoietic cells including blasts obtained from patients with AML (Fig. 1; Table 1). Therefore, we analyzed the presentation of MUC1-derived peptides by these primary AML blasts and used them as targets in a standard 51Cr-labeled release assay. As shown in Fig. 4, CTL.M1.1 and CTL.M1.2 did lyse primary MUC1-expressing AML blasts obtained from HLA-A2-positive patients, suggesting that MUC1 peptides are presented by these leukemias. In contrast, there was no lysis of control cell lines.

The antigen specificity and MHC restriction mediated by the in vitro induced CTL lines was further confirmed in a cold target inhibition assay (Fig. 5). The lysis of the AML cells (AMLs;Ch) could be blocked by addition of Croft cells pulsed with the cognate peptide, whereas cells pulsed with an irrelevant peptide showed no effect.

**DISCUSSION**

Recently, several attempts have been made to define possible leukemia-specific CTL epitopes. Fusion proteins such as BCR-ABL

**Fig. 4.** Lysis of AML blasts endogenously expressing MUC1 by CTL.M1.1 and CTL.M1.2. Human renal carcinoma cell line A498 (HLA-A2+/MUC1+), ovarian cancer cell line SK-OV-3 (HLA-A2−/MUC1+), and primary MUC1-expressing allogeneic AML blasts from four different HLA-A2-positive patients were used as targets in a standard 51Cr-labeled release assay.

**Fig. 5.** Cytotoxic activity of induced CTL (CTL.M1.1 and CTL.M1.2) against human tumor cell lines. The MUC1-positive, HLA-A2-expressing cell lines U266 (multiple myeloma) and A498 (renal cell carcinoma) were used as target cells in a standard 51Cr-labeled release assay. As demonstrated in Fig. 3 and Table 2, both CTL lines were able to efficiently lyse U266 and A498 cells (both HLA-A2+/MUC1+). There was no lysis of the ovarian cancer cells SK-OV-3 (MUC1+/HLA-A3+), IM9 cells (multiple myeloma; HLA-A2+, MUC1−), or Croft cells (HLA-A2+, MUC1−). These results demonstrate that the presentation of MUC1 epitopes in context of HLA-A2 molecules on the target cells is necessary for the efficient lysis of target cells and confirm the antigen specificity and MHC restriction of the CTL. Furthermore, these data show that the MUC1 peptides can be presented in an HLA-restricted manner by multiple myeloma cells on the cell surface.

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**DISCUSSION**

Recently, several attempts have been made to define possible leukemia-specific CTL epitopes. Fusion proteins such as BCR-ABL
in chronic myelogenous leukemia, ETV6-AML1 in pre-B acute lymphoblastic leukemia, PML-RARα in acute promyelocytic leukemia, and DEK-CAN in AML resulting from chromosomal translocations are potential targets because they are expressed only in malignant cells (18). Furthermore, they represent novel antigens and, in contrast to self-proteins, are not associated with the phenomenon of tolerance.

An alternative strategy to identify tumor- or leukemia-specific T-cell epitopes is the use of synthetic antigenic peptides derived from proteins that are preferentially expressed or overexpressed in malignant cells like MAGE, HER-2/neu, p53, or MUC1. However, thus far, only peptides from proteinase 3 and WT1 proteins have been demonstrated to elicit antigen-specific lysis of leukemic cells by cytotoxic T cells (19, 20).

We now demonstrate that the epithelial mucin, MUC1, is a novel tumor antigen in AML that is recognized by MUC1 peptide-specific CTLs. Recently, we identified two HLA-A2-binding peptides, M1.1 and M1.2, derived from the MUC1 protein. These peptide epitopes were expressed on various epithelial malignancies and were recognized by MUC1-specific CTLs (4). To extend the possible use of these peptides in vaccination therapies, we analyzed the expression of the MUC1 tumor antigen on various hematopoietic malignancies including AML, multiple myeloma, follicular lymphoma, hairy cell leukemia, and CLL, using MUC1-specific monoclonal antibodies. We found that MUC1 is expressed in 92% of samples from patients with multiple myeloma and about 67% of blast samples obtained from patients with AML, especially on AML FAB M4 and M5 subtypes. In addition, MUC1 protein expression was also observed on blasts from chronic myelogenous leukemia patients with myeloid blast crisis. Finally, MUC1 expression was detected on some follicular lymphomas, CLLs, and hairy cell leukemia samples (see Table 1).

To analyze whether MUC1-derived T cell epitopes are presented by AML cells endogenously expressing MUC1, we induced MUC1-specific CTLs. In our study, both M1.1- and M1.2-specific CTLs efficiently lysed primary allogeneic AML blasts from HLA-A2-positive patients. The antigen specificity of this cytotoxic effect was confirmed in a cold target inhibition assay.

Although not experimentally demonstrated here, it is likely that the MUC1-derived T-cell epitopes M1.1 and M1.2 might also be expressed by some B-CLL cells, hairy cells, and follicular lymphoma cells. Interestingly, MUC1 has been shown recently (21) to be rearranged and amplified in B-cell lymphomas by the t(1;14) translocation. The authors have shown that up to 16% of B-cell lymphomas show a molecular perturbation of the MUC1 region that can potentially lead to its deregulated overexpression. According to our results, the aberrant expression of MUC1 on AML blasts could also represent an oncogenic transformation, particularly because we could not observe MUC1 expression on the normal CD14+ CD57+, and the CD34+ cell populations (14).

There is now growing evidence that in vivo application of DC-presenting tumor-associated antigens or adoptive transfer of tumor-reactive CTLs generated ex vivo can induce antitumor immunity in patients with malignant diseases (13, 22–26). In a Phase I study using DCs pulsed with HLA-A2-binding peptides derived from Her-2/neu or MUC1 tumor antigens, 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, especially no induction of anemia, demonstrating that MUC1 peptides can be safely and efficiently applied in clinical studies (13). In addition, although MUC1 is expressed on normal cells in the gastrointestinal tract and several other tissues including breast and kidney, we did not observe any side effects during DC vaccinations. This might be related to the lower affinity of the induced MUC1-specific T cells (4) or because of the higher presentation of MUC1-derived peptides by tumor cells.

In conclusion, our results extend the list of malignancies including AML and multiple myeloma that present MUC1-derived T cell epitopes, which increases the possible clinical application of MUC1-derived peptides in vaccination studies.

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MUC1 IS EXPRESSED IN HEMATOLOGICAL MALIGNANCIES


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*Cancer Res* 2001;61:6846-6850.

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