Antilung Cancer Effect of WT1-specific Cytotoxic T Lymphocytes


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ABSTRACT

We and other groups have recently reported that CTLs that specifically recognize a peptide derived from WT1 lyse leukemia cells in a HLA class I-restricted manner. Because WT1 is expressed in numerous solid tumors as well as in leukemic cells, we investigated whether WT1-specific CTLs can also inhibit the growth of lung cancer by examining their cytotoxic activity against lung cancer cell lines in vitro and their inhibitory effect on the growth of human lung cancer cells engrafted into nude mice. The WT1 transcript was detected in most of the lung cancer cell lines examined. A WT1-specific, HLA-A24-restricted CTL clone (designated TAK-1) exhibited cytotoxicity against lung cancer cell lines bearing HLA-A24 but did not lyse cells lacking this HLA. This suggests that the target antigen for TAK-1 on HLA-A24-positive lung cancer cells is the naturally processed WT1 peptide. Adoptive transfer of TAK-1 into nude mice that had been engrafted with a HLA-A24-positive lung cancer cell line resulted in inhibition of cancer cell growth and prolonged survival. These findings strongly suggest that WT1 is a universal tumor-associated antigen and that WT1-targeting immunotherapy offers a potentially effective treatment option for lung cancer as well as leukemia.

INTRODUCTION

Despite recent progress in conventional chemotherapeutic, radiotherapeutic, and surgical approaches to anticancer treatment, the 5-year survival rate for most patients with lung cancer is still low, especially in those with advanced disease. New therapeutic strategies are therefore required. One recent development in this field is immunotherapy targeted against lung cancer-associated antigens. The identification of tumor-associated antigens is essential to the development of efficacious immunotherapy; however, to date, only a limited number of human lung cancer-associated antigens have been identified.

The WT1 gene encodes a zinc finger transcription factor (1), and WT1 binds to the early growth response-1 DNA consensus sequence present in various growth factor gene promoters (2). Although WT1 was initially shown to act as a transcriptional repressor, its specific functions in normal and neoplastic tissues remain to be fully elucidated. During normal ontogenesis, the WT1 gene is expressed in a time- and tissue-dependent manner, mainly in the fetal kidney, testis, ovary, and supportive structures of mesodermal origin (3, 4). In contrast, in adults, WT1 gene expression is limited to very few tissues, including the splenic capsule and stroma, the Sertoli cells of the testis, and the granulosa cells of the ovary (5, 6). With regard to malignant cells, it has been reported that most patients with leukemia aberrantly overexpress WT1, regardless of their leukemia subtype (7–10). WT1 has also recently been reported to be expressed in various solid tumors, including lung cancer (11, 12). These findings suggest that WT1 would be an attractive target for immunotherapy against various solid tumors as well as leukemia.

Recently, we have succeeded in establishing CD8+ CTL clones that recognize a 9-mer peptide derived from WT1 (13, 14). These WT1-specific CTLs efficiently lyse HLA-A24-positive but not HLA-A24-negative leukemic cells and do not lyse normal cells, regardless of their HLA-A24 expression status. Because WT1 is expressed in most types of lung cancer, we investigated whether WT1-specific CTLs can inhibit the growth of lung cancer cells by examining the cytotoxic activity of our WT1-specific CTL clone against lung cancer cells in vitro and the inhibitory effect of adoptive transfer of this clone on the growth of human lung cancer cells engrafted into nude mice. Our results strongly suggest that cell-mediated WT1-targeting immunotherapy will be effective against lung cancer as well as leukemia.

MATERIALS AND METHODS

Lung Cancer Cell Lines. Ten human lung cancer cell lines were used in the study. Of these, OU-LC-A2 was established in our laboratory; the others were kindly provided by Dr. E. Nakayama (Okayama University Graduate Schools, Okayama, Japan). All of the cell lines were cultured in RPMI 1640 supplemented with 10% FCS. The HLA class I genotypes of the cell lines were determined as described previously (15). Their HLA-A24 expression status was examined by flow cy-
to determine whether WT1-specific CTLs lyse lung cancer cells via recognition of the WT1 peptide, which is naturally processed in lung cancer cells and expressed in the presence of HLA-A24, cold target inhibition assays were performed as follows. Autologous LCL cells were incubated with one of the WT1-derived peptides at a concentration of 10 μM for 2 h. After extensive washing, the peptide-loaded cells were used as cold target cells. Various numbers of these cells were incubated with 5 × 10^5 cytotoxic effector cells for 1 h, and then 5 × 10^5 51Cr-labeled lung cancer cells were added to the wells. Cytotoxicity assays were then performed as described above. The percentage of specific lysis was calculated as follows: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm).

Adoptive Immunotherapy Model. Six-week-old BALB/c-nu/nu female mice were purchased from Nippon Clea (Tokyo, Japan) and maintained at the Animal Center of the Ehime University School of Medicine. For xenografting, 5 × 10^6 human lung cancer cells were injected s.c. into the right midabdomen of each mouse. Four days later, 5 × 10^6 WT1 peptide-specific CTL clone cells, suspended in PBS, were injected i.v. via the orbital vein. Control mice received an equal volume of PBS alone i.v. Each CTL-treated and control group contained five mice. Each week, the mice were injected with an additional dose of 5 × 10^6 CTL clone cells or PBS alone, and the groups were monitored for tumor growth until all of the mice in the control group had died. The tumors were measured at 10-day intervals, and tumor volumes were calculated using the ellipsoid formula (length × width × height).

Statistical Analysis. The significance of differences between the mean values for the CTL-treated and control groups was analyzed using the Mann-Whitney exact test. Differences were considered significant at P < 0.05.

RESULTS

WT1 Expression in Lung Cancer Cell Lines. WT1 expression levels in the human lung cancer cell lines were determined by quantitative RT-PCR and calculated relative to the WT1 expression level in the human leukemia cell line K562. Because relative WT1 expression levels in most normal tissues are <10^-6, levels of >10^-5 were considered positive. As shown in Table 1, 2 of the 10 lung cancer cell lines (LC99A and Sq-1) expressed high levels of WT1, whereas 4 (RERF-LC-AI, LK79, LK87, and QG56) expressed intermediate levels of WT1 (10^-1 to 10^-3), and 3 (11-18, LC65A, and OU-LC-A2) expressed low levels of WT1 (10^-3 to 10^-5). WT1 expression in the remaining cell line (PC-9) was considered negative (<10^-6).

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The abbreviations used were: mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; DC, dendritic cell; MMC, mitomycin C; IL, interleukin; LCL, lymphoblastoid cell line.

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RT-PCR for WT1 Gene Expression Analysis. A quantitative RT-PCR procedure for determining WT1 gene expression in lung cancer cells was performed as described previously (8), with some modifications. Briefly, 2 μg of total RNA were isolated from each sample and converted into cDNA in 30 μl of reaction buffer. PCR was performed for 22–35 cycles for quantification of WT1 mRNA and for 16 cycles for quantification of β-actin mRNA. All analyses were performed in duplicate. To normalize differences in RNA degradation between the individual samples and in RNA loading for the RT-PCR procedure, the WT1 expression level for a particular sample was defined as its WT1 gene expression level divided by its β-actin gene expression level. The WT1 gene expression level of K562 leukemia cells, which strongly express WT1, was designated 1.0, and the levels for the experimental samples were calculated relative to this value.

Generation of the WT1 Peptide-specific CD8+ CTL Clone. A CTL clone that specifically recognizes a peptide derived from WT1, designated TAK-1, was generated as described previously (13). Briefly, WT1-derived peptides containing the binding motifs for HLA-A24 were synthesized. The peptide sequences of these synthetic peptides, designated WT1-T1, WT1-T2, WT1-T3, and WT1-T4, were as follows: (a) WT1-T1, QMTSQLECM (residues 228-236); (b) WT1-T2, CMTWNQMNL (residues 235-243); (c) WT1-T3, DFKD-CERRF (residues 356-364); and (d) WT1-T4, RWPSCKQKF (residues 417-425). DCs were generated from peripheral blood monocytes as described previously (16) and treated with MMC (Kyowa Hakko, Tokyo, Japan). One million CD8+ T lymphocytes were isolated from the peripheral blood lymphocytes of the same donor and cultured with 1 × 10^5 MMC-treated DCs in RPMI 1640, supplemented with 10% human AB-type serum and 5 ng/ml recombinant human IL-7 (Genzyme, Boston, MA) and containing 10 μM of one of the WT1 synthetic peptides, designated WT1-T1, WT1-T2, WT1-T3, and WT1-T4, were exchanged for fresh IL-7-supplemented medium, and the cells were restimulated in the same way, except that no IL-7 was added. Four days later, recombinant human IL-2 (10 units/ml; Boehringer Mannheim, Mannheim, Germany) was added to each well. The cytotoxicity of the growing cells was then examined, and cells that exhibited a cytotoxic effect on a WT1 peptide-loaded autologous B-LCL were cloned using a limiting dilution method as described previously (17).

Cytotoxicity Assays. Chromium-51 release assays were performed as described previously (18). Briefly, 1 × 10^4 51Cr (Na_2^51CrO_4; New England Nuclear, Boston, MA)-labeled target cells, suspended in 100 μl of RPMI 1640 supplemented with 10% FCS (assay medium), were seeded into round-bottomed microtiter wells and incubated with or without synthetic peptide for 2 h. In some experiments, the target cells were incubated with an anti-HLA class I framework mAb (w6/32; American Type Culture Collection, Manassas, VA) or an anti-HLA-DR mAb (L243; American Type Culture Collection) at an optimal concentration (10 μg/ml) for 30 min to determine whether cytotoxicity was restricted by HLA class I. Various numbers of effector cells, suspended in 100 μl of assay medium, were added to the well and incubated for 4 h, and then 100 μl of supernatant were collected from each well.

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3 The abbreviations used are: mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; DC, dendritic cell; MMC, mitomycin C; IL, interleukin; LCL, lymphoblastoid cell line.
WT1-targeting Immunotherapy for Lung Cancer

The presence of WT1-T2 peptide, as demonstrated previously (13), was shown in cell line C1R-A*2402 (but not its parent cell line, C1R) in the 24-positive allogeneic LCLs and the HLA-A*2402 transfectant WT1-T4. TAK-1 appeared to be cytotoxic only to HLA-A24-positive cells, as poducedly lysed LC99A, RERF-LC-AI, and 11-18 cell lines, which expressed WT1 at intermediate or low levels, were also efficiently lysed by TAK-1, but the degree of cytotoxicity against these cell lines was not as great as the degree of cytotoxicity against LC99A. In contrast, PC-9, in which WT1 expression was decreased, did not show significant cytotoxicity against these cell lines. Taken together with the data shown in Table 1, these findings demonstrate that the cytotoxicity of TAK-1 against lung cancer cells is mediated by specific recognition of endogenously processed WT1, and the cytotoxicity of TAK-1 against lung cancer cells is restricted by HLA-A24 status.

Cold Target Inhibition Assays. To further confirm that the cytotoxicity of TAK-1 against lung cancer cells was mediated by specific recognition of endogenously processed WT1, we performed cold target inhibition experiments. As shown in Fig. 3, the addition of WT1-T2-loaded allogeneic LCLs decreased the cytotoxicity of TAK-1 against LC99A and RERF-LC-AI.

Table 1. Cytotoxicity of TAK-1 against various lung cancer cell lines

<table>
<thead>
<tr>
<th>Target cells</th>
<th>WT1 expression level</th>
<th>Origin</th>
<th>HLA-A24</th>
<th>E:T ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC99A</td>
<td>$2.0 \times 10^{-1}$</td>
<td>lc</td>
<td>HLA-A24</td>
<td>20:1</td>
</tr>
<tr>
<td>LK79</td>
<td>$7.9 \times 10^{-2}$</td>
<td>sc</td>
<td>+</td>
<td>90.1</td>
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<tr>
<td>RERF-LC-AI</td>
<td>$8.0 \times 10^{-2}$</td>
<td>sq</td>
<td>+</td>
<td>68.6</td>
</tr>
<tr>
<td>11-18</td>
<td>$7.0 \times 10^{-4}$</td>
<td>ad</td>
<td>+</td>
<td>50.1</td>
</tr>
<tr>
<td>PC-9</td>
<td>$5.0 \times 10^{-7}$</td>
<td>ad</td>
<td>+</td>
<td>9.6</td>
</tr>
<tr>
<td>Sq-1</td>
<td>$1.9 \times 10^{-1}$</td>
<td>sq</td>
<td>+</td>
<td>5.8</td>
</tr>
<tr>
<td>LC65A</td>
<td>$1.0 \times 10^{-4}$</td>
<td>sc</td>
<td>–</td>
<td>6.1</td>
</tr>
<tr>
<td>QG56</td>
<td>$2.0 \times 10^{-2}$</td>
<td>sq</td>
<td>–</td>
<td>7.8</td>
</tr>
<tr>
<td>LK87</td>
<td>$5.0 \times 10^{-2}$</td>
<td>ad</td>
<td>–</td>
<td>6.8</td>
</tr>
<tr>
<td>OU-LC-A2</td>
<td>$4.0 \times 10^{-5}$</td>
<td>ad</td>
<td>–</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*a* ad, adenocarcinoma; lc, large cell carcinoma; sc, small cell carcinoma; sq, squamous cell carcinoma.

*b* The cytotoxicity of TAK-1 against the various lung cancer cell lines in the absence of the WT1 peptide was determined by 4-h $^{51}$Cr release assays at E:T ratios of 20:1, 10:1, and 5:1.

These data findings confirmed that TAK-1-mediated cytotoxicity is WT1-T2 peptide specific and restricted by HLA-A24.

Cytotoxicity of TAK-1 against Lung Cancer Cell Lines. HLA-A24 expression in the lung cancer cell lines was examined by flow cytometry and genotyping. Among the 10 cell lines examined, 5 cell lines appeared to be positive for HLA-A24 (HLA-A*2402).

The cytotoxicity of TAK-1 against the lung cancer cell lines is shown in Table 1. TAK-1 exhibited cytotoxicity only against HLA-A24-positive lung cancer cell lines and not against HLA-A24-negative cells. Interestingly, the degrees of TAK-1-mediated cytotoxicity against the lung cancer cell lines reflected their WT1 expression levels. That is, TAK-1 lysed LC99A, in which WT1 is expressed at the highest WT1 level, most efficiently. The LK79, RERF-LC-AI, and 11-18 cell lines, which expressed WT1 at intermediate or low levels, were also efficiently lysed by TAK-1, but the degrees of cytotoxicity against these cell lines were not as great as the degree of cytotoxicity against LC99A. In contrast, PC-9, in which WT1 expression was undetectable by quantitative RT-PCR, was hardly lysed by TAK-1. These results strongly suggest that WT1-specific CTLs can lyse lung cancer cells via recognition of their WT1-derived peptide in the context of HLA-A24.

Inhibition of TAK-1-mediated Cytotoxicity against Lung Cancer Cells by an Anti-HLA Class I mAb. To confirm that the cytotoxicity of TAK-1 against lung cancer cells is restricted by HLA-A24 status, inhibition assays using mAbs were performed. As shown in Fig. 2, the addition of an anti-HLA class I framework mAb, but not a control HLA-DR mAb, to the assay medium inhibited the cytotoxic effect of TAK-1 on HLA-A24-positive lung cancer cells. Taken together with the data shown in Table 1, these findings demonstrate that the cytotoxicity of TAK-1 against lung cancer cells is restricted by HLA-A24.

Cytotoxic Activity of the WT1 Peptide-specific CTL Clone. We previously established a WT1 peptide-specific, HLA-A24-restricted CTL clone, designated TAK-1. Flow cytometric analysis demonstrated that >99% of TAK-1 cells were CD3+, CD4+, CD8+, and CD56+. The TAK-1 clone cells had been stored frozen in liquid nitrogen and were thawed for use in the present study. To confirm that the freezing and thawing procedures had not affected the antigen specificity and HLA restriction of the TAK-1 cells, we first investigated their cytotoxic activity against peptide-loaded and unloaded cells. As shown in Fig. 1, TAK-1 lysed autologous LCLs that had been loaded with the WT1-T2 peptide but was not cytotoxic to unloaded LCLs or to those loaded with WT1-T1, WT1-T3, or WT1-T4. TAK-1 appeared to be cytotoxic only to HLA-A24-positive allogeneic LCLs and the HLA-A*2402 transfectant cell line C1R-A*2402 (but not its parent cell line, C1R) in the presence of WT1-T2 peptide, as demonstrated previously (13).
LC-AI, whereas the addition of WT1-T2-loaded HLA-A24-negative LCLs had no effect on cytotoxicity. These findings strongly suggest that WT1 is naturally processed in lung cancer cells, expressed in the context of HLA-A24, and recognized by WT1-specific CD8\(^+\) CTLs.

Inhibition of Lung Cancer Cell Growth in Nude Mice by TAK-1. Because TAK-1 showed highly WT1-specific cytolytic activity against lung cancer cells in vitro, its therapeutic efficacy was assessed in an experimental lung cancer xenograft model. After engrafting nude mice with a s.c. dose of a human lung cancer cell line, we performed adoptive transfer experiments in which TAK-1 or PBS alone was administered. The resulting lung cancer cell line growth curves are shown in Fig. 4. Adoptive transfer of TAK-1 resulted in significant inhibition of tumor growth. Representative examples of tumor formations in TAK-1- and PBS-treated mice are shown in Fig. 5. The tumors in the mice treated with TAK-1 were significantly smaller than those in control mice (Fig. 5A), and complete tumor regression occurred in one mouse that received TAK-1 (Fig. 5B).
Survival of Mice Engrafted with Human Lung Cancer Cells and Treated with or without TAK-1. Survival curves for nude mice that had been engrafted with a human lung cancer cell line and received adoptive transfer of TAK-1 or control treatment with PBS alone are shown in Fig. 6. A significant difference was observed between the two groups in that all of the mice in the control group died within 113 days, whereas only one mouse in the TAK-1-treated group died within the observation period. Because the growth rate of the TAK-1 cells in the in vitro culture slowed as time progressed and sufficient numbers of TAK-1 cells to continue transfer could not be obtained, the adoptive transfer procedure was discontinued on day 120.

DISCUSSION

In the present study, we demonstrated that WT1-specific CTLs exert a strong cytotoxic effect against human lung cancer cells in a HLA class I-restricted manner. The cytotoxicity of WT1-specific CTLs against lung cancer cells was shown to be mediated by recognition of the WT1-derived peptide in the context of HLA-A24 in several ways. Firstly, the WT1-specific CTL clone TAK-1 lysed HLA-A24-positive but not HLA-A24-negative lung cancer cells, and its cytotoxicity was inhibited by an anti-HLA class I mAb. Secondly, the degree of cytotoxic activity exhibited by TAK-1 against various lung cancer cell lines reflected the WT1 expression level in the particular cell line. Thirdly, cold target inhibition assays demonstrated that the addition of WT1 peptide-loaded autologous cells but not HLA-mismatched allogeneic cells inhibited the cytotoxic effect of TAK-1 against lung cancer cells. Although previous studies by us and other groups have demonstrated that WT1-specific CTLs exert a cytotoxic effect against leukemic cells in a HLA class I-restricted manner (13, 19, 20), the present study is the first to demonstrate that WT1 protein is naturally processed in human lung cancer cell lines, becomes apparent in the context of HLA class I molecules, and is recognized by CD8+ CTLs.

It has previously been shown that WT1 is essential for the formation of the urogenital system during fetal development (21); however, in adults, WT1 expression is extremely limited, occurring in only a few tissues and at a low level. To ensure that WT1-targeting immunotherapy is safe, it is essential to demonstrate that WT1-specific CTLs are not cytotoxic to normal tissues. In this respect, Oka et al. (22) generated WT1-specific CTLs by vaccinating mice with a WT1-derived peptide. The mice remained quite healthy, and histopathological investigations demonstrated no adverse effects on any of the organs examined, including the kidney and bone marrow. These data strongly suggest that WT1 is a tumor-specific antigen and that WT1-targeting immunotherapy for lung cancer can be performed safely.

There are several methods of delivering cell-mediated cancer immunotherapy, including peptide vaccination (23), immunization with DCs that have been pulsed with a peptide or tumor cell lysate (24, 25), immunization with DC/tumor cell hybrids (26), and adoptive transfer of tumor-specific CTLs (27). One of the most important factors governing the success of adoptive transfer is the effectiveness of CTL migration toward the tumor cells. In the present study, we examined the distribution of transferred WT1-specific CTLs in nude mice that had been engrafted with human lung cancer cells and sacrificed 6–12 h after subsequent CTL transfer. Immunohistochemistry demonstrated that only a few human T lymphocytes were detectable in the lung cancer lesions (data not shown). This might have been due to the relatively small number of CTLs transferred. Interactions between species-specific adhesion molecules and chemokine systems may also be important for effective migration of CTLs toward tumor cells. It is therefore possible that, during the present study, the human CTLs could not accumulate to effective levels in the human lung cancer lesions because the environment surrounding the lung cancer cells was not human but murine. Gene therapy targeted against adhesion molecules and chemokines might be able to overcome this problem and increase the efficacy of adoptive immunotherapy using CTLs. On the other hand, our findings also suggest that transfer of a large number of CTLs will not necessarily be needed to exert an antitumor effect in vivo because the CTLs may be actively recruited to tumor lesions. The most effective number of anti-tumor CTLs for transfer to cancer patients will need to be determined in future studies.

In conclusion, we have demonstrated that WT1-specific CTLs can efficiently lyse human lung cancer cells in a HLA class I-restricted manner. We also found that adoptive transfer of WT1-specific CTLs inhibits the growth of human lung cancer cells engrafted into nude mice. To the best of our knowledge, this is the first report to describe the efficacy of WT1-specific CTLs against human solid tumors. The present findings may contribute to the development of novel immunotherapeutic methods for lung cancer and suggest that vaccination with a WT1-derived peptide or with WT1-coding DNA (28) and adoptive immunotherapy using WT1-specific CTLs may provide an effective treatment option for solid tumors as well as leukemia.

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