Induction of γδ T Cells Using Zoledronate Plus Interleukin-2 in Patients with Metastatic Cancer

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ABSTRACT

A loss of human leukocyte antigen (HLA) expression in clinical tumors is one of their escape mechanisms from immune attack by HLA-restricted effector cells. In this study, the induction of HLA-unrestricted effector cells, γδT cells, using zoledronate (ZOL) and interleukin (IL)-2 in vitro was investigated in patients with metastatic cancer. Peripheral blood mononuclear cells (PBMCs) from 10 cancer patients (8 colorectal and 2 esophageal) with multiple metastases and ascites lymphocytes from 3 cancer patients (1 gastric and 2 colorectal) were stimulated with varied concentrations of ZOL plus 100 U/ml IL-2 for 48 hr followed by culturing with IL-2 alone for 12 days. Lymphocyte proliferative responses were determined using 3H-TdR uptakes and interferon (IFN)-γ production was evaluated using enzyme-linked immunosorbent assay. Surface phenotyping was performed using flow cytometry. Cytotoxic activity of effector cells was determined using 51Cr-releasing assay. It was found that proliferative responses of PBMCs were significantly stimulated with ZOL plus IL-2 when compared with IL-2 alone, showing 200 to 500-fold expansions for 2 weeks, although ZOL alone induced no response. The optimal concentration of ZOL was 1-5 µM. Ascites lymphocytes could also be stimulated with ZOL plus IL-2. The proliferative responses were remarkable in patients whose PBMCs could produce high levels of IFN-γ during an initial 48-hr stimulation using ZOL plus IL-2. Removal of an adherent cell fraction before the induction augmented the proliferative responses in patients who otherwise had low-grade proliferative responses. Generated cells comprising approximately 90% or 20% in PBMCs from healthy donors or cancer patients, respectively, expressed γδ-type T-cell receptor. γδT cells showed high cytotoxic activity against CD166-positive TE12 and TE13 cancer cells but not against CD166-negative MKN45 cells. The cytotoxic activity against TE13 cells was augmented when target cells were pre-treated overnight with ZOL. These results suggest that ZOL in the presence of IL-2 can efficiently stimulate the proliferation of γδT cells, which have cytotoxic properties against cancer cells. The use of zoledronate-activated killer (ZAK) cells should be encouraged in possible adoptive immunotherapy trials for patients with incurable cancer.

Key words: γδT cells, Zoledronate, Interleukin-2, Adoptive immunotherapy, Cancer

Anti-tumor T lymphocytes exhibit two types of immune responses: human leukocyte antigen (HLA)-unrestricted or -restricted responses. These responses are representative of innate or adaptive immunity, respectively. After the discovery of tumor antigen peptides and professional antigen presenting cells, dendritic cells (DCs), most current immunotherapeutic approaches have aimed at inducing antitumor response via stimulation of the adaptive immune system. Clinical trials, including vaccine strategies and adoptive immunotherapy protocols, have been conducted using this antigen/DC system for treating patients with antigen-positive metastatic cancer. However, clinical results have shown limited efficacy. These unfavorable results were explained by the escape mechanisms of tumors from immune attack by HLA-restricted effector cells, one of which is a loss of HLA expression in clinical tumors. Tumor cells have been shown to lose HLA expression on
their surface in parallel with disease progression, resulting in their escaping from immune recognition\(^6,27\). Hence, it is important to investigate the induction of HLA-unrestricted immune effector cells in order to overcome this type of escape mechanism of the tumor.

We have been engaged in developing adoptive immunotherapy of cancer using novel autologous activated lymphocytes *in vitro*\(^{27}\). T cells expressing the V\(_\gamma\)9V\(\delta\)2 T cell receptor (TCR) were identified as \(\gamma\delta\) T cells about two decades ago. These cells play an important role in the innate immunity of immune surveillance and defense, and exhibit potent HLA-unrestricted lytic activity against different tumor cells *in vitro*\(^6,8,11\), suggesting their potential utility in anticancer therapy. Recently, it has been reported that pyrophosphomonoesters can easily and exponentially expand \(\gamma\delta\) T cells\(^{29}\). More recently, synthetic nitrogen-containing bis-phosphonate compounds have also been shown to be capable of activating the same subset of human T cells *in vitro* and *in vivo*\(^{7,10,28}\). Bis-phosphonates have already been used widely to treat bone diseases and hypercalcemia in clinics, so the use of bis-phosphonates may facilitate adoptive immunotherapy of cancer using \(\gamma\delta\) T cells. Although clinical results of the *in vivo* administration of ZOL plus IL-2 have been published\(^7,8\), adoptive immunotherapy protocols using \(\gamma\delta\) T cells have not been well-established, so far\(^{17,18}\). In the present study, we attempted to induce \(\gamma\delta\) T cells *in vitro* in patients with metastatic cancer using the most potent currently used third generation bis-phosphonate, zoledronate (ZOL)\(^{26}\), for developing a novel adoptive immunotherapy protocol.

**MATERIALS AND METHODS**

**Collection of lymphocytes.** Heparinized venous blood was obtained from 10 patients (8 colorectal and 2 esophageal) and healthy volunteers, and theuffy coat was immediately separated by centrifugation (2,000 rpm, 30 min). Citrated ascites from 3 cancer patients (1 gastric and 2 colorectal) was collected by paracentesis and ascites cells were pelleted. Theuffy coat and ascites cells were then resuspended in RPMI-1640 medium and layered on a Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient. Peripheral blood mononuclear cells (PBMCs) and ascites lymphocytes were isolated by gradient centrifugation (2,000 rpm, 30 min), washed twice, and resuspended in the medium containing 2% autologous serum at a density of \(1 \times 10^6 / \text{ml}\). Numbers of lymphocytes were counted using trypan blue exclusion assay.

**Lymphocyte activation using zoledronate plus IL-2.** PBMCs and ascites lymphocytes were stimulated with 0-10 \(\mu\)M ZOL (Novartis, Japan) in the presence or absence of 100 U/ml interleukin (IL)-2 (Sionogi Pharmaceutical Co. Ltd., Osaka, Japan) at 37\(^{\circ}\)C for 2 days. Cells were washed, resuspended in the medium containing 100 U/ml IL-2, and further cultivated for 12 days. In some experiments, the isolated PBMCs were incubated in a medium containing 2% autologous serum alone at 37 oC for 4hr, and then adherent cells were removed. Non-adherent cells were stimulated with ZOL plus IL-2 exactly as mentioned above.

**Proliferation assay.** Lymphocyte proliferative responses were determined using \(^3\)H-TdR uptake assay. Briefly, PBMCs were stimulated with ZOL plus IL-2 in the medium supplemented with 2% autologous serum. On day 4, half of the cells were put in 96-well flat-bottomed microtiter plates in triplicate, pulsed with \(^3\)H-TdR, and further incubated for 8 hr. Cells were harvested, and their radioactivity was determined by liquid scintillation counter (Beckmann, USA). Fresh medium containing autologous serum and IL-2 was added to the remaining half of the cells, and they were further cultured for a total of 7 days. \(^3\)H-TdR uptake was determined as mentioned above.

**Interferon-\(\gamma\) assay.** The function of activated cells was examined using interferon (IFN)-\(\gamma\) assay. Briefly, PBMCs (1 \(\times 10^6 / \text{ml}\)) were stimulated with ZOL plus IL-2 for 4 or 7 days, and the supernatants were collected. The culture supernatants were subjected to enzyme-linked immunosorbent assay (ELISA) specific for IFN-\(\gamma\) (R & D Systems, USA). The assay was performed in triplicate according to the manufacturer’s instructions.

**Flow cytometric analysis.** Fifty \(\mu\)l of the PBMC suspension (5 \(\times 10^6\)) was stained with 10 \(\mu\)g/ml monoclonal antibodies at 4 \(\circ\)C for 30 min. The monoclonal antibodies used were Per-CP-labeled anti-CD3, fluorescence isothiocyanate (FITC)-labeled anti-\(\gamma\delta\) TCR, and phycoerythrin (PE)-labeled anti-CD56 antibodies (Becton Dickinson, Mountain View, CA). The cells were washed, and flow cytometric analysis was performed on FACSCalibur (Becton Dickinson, Mountain View, CA). Data acquisition was stopped at 100,000 events, and positive cell populations were analyzed by the attached program after being gated on CD3\(^+\) T cells\(^{30}\).

**Cytotoxicity assay.** The cytotoxic activity of activated lymphocytes was determined by a standard \(^{51}\)Cr-releasing assay. The target cells used were the gastric cancer cell line MKN45 (the American Type Culture Collection), and the esophageal cancer cell lines TE12 and TE13\(^{14}\). Target cells were labeled with \(^{51}\)Cr for 2 hr. In some experiments, target cells were pretreated for 12 hr with
1 µM ZOL prior to $^{51}$Cr labeling. Target cells were admixed with effector lymphocytes in 96-well round-bottomed microtiter plates in triplicate at effector-to-target (E/T) ratios of 10 and 20 in a volume of 200 µl. After overnight incubation, the radioactivity of the supernatants was measured using an auto-gamma scintillation counter (Packard, USA). Spontaneous release was determined in wells containing the target cells alone, and maximum release was obtained by adding 100 µl of 1% Triton X-100 solution to the target cells instead of the effector cells. Cytotoxic activity was calculated from triplicate samples by the following formula: cytotoxic activity (percent) = (experimental release [cpm] - spontaneous release [cpm])/(maximal release [cpm] - spontaneous release [cpm]) × 100.

**Statistics.** Results are expressed as the mean ± standard deviation (SD). Statistical analysis was conducted by un-paired Student's t-test using StatView software (Version 5) on a Macintosh computer. A value of p<0.05 was considered statistically significant.

**RESULTS**

**Mitogenic activity of ZOL in the presence of IL-2.** Mitogenic activity of ZOL was examined by measuring $^3$H-TdR uptake of PBMCs (Fig. 1). ZOL alone (3 µM) did not show any stimulating activity of PBMCs. IL-2 alone showed a 2- to 3-fold higher stimulating activity of PBMC growth over 4 days. On the other hand, ZOL plus IL-2 augmented the proliferative activity of PBMCs 5-fold or more when compared with PBMCs alone. This augmentation was obvious when cultured over 7 days, showing a significant difference from IL-2 alone (p<0.01). Another 2 independent experiments showed similar results.

**Optimal concentration of zoledronate.** The optimal concentration of ZOL was examined. PBMCs were stimulated with varied concentrations of ZOL in the presence of IL-2, and IFN-γ production was measured on day 7 (Fig. 2). One and five µM zoledronate were most effective for stimulating PBMCs in experiment 1 and 2, respectively, showing significant differences from IL-2 alone (p<0.01). Higher concentrations of ZOL, however, seemed rather less stimulatory for the IFN-γ production of PBMCs. Another 3 independent experiments showed similar results.

**Expansion of ZOL-stimulated PBMCs and ascites lymphocytes in the presence of IL-2.** The expansion of PBMCs and ascites lymphocytes stimulated with ZOL in the presence of IL-2 was examined (Fig. 3a, b). ZOL-stimulated PBMCs showed a highly proliferative response in the presence of IL-2, and approximately $10^7$ cells could be generated from 10 ml blood (approximately $10^7$ cells), indicating 200- to 500-fold growth of PBMCs over a 14-day culture. ZOL-stimulated ascites lymphocytes also showed similar proliferative responses compared with PBMCs. IFN-γ levels in the supernatant of the PBMC culture on day 2 were predictive for the cell number yielded on day 14 (Table 1).

![Fig. 1. Mitogenic activity of zoledronate in the presence of IL-2.](image)

![Fig. 2. Optimal concentration of zoledronate for stimulating PBMCs.](image)
**Removal of adherent cells for ZOL-induced activation of PBMCs.** The effect of removing an adherent cell fraction from whole PBMCs was examined (Fig. 4). Removal of adherent cells significantly augmented IFN-γ production of PBMCs stimulated with ZOL plus IL-2 in 3 of 5 patients who had low responses of IFN-γ production of less than 3000 pg/ml over a 2-day stimulation with zoledronate plus IL-2. This augmentation by the removal of adherent cells was not apparent in PBMCs from high responders, who could produce more than 7000 pg/ml IFN-γ during the stimulation.

**γδ-type T cell receptor expression on ZOL-activated PBMCs.** T cell receptor (TCR) expression in PBMCs activated with ZOL plus IL-2 was examined using flow cytometry (Fig. 5, Table 2). γδ TCR-expressing cells were less than 5% in healthy donors and cancer patients before stimulation. However, approximately 90% or more of ZOL-activated cells from healthy donors expressed γδ TCR after stimulation with ZOL plus IL-2. Half or more of the cells in healthy donors co-expressed CD56 molecules. ZOL-activated cells from cancer patients expressed γδ TCR to a varied extent; two showed 80% < γδ TCR expression with co-expression of 50% > CD56 expression, and the other showed 25% > γδ TCR. This variation seemed to correlate with the proliferative responses and IFN-γ production of ZOL-activated PBMCs (data not shown).

**Table 1.** Relationship between IFN-γ production on day 2 and cell yield on day 14 of PBMCs stimulated with ZOL plus IL-2

<table>
<thead>
<tr>
<th>Patient</th>
<th>IFN-γ on day 2 (pg/ml)</th>
<th>cells yielded on day 14 (x 10⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>396</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>1015</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>6774</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>8392</td>
<td>4.2</td>
</tr>
</tbody>
</table>

PBMCs from cancer patients were stimulated with 1 µM ZOL plus 100 U/ml IL-2. Culture supernatant was collected on day 2, and IFN-γ levels were determined. Cells were further cultured and harvested on day 14.

**Table 2.** Expression of γδ TCR on PBMCs stimulated with ZOL plus IL-2

<table>
<thead>
<tr>
<th>subjects</th>
<th>γδ TCR+ cell population (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donor</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.2</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>Cancer patient</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
</tr>
<tr>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>4.4</td>
</tr>
<tr>
<td>5</td>
<td>2.9</td>
</tr>
<tr>
<td>6</td>
<td>4.2</td>
</tr>
</tbody>
</table>

PBMCs from healthy donors and cancer patients were stimulated with 1 µM ZOL plus 100 U/ml IL-2 for 14 days. Cells were stained with labeled anti-γδ TCR antibody prior to and after the stimulation. Flow cytometry was performed on FACSCalibur.

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**Fig. 3.** Growth of PBMCs and ascites lymphocytes stimulated with zoledronate plus IL-2. PBMCs (a) and ascites lymphocytes (b) from cancer patients were stimulated with 1 µM ZOL plus 100 U/ml IL-2. Cell numbers were counted on the days indicated.

**Fig. 4.** Effect of removing an adherent cell fraction on zoledronate-induced activation of PBMCs from cancer patients. Whole or adherent cell fraction-removed PBMCs were stimulated with 1 µM ZOL plus 100 U/ml IL-2. IFN-γ levels in the supernatant were determined on day 2. Experiments were performed in triplicate. Significant difference, *p<0.05.
Cytotoxic activity of ZOL-activated PBMCs. The cytotoxic activity of ZOL-activated PBMCs was examined against cultured cancer cells (Table 3). ZOL-activated PBMCs showed lower levels of cytotoxic activity of 10.0% and 4.4% against the gastric cancer cell line MKN45 in the 2 patients examined. However, ZOL-activated PBMCs exerted higher levels of cytotoxic activity of 29.8% and 22.2% against esophageal cancer cell lines TE12 and TE13, respectively. These activities were significantly augmented to 41.6% (p<0.05) and 71.3% (p<0.01), respectively, when target cells were pre-treated overnight with ZOL. This augmentation was not found in the MKN45 cancer cell line. Another 3 independent experiments showed similar results.

CD166 expression of cancer cell lines. CD166 expression of cancer cell lines was examined and compared with the cytotoxic activity of ZOL-activated PBMCs (Fig. 6). CD166 expression was not detected in MKN45 cancer cells, which were resistant to the killing of ZOL-activated PBMCs. On the other hand, CD166 expression was obvious in TE12 or TE13 cancer cell lines, which were highly susceptible to ZOL-activated PBMCs.

**Fig. 5.** Surface expression of γδ TCR and CD56 on PBMCs stimulated with ZOL plus IL-2. PBMCs from healthy donors and cancer patients were stimulated with 1 µM ZOL plus 100 U/ml IL-2 for 14 days. Cells were stained with labeled anti-CD3, -γδ TCR, and -CD56 antibodies, and flow cytometry was performed on FACSCalibur.

**Fig. 6.** CD166 expression on tumor cell lines. Tumor cells were stained with labeled anti-CD166 antibody, and flow cytometry was performed on FACSCalibur.

**Table 3. Cytotoxic activity of PBMCs stimulated with ZOL plus IL-2**

<table>
<thead>
<tr>
<th>patient</th>
<th>Target cells</th>
<th>Treatment</th>
<th>cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MKN45</td>
<td>none</td>
<td>10.0 +/- 2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ ZOL</td>
<td>9.8 +/- 2.9</td>
</tr>
<tr>
<td></td>
<td>TE12</td>
<td>none</td>
<td>29.8 +/- 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ ZOL</td>
<td>41.6 +/- 4.2*</td>
</tr>
<tr>
<td>2</td>
<td>MKN45</td>
<td>none</td>
<td>4.4 +/- 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ ZOL</td>
<td>6.6 +/- 0.8</td>
</tr>
<tr>
<td></td>
<td>TE13</td>
<td>none</td>
<td>22.2 +/- 2.2**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ ZOL</td>
<td>71.3 +/- 1.7**</td>
</tr>
</tbody>
</table>

PBMCs from cancer patients were stimulated with 1 µM ZOL plus 100 U/ml IL-2 for 14 days. Cytotoxic activity against ZOL-treated or -untreated cancer cells was determined using 51 Cr-releasing assay. Experiments were performed in triplicate and repeated in independent 5 patients. Two representative data were shown. E/T=20. Significant differences, *p<0.05, **p<0.01.
DISCUSSION

The present study demonstrated that the currently most potent third generation bis-phosphonate ZOL plus IL-2 could stimulate a 200 to 500-fold proliferation of PBMCs and ascites lymphocytes from cancer patients in a 2-week cultivation, and that ZOL-stimulated PBMCs expressed IFN-γ-producing potential, γδ TCR and CD56 to a varied extent, and cytotoxic activity against cancer cell lines. The optimal concentration of ZOL was 1-5 µM in this study. It has been shown that nitrogen-containing bis-phosphonate is internalized by cells and inhibits farnesyl pyrophosphate (FPP) synthase, resulting in intracellular accumulation of isopentenyl pyrophosphate (IPP) upstream of FPP in the mevalonate pathway, and that accumulated IPP may act as a powerful non-peptide antigen to activate the immune response. Activated T cells form stable conjugates with tumor cells pulsed with bis-phosphonates. Sato et al. have reported that 1 µM ZOL plus IL-2 can stimulate γδ T cell expansion of 298-fold to 768-fold over a 14-day incubation, and that the small cell lung cancer and fibrosarcoma cell lines pretreated with 5 µM ZOL showed a marked increase in sensitivity to lysis by γδ T cells, consistent with our results. These results suggest that ZOL plus IL-2 can generate potent effector cells expressing γδ TCR with ease. Therefore, these effector cells can be introduced into adoptive immunotherapy trials for patients with incurable cancer. We would propose to call these effector cells “zoledronate-activated killer (ZAK) cells.”

Surprisingly, we observed in this study that the removal of adherent cells was effective for stimulating PBMCs from cancer patients with ZOL plus IL-2, especially from patients in whom only low IFN-γ production could be induced with ZOL plus IL-2. Miyagawa et al. have reported in healthy donor experiments that the activation of γδ T cells by bis-phosphonate in vitro essentially requires the presence of accessory cells such as macrophages, and that human γδ T cells are functionally activated via γδ TCR by bis-phosphonate Ag presented on the surface of monocyte lineage cells rather than directly by its free form. Where does this difference in the requirement of adherent cells for γδ T cell induction come from? It has been reported in cancer patients that immune responses are suppressed by adherent cell fraction, and that suppressor macrophages exist and suppress an antitumor lymphocyte response by producing nitric oxide. Saitoh et al. have found the optimal ratio between nylon wool-passed T lymphocytes and nylon wool-adherent accessory cells to induce cytotoxic T cells in the patient’s PBMCs to be 25 to 1. Taken together, these results suggest that the adherent cell fraction of PBMCs from cancer patients may have 2 types of cells: one type is responsible for antigen presentation, and the other is responsible for the suppression of lymphocyte activation. Therefore, the removal of the adherent cell fraction is rather effective for augmenting ZAK cell induction in some cancer patients in whom the adherent fraction may have a preferential suppressive function to produce only low-level IFN-γ with ZOL plus IL-2. The suppressive activity of adherent cells remained to be explored.

Flow cytometric analysis revealed heterogenous phenotypes of ZAK cells in this study. γδ TCR and CD56 expressions in ZAK cells varied among the patients examined. Alexander et al. demonstrated that 30 to 70% of expanded γδ T cells express CD56 on their surface, and that although both CD56+ and CD56- γδ T cells express comparable levels of γδ TCRs, only CD56+ γδ T cells are capable of killing squamous cell carcinoma and other solid tumor cell lines, indicating the importance of CD56 expression for the killing activity of γδ T cells. The CD56 molecule has been demonstrated to be expressed on natural killer (NK) cells, the expression of which on γδ T cells may indicate the existence of γδ TCR-expressing NKT cells, although NKT cells usually express invariant or non-invariant γδ TCRs. Moreover, it has been reported that effector molecules, such as IFN-γ, tumor necrosis factor (TNF)-α, perforin, granzyme B, FasL and TNF-related apoptosis-inducing ligand (TRAIL) are expressed in ZOL-stimulated γδ T cells. Further investigations are required to elucidate the regulation mechanisms of cytotoxic activity, effector molecules and CD56 expressions of γδ T cells, and the differences from NKT cells.

Interestingly, CD166 expression in target cells was correlated with the cytotoxic activity of ZAK cells in this study. It has been demonstrated that the expression of CD166 closely parallels the capacity to activate γδ T cells upon bis-phosphonate treatment, and that the engagement of CD6, a receptor of CD166, in γδ T cells with CD166 in tumor cells plays an important role in the activation of and tumor recognition by γδ T cells. It is suggested that we may predict the clinical responses of adoptive immunotherapy using ZAK cells by evaluating the CD166 expression in tumor cells, leading to the possibility of personalized medicine.

In conclusion, we confirmed and demonstrated the possible induction of “zoledronate-activated killer (ZAK) cells” in vitro. ZAK cells show extraordinary growth with easy manipulation of culture and have potent cytotoxic activity against cancer cells. The use of ZAK cells should be encouraged in novel adoptive immunotherapy trials for patients with incurable cancer.
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