Requirement of Species-Specific Interactions for the Activation of Human γδ T Cells by Pamidronate

Yu Kato,* Yoshimasa Tanaka,*† Hidenori Tanaka,† Seiji Yamashita,* and Nagahiro Minato**†

Human γδ T cells bearing Vγ2Vδ2-TCR recognize various kinds of small nonpeptide Ags, and activation of them by a nitrogen-containing bisphosphonate Ag, pamidronate, requires Ag presentation by cells other than γδ T cells, including many human tumor cells. Present results demonstrated that tumor cell lines of nonhuman origins pulsed with pamidronate failed to activate human γδ T cells without exception, whereas most if not all human tumor cell lines could do so. γδ T cells formed stable conjugates with pamidronate-pulsed human tumor cells and both conjugate formation and γδ T cell activation were inhibited significantly by anti-LFA-1 mAb, suggesting the requirement of LFA-1-mediated interaction with APC for efficient γδ T cell activation. Consistently, ICAM-1low tumor cell lines pulsed with pamidronate induced no or only weak activation of γδ T cells, whereas similarly treated ICAM-1high cell lines could activate them. One of the two ICAM-1low tumor cell lines pulsed with pamidronate induced strong γδ T cell activation after ICAM-1 gene transfer. However, another ICAM-1low human cell line as well as murine tumor cell lines pulsed with pamidronate remained totally defective in γδ T cell activation even after expression of human ICAM-1. These results suggested that activation of human γδ T cells by nonpeptide Ags required species-specific interactions in addition to LFA-1/ICAM-1-mediated cell adhesion with APC. The Journal of Immunology, 2003, 170: 3608–3613.

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uman γδ T cells bearing Vγ2Vδ2-γδ TCR, a predominant γδ T cell subset found in adult peripheral blood, specifically recognize various kinds of nonpeptide Ags (reviewed in Ref. 1), such as bacterial pyrophosphonomonolactones (2–5) and alkyl amines (6) as well as synthetic nitrogen-containing bisphosphonates (7–9) in the Vγ2Vδ2-γδ TCR-dependent manner (2–11). However, it remains unknown how the γδ TCR recognizes such nonpeptide Ags of small molecular masses. We previously reported that the recognition was dependent on the lysine residues encoded by a germline Jγ1.2 gene segment proximal to the junc-tional region (11) located in a putative Ag-binding pocket (12). These lysine residues are not conserved in other human nor murine γγ segments, which may in part explain why recognition of these nonpeptide Ags is a unique feature of human Vγ2γδ1Vδ2-γδ T cells. Among nonpeptide Ags, nitrogen-containing bisphosphonates such as pamidronate were rather unique, in that activation of the primary γδ T cells required the presence of monocytes (9). Furthermore, many if not all human tumor cell lines prepulsed with pamidronate could efficiently activate human γδ T cells, whereas no TCR-dependent recognition was observed in mock-treated tumor cells (13). These results strongly suggested that pamidronate had to be “presented” by selected cells other than γδ T cells per se for effective γδ T cell activation.

In the present study, we show that the optimal activation of γδ T cells by pamidronate-pulsed human tumor cells was dependent on the stable cell adhesion mediated by LFA-1 expressed on γδ T cells and ICAM-1 on the Ag-pulsed tumor cells. Although human tumor cell lines with high ICAM-1 expression (ICAM-1high) could efficiently present pamidronate to γδ T cells, a minor population with little or no expression of ICAMs (ICAM-1low) as well as any of the tumor cell lines of nonhuman origins pulsed with pamidronate failed to activate human γδ T cells. Human ICAM-1 expression by cDNA transfer could confer the strong γδ T cell-activating capacity in one ICAM-1low human tumor cell line. However, no such induction was observed in another ICAM-1low human tumor cell as well as mouse tumor cell lines, suggesting the requirement of additional species-specific interaction(s) with Ag-presenting tumor cells for the effective activation of γδ T cells by a nonpeptide Ag, pamidronate. These results argue for the presence of a specific mechanism for nonpeptide Ag presentation to human γδ T cells, complementing that for αβ T cell activation by peptide Ags.

Materials and Methods

Nonpeptide Ags

Monoethyl pyrophosphate was synthesized as described previously (4) and pamidronate was purchased from Novartis Pharma (Nurnberg, Germany).

Human γδ T Cells

PBMC were separated from healthy volunteers’ blood using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and stimulated with 0.2 mM monoethyl pyrophosphate in Yssel’s medium supplemented with 2.5% human serum albumin for 2 days. The cells were further cultured in Yssel’s medium containing 10% human AB sera and IL-2 (100 U/ml) for 14 days. The proportions of Vγ2Vδ2-bearing γδ T cells in such populations were consistently 95% or more in CD3-positive cells and were used as polyclonal γδ T cell lines in the present study.

Tumor cell lines and pulsing with nonpeptide Ag

Tumor cell lines of human and nonhuman origins were supplied from Human Science Research Resource Bank (Osaka, Japan) and cultured according to the original instructions. To pulse the tumor cells with pamidronate, 1.0 × 10^5 tumor cells were incubated with 0.1 mM pamidronate for 2 h at 37°C and washed extensively with RPMI 1640 containing 10% FCS before use.

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Flow cytometry and mAbs

Single- and two-color flow cytometric analyses were conducted using FACScan (BD Biosciences, Mountain View, CA) as described previously (13). mAbs used in the present study were anti-TCR-β (Immunotech, Marseille, France), anti-IFN-γ (BD Pharmingen, San Diego, CA), anti-ICAM-1, -2, and -3 (BD Pharmingen), anti-LFA-1 (TSI/22), and anti-LFA-1 specific for an activated form (NKL-I16), kindly provided by Dr. T. Kinashi (Kyoto University, Kyoto, Japan).

Proliferation assay

Human γδ T cells were cocultured at 1.0 × 10^5/well with varying numbers (1.2 × 10^5–2.0 × 10^5) of mitomycin C (100 μg/ml)-treated tumor cells at 37°C for 36 h and pulsed with [3H]thymidine (2 μCi) for 12 h, and radioactivity incorporated in DNA was counted by a MicroBeta scintillation counter (PerkinElmer Life Sciences, Norwalk, CT).

Cytotoxicity assay

Tumor cells (1.0 × 10^5) pretreated with 0.1 mM pamidronate were labeled with 100 μCi of 51Cr sodium chromate for 1 h at 37°C. Varying numbers of human γδ T cells (0.625 × 10^5–4.0 × 10^5) were cocultured with 1.0 × 10^6 tumor cells for 5 h, and 51Cr release was counted by a gamma counter (PerkinElmer Life Sciences).

Intracellular IFN-γ staining

Human γδ T cells (5.0 × 10^5/well) were cocultured with pamidronate-pulsed tumor cells (5.0 × 10^5/well) at 37°C. After 2 h, brefeldin A (10 μg/ml) (Sigma-Aldrich, St. Louis, MO) was added in the culture and the cells were incubated for another 5 h. The cells were then washed and stained with FITC-conjugated anti-TCR-Vδ2 mAb on ice for 20 min. The cells were washed twice, fixed with 1% paraformaldehyde, permeabilized with 0.5% saponin, and stained with PE-conjugated anti-IFN-γ mAb for 20 min. After washing three times, the cells were analyzed using FACScan (BD Biosciences).

Conjugation assay

Tumor cells were stained with PKH26 (Molecular Probes, Eugene, OR) at room temperature for 5 min and human γδ T cells were stained with CFSE (Molecular Probes) at 37°C for 15 min. After washing, the labeled γδ T cells and tumor cells were incubated for varying periods and were two-color analyzed after gentle vortexing using FACScan.

Human ICAM-1 cDNA transfection

A full-length human (h)1 ICAM-1 cDNA was kindly provided by Dr. O. Takashi (Daichi Pharmaceutical, Tokyo, Japan). Tumor cell lines were transfected with hICAM-1 cDNA constructed in a pEF-BOS expression vector, kindly provided by Dr. S. Nagata (Osaka University, Suita, Osaka, Japan), by electroporation using ECM830 (BTX). The cells were cultured in the medium containing 1 mg/ml G418 (NacalaiTesque, Osaka, Japan) and screened for the expression using anti-ICAM-1 mAb to establish stable clones highly expressing hICAM-1.

Results

Pamidronate-pulsed tumor cells derived from nonhuman species fail to activate human γδ T cells

Most human tumor cell lines from various tissues pulsed with pamidronate for 2 h activated human γδ T cells in terms of IFN-γ production, proliferation, and specific cytotoxicity to varying degrees, while a minor population failed to do so, with representative data being shown in Table I. Without Ag pulsing, none of them could induce IFN-γ production or specific proliferation. In contrast, tumor cell lines from nonhuman species similarly pulsed with pamidronate at any doses totally failed to activate human γδ T cells (Table I). As summarized in Table II, none of the 33 tumor cell lines pulsed with pamidronate from 12 different species other than human could activate human γδ T cells, whereas a significant level of IFN-γ was observed in human γδ T cells in response to

Table II. Summary of the human Vγ2Vδ2 γδ T cell activation by pamidronate-pulsed tumor cell lines originated from human and other animal species

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of Tumor Cell Lines Examined</th>
<th>No. of Tumor Cell Lines Capable of Activating γδ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Mouse</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Rat</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Hamster</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Monkey</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Human tumor cell lines included two bladder cancer lines, two lymphoma lines, four myelomonocytic leukemia lines, two renal cancer lines, two lung cancer lines, two melanoma lines, six osteosarcoma lines, three breast cancer lines, and three stomach cancer lines. Mouse tumor cell lines tested were two mammary cancer lines, four myelomonocytic leukemia lines, three fibrosarcoma lines, a melanoma line, and a teratocarcinoma line. Rat tumor cell lines included a melanoma line, a lung cancer line, and a kidney cancer line. Hamster tumor cell lines were two lung cancer lines, an ovary cancer line, a melanoma line, and a kidney cancer line. Monkey tumor cell lines consisted of four kidney cancer lines and others included kidney cancer lines derived from pig, dog, cat, rabbit, bovine, marsupial, chicken, and Indian muntjac.
21 of 26 human tumor cell lines pulsed with pamidronate. An effective Ag-pulsing effect was detected by the incubation with pamidronate for as little as 2 h. After the pulsing with 0.1 mM pamidronate for 2 h, no significant toxic effect was observed as judged by propidium iodide staining in both human and nonhuman tumor cell lines (data not shown), excluding a possibility that the different "pulsing effect" reflected differential sensitivity of each cell line to the toxic metabolic effects by pamidronate. The results strongly suggested that effective presentation of pamidronate to human γδ T cells by tumor cells required species-specific cellular interactions.

**Requirement of LFA-1/ICAM-1-mediated adhesive interaction for effective activation of human γδ T cells by pamidronate-pulsed human tumor cells**

We previously reported that pamidronate specifically induced the clustering between human γδ T cells and accessory monocytes, suggesting the requirement of direct cellular adhesion for effective γδ T cell activation (9). As indicated in Fig. 1A, both primary and activated human γδ T cells highly expressed LFA-1 in an active form. On the other hand, human tumor cell lines, which could efficiently present pamidronate to γδ T cells, expressed ICAM-1, with representative examples being indicated in Fig. 1B. In contrast, a minor population of human tumor cell lines with no or only weak capacity to activate γδ T cells exhibited only marginal LFA-1 ligand expression including ICAM-1, -2, and -3 (Fig. 1B). Although not shown, ICAM expression levels on tumor cells were not affected by pamidronate-pulsing. γδ T cells and ICAM-1 high EJ-1 tumor cells either mock treated or pulsed with pamidronate formed significant cellular conjugates by 30 min after the mixture (Fig. 2A). The conjugate formation with untreated EJ-1 cells was only transient and dissolved nearly to the background level in 2 h. On the other hand, the cellular conjugates between γδ T cells and pamidronate-pulsed EJ-1 cells lasted stably at least for 2 h (Fig. 2A). Inclusion of a blocking anti-LFA-1 mAb in the cocultures of γδ T cells and pamidronate-pulsed EJ-1 cells significantly inhibited the conjugate formation and subsequent IFN-γ production as well (Fig. 2B). These results strongly suggested that LFA-1/ICAM-1-mediated adhesive interaction with pamidronate-pulsed human tumor cells was one of the requirements for effective activation of γδ T cells.

**LFA-1/ICAM-mediated adhesive interaction is necessary but not sufficient for γδ T cell activation by pamidronate-pulsed human tumor cells**

These results raised a possibility that failure of γδ T cell activation by a minor population of human tumor cell lines pulsed with pamidronate was due to the insufficient expression of ICAM-1 on the cell surface. To examine this, we transfected a human ICAM-1 cDNA into ICAM-1low LK-2 and MKN45 cells, which induced only weak or no detectable activation in γδ T cells (see Fig. 1B and Table I), and established ICAM-1high clones (Fig. 3A). A pamidronate-pulsed ICAM-1high LK-2 clone induced a much stronger activation of γδ T cells than wild-type cells in terms of both IFN-γ
production and specific proliferation (Fig. 3A, b–d). The results confirmed that inefficient activation of γδ T cells by pamidronate-pulsed LK-2 tumor cells was primarily due to the low expression of ICAM-1. In quite a contrast, an ICAM-1 high MKN45 clone pulsed with pamidronate remained defective in activating γδ T cells (Fig. 3B). Essentially the same results were obtained by using other independent ICAM-1 high MKN45 clones. Thus, it was suggested that LFA-1/ICAM-mediated interaction was required but not sufficient for the effective activation of human γδ T cells by tumor cells pulsed with pamidronate.

Expression of human ICAM-1 does not confer the capacity of activating human γδ T cells to murine tumor cell lines

We finally examined the effect of human ICAM-1 expression on murine tumor cell lines. A mouse B16 melanoma clone transfected with human ICAM-1 cDNA (hiCAM-1 B16) expressed a high level of human ICAM-1 comparable to that of EJ-1 human tumor cells (Fig. 4A). However, hiCAM-1 high B16 cells pulsed with pamidronate failed to activate human γδ T cells with respect to either IFN-γ production or specific proliferation (Fig. 4, B and C). In addition, human ICAM-1 high B16 cells pulsed with pamidronate remained totally resistant to cytotoxicity elicited by human γδ T cells (data not shown). Identical results were obtained using hiCAM-1 high P3U1 mouse myeloma clones (data not shown). These results strongly suggested that the lack of human γδ T cell activation by pamidronate-pulsed tumor cells of nonhuman origins was not only due to the defective LFA-1/ICAM-1-mediated interaction with human γδ T cells, but also to the absence of additional human-specific element(s) for the effective activation.

Discussion

Human γδ T cells bearing Vγ2Jγ1.2Vδ2-TCR recognize several kinds of small nonpeptide Ags with distinct chemical features (1, 10), but the exact mode of Ag recognition remains unknown. Previous reports indicated that homologous aggregation among γδ T cells was required for their activation by some of the nonpeptide Ags (14). More recently, it was reported also that human γδ T cells formed stable conjugation with the susceptible tumor target cells with synaptic molecular transfer resembling immunological synapse between αβ T cells and specific APC (15). Although these results implied that cell-associated presentation of nonpeptide Ags might be required for γδ T cell activation, detailed analysis was hampered by the fact that most of the nonpeptide Ags could efficiently activate γδ T cells even in the absence of other specific
cells (14). In this aspect, we indicated that the primary human γδ T cells failed to respond to soluble pamidronate, but recognized the compound in the presence of accessory monocytes (9). In addition, we also showed that many human tumor cells pulsed with pamidronate could efficiently activate γδ T cells (13). Such a requirement of Ag presentation was a common feature of all of the antigenic nitrogen-containing bisphosphonate molecules (S. Kita, Y. Tanaka, and N. Minato, unpublished observation), which might provide an ideal system for detailed analysis of γδ T cell activation by nonpeptide Ags.

The present results indicated that the majority of pamidronate-pulsed human tumor cell lines of varying tissue origins (21 of 26 lines) activated γδ T cells, whereas none of the tumor cell lines of nonhuman origins could activate human γδ T cells (0 of 33 lines). After the pulsing procedure, no significant toxicity was detected in all of the tumor cell lines of both human and nonhuman origins as judged by propidium iodide staining. Thus, it was unlikely that γδ T cells were activated simply by pamidronate nonspecifically bound to the target tumor cells. Incidentally, the present results also suggested that most human Vγ2Vδ2 γδ T cells could not recognize xenogeneic MHC Ags, while human αβ T cells were shown to recognize xenogeneic MHC Ags directly and exhibit comparable levels of responses to allogeneic cells (16).

Human γδ T cells highly expressed LFA-1 molecules on their cell surface, and ICAM-1 was detected on most of the human tumor cell lines that could present pamidronate. Then, γδ T cells formed tight conjugates with the pamidronate-pulsed tumor cells. Inclusion of a blocking anti-LFA-1 mAb in the cocultures significantly inhibited the conjugate formation, resulting in the inhibition of IFN-γ production by γδ T cells. The results suggested that LFA-1/ICAM-1-mediated adhesive interaction was required for effective γδ T cell activation. Consistently, ICAM-1low LK-2 lung tumor cell line gained a strong capacity of activating γδ T cells by ICAM-1 cDNA transfection upon pretreatment with pamidronate. Although cell clusters between γδ T cells and mock-treated tumor cells were only transient, γδ T cells formed stable conjugates with pamidronate-pulsed tumor cells, lasting for at least 2 h. In αβ T cells, it is indicated that LFA-1 is activated and reorganized to form stable immunological synapse with APC via “inside-out” signaling mediated by TCR (17–20). In analogy, we speculate that the γδ TCR signaling via cell-associated pamidronate may induce reorganization of LFA-1 to form stable synapse with the Ag-presenting tumor cells.

The present results further indicated that the integrin-mediated interaction was not sufficient for pamidronate-pulsed tumor cells to activate human γδ T cells. Thus, a high level of ICAM-1 expression by cDNA transfection failed to confer the capacity of γδ T cell activation to the ICAM-1low MKN45 human tumor cell line. Furthermore, expression of human ICAM-1 in murine tumor cell lines could not induce the capacity to activate human γδ T cells at all. These results strongly suggested that additional specific interaction other than integrin-mediated adhesion was required for γδ T cell activation by pamidronate-pulsed tumor cells.

Although the exact nature of interaction between γδ T cells and pamidronate-presenting cells remains to be investigated, at least two possible models are considered, which are not exclusive to each other. First, although the γδ TCR may directly interact with the native nonpeptide Ags presented on the target cells, additional costimulatory signals via specific ligands may be needed for the efficient γδ TCR signaling like the B7 family molecules in the case of αβ T cell activation (21, 22). Although human γδ T cells express a significant level of CD28 (our unpublished data), few tumor cell lines exhibit B7-1 or B7-2 expression, and the candidate molecules remain to be identified. Alternatively, γδ TCR may recognize nitrogen-containing bisphosphonate Ags only in association with specific molecules on the Ag-presenting tumor cells. Our unpublished results indicated that, in addition to the Jy1.2-encoded lysine residues (11), at least two more additional residues in the putative Ag-binding pocket in Vγ2Jy1.2Vδ2 TCR were required for γδ T cell activation by diverse nonpeptide Ags (S. Yamashita, Y. Tanaka, and N. Minato, manuscript in preparation). Thus, it is possible that nonpeptide Ags along with specific Ag-presenting molecules optimally fit to the pocket via anchoring residues in much the same way as peptide Ags in complex with MHC molecules for αβ TCR. A number of previous studies provided no evidence for involvement of MHC in γδ T cell activation by nonpeptide Ags (14, 23, 24) and again the candidate molecules remain to be seen.

The present results strongly support the hypothesis that effective activation of human γδ T cells by nonpeptide Ags depends on the immunological synapse formation in which species-specific interactions take place. Nitrogen-containing bisphosphonate Ags are particularly intriguing, in that the vast majority of human tumor cells can efficiently present them to γδ T cells, resulting in the targeted lysis of tumor cells by γδ T cells. It is suggested that the putative molecules required for Ag presentation and effective γδ T cell activation are rather ubiquitously expressed on many human tumor cells. Molecular understanding of the interaction may provide a new clue for potential immunotherapy of human malignancy by targeting tumor cells with chemically defined nonpeptide Ags for γδ T cells.

References


