TNF-related apoptosis-inducing ligand (TRAIL) frequently induces apoptosis in Philadelphia chromosome–positive leukemia cells


Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) have been implicated in antitumor immunity and therapy. In the present study, we investigated the sensitivity of Philadelphia chromosome (Ph1)–positive leukemia cell lines to TRAIL- or FasL–induced cell death to explore the possible contribution of these molecules to immunotherapy against Ph1-positive leukemias. TRAIL, but not FasL, effectively induced apoptotic cell death in most of 5 chronic myelogenous leukemia–derived and 7 acute leukemia–derived Ph1-positive cell lines. The sensitivity to TRAIL was correlated with cell-surface expression of death-inducing receptors DR4 and/or DR5. The TRAIL–induced cell death was caspase-dependent and enhanced by nuclear factor κB inhibitors. Moreover, primary leukemia cells from Ph1-positive acute lymphoblastic leukemia patients were also sensitive to TRAIL, but not to FasL, depending on DR4/DR5 expression. Fas-associated death domain protein (FADD) and caspase-8, components of death-inducing signaling complex (DISC), as well as FLIP (FLICE [Fas-associated protein with death domain–like interleukin-1–converting enzyme]/caspase-8 inhibitory protein), a negative regulator of caspase-8, were expressed ubiquitously in Ph1-positive leukemia cell lines irrespective of their differential sensitivities to TRAIL and FasL. Notably, TRAIL could induce cell death in the Ph1-positive leukemia cell lines that were refractory to a BCR-ABL–specific tyrosine kinase inhibitor imatinib mesylate (STI571; Novartis Pharma, Basel, Switzerland). These results suggested the potential utility of recombinant TRAIL as a novel therapeutic agent and the possible contribution of endogenously expressed TRAIL to immunotherapy against Ph1-positive leukemias. (Blood. 2003;101:3658-3667)

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stem cell transplantation (allo-SCT). IFN-α can prolong survival, but complete elimination of Ph1-positive leukemia cells has been attained in only 5% to 10% of the patients. Several reports have suggested a direct antileukemic effect of IFN-α on CML cells and indirect effects modulating the immune system. Allo-SCT is presently an only curative therapy not only for chronic phase of CML, but also for blast crisis of CML (CML-BC) and Ph1-positive ALL. A critical role for the immune system, termed graft-versus-leukemia (GVL) effect, in achieving a cure in patients with CML and Ph1-positive ALL has been well documented.

Although the clinical observations strongly suggest a pivotal role of cytotoxic T lymphocytes (CTLs) in suppressing Ph1-positive leukemias, the underlying effector mechanisms have not been well characterized. A role of FasL has been suggested, but its contribution to the elimination of Ph1-positive leukemia cells is still controversial. Selleri et al reported that Fas was expressed on CD34+ cells from CML patients and up-regulated by IFN-α. They also reported that CD34+ cells from CML patients who showed an optimal response to IFN-α therapy underwent apoptosis upon Fas triggering, whereas those derived from patients with a poor response were resistant to Fas-mediated killing. These results suggested a pivotal role of FasL in the elimination of CML clones by IFN-α therapy. However, Gora-Tybor et al reported that most of Ph1-positive leukemia cell lines did not express Fas and were resistant to Fas-mediated apoptosis. In a large-scale clinical study has indicated that the higher Fas expression on CD34+ cells from CML patients was associated rather with poor cytogenetic response to IFN-α therapy. Moreover, using a retrovirally induced murine CML model, Shlomchik and Pear reported that GVL effect was not impaired in Fas-deficient mice, suggesting that the mechanism other than the Fas/FasL interaction may be sufficient for the immune-mediated eradication of CML clones. Given the IFN-α-inducible expression of TRAIL on human T cells, TRAIL may participate in the process of antileukemic effects against Ph1-positive leukemias. In the present study, by using Ph1-positive leukemia cell lines and primary leukemia cells, we showed that TRAIL, but not FasL, effectively induced apoptosis in Ph1-positive leukemia cells, expressing surface DR4 and/or DR5. Notably, TRAIL was also effective in suppressing leukemia cells, we showed that TRAIL, but not FasL, effectively induced apoptosis in Ph1-positive leukemia cells, expressing surface DR4 and/or DR5. Notably, TRAIL was also effective in increasing the cytoxicity of anti-phosphotyrosine antibodies, as shown by the increased death of Ph1-positive leukemia cells. These results suggest not only the potential utility of recombinant TRAIL for clinical treatment of Ph1-positive leukemias but also shed light on the possible involvement of the TRAIL/TRAIL receptor interaction in the IFN-α therapy for CML and/or the GVL effect after allo-SCT for CML and Ph1-positive ALL.

Table 1. Characteristics and sensitivity to FasL in Ph1-positive leukemia cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BCR-ABL</th>
<th>Linage</th>
<th>Control</th>
<th>FasL</th>
<th>% inhibition by FasL</th>
<th>Expression of Fas, RFI†</th>
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<tbody>
<tr>
<td>KOPM28</td>
<td>p210</td>
<td>Myeloid</td>
<td>226 181 ± 2256</td>
<td>244 361 ± 1326</td>
<td>–8</td>
<td>3.0</td>
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<tr>
<td>KOPM53</td>
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<td>Myeloid</td>
<td>178 218 ± 1645</td>
<td>174 291 ± 1364</td>
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<td>KOPN55bi</td>
<td>p210</td>
<td>Lymphoid</td>
<td>8 184 ± 340</td>
<td>7 969 ± 493</td>
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<tr>
<td>Nalm1</td>
<td>p210</td>
<td>Lymphoid</td>
<td>40 925 ± 269</td>
<td>36 365 ± 1361</td>
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<td>K562</td>
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<td>9 611 ± 110</td>
<td>9 906 ± 295</td>
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<td>p190</td>
<td>Myeloid</td>
<td>10 849 ± 141</td>
<td>7 511 ± 375</td>
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<td>180 406 ± 3438</td>
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<td>86 368 ± 5687</td>
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<td>117 272 ± 2663</td>
<td>114 914 ± 2197</td>
<td>2</td>
<td>1.3</td>
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<tr>
<td>KOPN72bi</td>
<td>p190</td>
<td>Lymphoid</td>
<td>145 957 ± 3415</td>
<td>140 332 ± 4242</td>
<td>4</td>
<td>1.2</td>
</tr>
<tr>
<td>YAMN73</td>
<td>p203§</td>
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<td>60 397 ± 991</td>
<td>59 396 ± 1840</td>
<td>2</td>
<td>1.0</td>
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<tr>
<td>YAMN91</td>
<td>p190</td>
<td>Lymphoid</td>
<td>13 884 ± 246</td>
<td>14 143 ± 167</td>
<td>–2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Data are shown as means ± SE of triplicate cultures.
†% inhibition by FasL was determined by the % inhibition by FasL assay in the presence or absence of 100 ng/mL rhsFasL as described in "Materials and methods."
‡Relative fluorescence intensity (RFI) was determined by the ratio of mean fluorescence intensity for specific staining to that for control staining.
§p203 corresponds to the Abl exon2-spliced BCR-ABL protein.
N-terminal to linker peptides and FRAG- and His-tags, respectively; and do not require a cross-linker for biologic activities. z-VAD-fmk, a caspase inhibitor with broad spectrum, was purchased from Enzyme Systems Products (Livermore, CA). A proteasome inhibitor N-acetyl-Leu-Leu-Leu-norLeu-al (LLnL), a cell-permeable nuclear factor κB (NF-κB) inhibitory peptide SN50, 36 and its control peptide SN50M were purchased from Sigma-Aldrich (Tokyo, Japan) and BIOMOL (Plymouth Meeting, PA), respectively. Imatinib mesylate (Gleevec) was kindly provided by Novartis Pharma (Basel, Switzerland).

**3H-thymidine uptake assay**

Leukemia cell lines (2–5 × 10^5 cells/well) were cultured in triplicate in 200 μL RPMI1640 medium supplemented with 10% FCS in a flat-bottomed 96-well plate (Costar, Cambridge, MA). The plates were incubated for the indicated periods, pulsed for the last 6 hours with 10^3Ci/well [0.037 MBq/well], and harvested onto glass-fiber filters. Radioactivity incorporated into DNA was measured by liquid scintillation counting.

The effects of rhsFasL and rhsTRAIL were determined by the last 6-hour pulse of the 42-hour culture in the absence or presence of 3-fold diluted concentrations (3.7, 11, 33, 100, and 300 ng/mL) of rhsFasL or rhsTRAIL. In some experiments, a neutralizing anti-TRAIL monoclonal antibody (mAb) (RIK-2; 10 μg/mL) 13 or z-VAD-fmk (20 μM) was used to block the activity of rhsTRAIL and caspases, respectively. In other experiments, cell lines were preincubated for 3 hours with LLnL (2.5 μM), SN50, or SN50M (100 μg/mL) and then cultured in the absence or presence of rhsTRAIL. The effect of imatinib mesylate was also determined after 42-hour incubation in the absence or presence of imatinib mesylate (1.0 μM). The percent inhibition by TRAIL or imatinib mesylate was calculated as follows: \[ \left(1 - \frac{\text{cpm of treated wells}}{\text{cpm of untreated well}}\right) \times 100. \]

**Viability and apoptosis assays**

The cytotoxic effects of FasL and TRAIL were examined by the dye exclusion assay. Cell lines (1 × 10^5 cells/well) were cultured in the presence of rhsFasL or rhsTRAIL at 100 ng/mL, harvested at 12, 24, and 36 hours, and the viability was determined by staining with trypan blue. The early apoptotic event in leukemia cell lines was examined by binding of Annexin-V to surface-exposed phosphatidylserine. Cell lines (4 × 10^5 cells/mL) were cultured in the absence or presence of rhsTRAIL (100 ng/mL), harvested at 12 hours, stained with fluorescein isothiocyanate (FITC)–conjugated Annexin-V (MBL, Nagoya, Japan), and analyzed by flow cytometry (EPICS PROFILE; Coulter, Miami, FL). In experiments with primary leukemias, cells (2 × 10^5 cells/well) were incubated in the absence or presence of rhsFasL or rhsTRAIL at 100 ng/mL with or without a neutralizing anti-TRAIL mAb RIK-2 (10 μg/mL) in triplicate in a 96-well plate for 24 hours, and the viability was determined by staining with trypan blue. In some patient samples, apoptosis was also examined by binding of FITC–conjugated Annexin-V after 12-hour culture.

**Cell-surface expression of TRAIL receptors and Fas**

mAbs specific for DR4 (DJR1, mouse immunoglobulin G 1 [IgG 1]), DR5 (DJR2, mouse IgG 1), DcR1 (DJR3, mouse IgG 1), and DcR2 (DJR4, mouse IgG 1) were raised against soluble human IgG1 Fc fusion proteins containing the extracellular domain of each TRAIL receptor and identified by their specific reactivity with the respective fusion protein in enzyme-linked immunosorbent assay (ELISA). Leukemia cell lines, primary leukemias, and baby hamster kidney (BHK) cell lines stably expressing DR4, DR5, DcR1, or DcR2 cDNA (1 × 10^6 cells) were incubated with 1 μg of biotinylated control mouse IgG1 or mAb for 30 minutes on ice. After washing, the cells were incubated with phycoerythrin-conjugated streptavidin (Biomeda, Foster City, CA) for 30 minutes on ice, and then analyzed by flow cytometry. The relative fluorescence intensity (RFI) was determined by calculating the ratio of mean fluorescence intensity for specific staining to that for control staining. For the Fas expression, each cell line was incubated with mouse anti-human Fas (4A5; MBL) or irrelevant mouse IgG for 30 minutes on ice, and subsequently with FITC-conjugated anti-mouse IgG, and analyzed by flow cytometry.

![Figure 1. Fas expression and cytotoxic effect of FasL against Ph1-positive leukemia cell lines.](image)
Western blot analysis

The nonidet P-40 lysates of cell lines were separated on a sodium dodecyl sulfate–polyacrylamide gel under reducing conditions and then transferred to polyvinyl difluoride membranes as previously described. After blocking with 5% nonfat dry milk in 0.05% Tween-20 Tris (tris(hydroxymethyl) aminomethane)-buffered saline (TBS), membranes were incubated with mouse anti-human FADD (1:250 dilution; BD Transduction Laboratories, Lexington, KY), antihuman caspase-8 (1:1000 dilution; MBL), antihuman kinase domain of c-ABL (1:400 dilution; Pharmingen, San Diego, CA), or rat anti-human FLIP (FLICE [Fas-associating protein with death domain–like interleukin–1–converting enzyme]/caspase-8 inhibitory protein; 1:1000 dilution; Kamiya Biochemical, Seattle, WA) antibodies in 5% milk TBS at 4°C overnight. Membranes were incubated with horseradish peroxidase–conjugated goat antimouse or rat IgG (1:1000 dilution; MBL) at room temperature for 1 hour and then developed using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Results

Cytotoxic effect of FasL against Ph1-positive leukemia cell lines

We first examined the susceptibility of 12 Ph1-positive leukemia cell lines to rhsFasL, by the ³H-thymidine uptake for the last 6 hours of the 42-hour culture with various concentrations of rhsFasL. As shown Figure 1A, a marked growth inhibition was observed in a dose-dependent manner well-characterized FasL-sensitive T-leukemia cell lines (Jurkat and MOLT4F). This growth inhibition was due to loss of cell viability, rather than cytostasis, as estimated by the trypan blue exclusion assay (Figure 1B). Although 1 of 5 CML-BC–derived (Nalm1) and 1 of 7 AL–derived (KOPM30) cell lines were moderately susceptible, the other 10 Ph1-positive cell lines were highly resistant to FasL as estimated by either the ³H-thymidine uptake (Figure 1A and Table 1) or the trypan blue exclusion assay (Figure 1B).

These results suggested that Ph1-positive leukemia cell lines are generally resistant to the FasL-induced cell death. Next, we analyzed the cell-surface expression of Fas by flow cytometry. As indicated in Figure 1C and summarized in Table 1, Fas was detectable on myeloid cell lines except for K562 but rarely detectable on lymphoid cell lines except for Nalm1. Accordingly, the relatively low expression of Fas could explain the resistance to FasL in lymphoid cell lines, while some factor other than the Fas expression might contribute to the resistance to FasL in myeloid cell lines.

Cytotoxic effect of TRAIL against Ph1-positive leukemia cell lines

We next investigated the cytotoxic effect of rhsTRAIL against Ph1-positive leukemia cell lines. Among the CML-BC–derived cell lines (Figure 2A, left panel), 2 cell lines (Nalm1 and KOPM28) showed a marked growth inhibition in a dose-dependent manner, whereas the other 3 cell lines (KOPM53, KOPN55bi, and K562) were rather resistant. Among the Ph1-positive AL–derived cell lines (Figure 2A, right panel), 3 cell lines (YAMN91, KOPN66bi, and KOPM30) were highly sensitive, 2 cell lines (YAMN73 and KOPN72bi) were moderately sensitive, but 2 cell lines (KOPN57bi and KOPN30bi) were resistant. To demonstrate that these antileukemic effects were really mediated by TRAIL, we performed the blocking experiment using a neutralizing anti-TRAIL mAb (RIK-2). As shown in Figure 2B, the growth inhibition in Nalm1 was totally abolished by RIK-2, substantiating the specific activity of rhsTRAIL. As summarized in Table 2, the sensitivity of Ph1-positive leukemia cell lines to TRAIL was not correlated with the type of disease (CML-BC or AL), the type of BCR-ABL fusion protein (p210, p203, or p190), or the type of lineage (myeloid or lymphoid).

To more directly evaluate the cytotoxic activity of TRAIL, we performed the dye exclusion assay (Figure 2C).

Consistent with the growth inhibition as estimated by the ³H-thymidine uptake assay, 7 of 12 Ph1-positive leukemia cell lines were moderately or highly sensitive to the TRAIL–induced cell death.

We also examined the binding of Annexin-V by flow cytometry after 12-hour treatment with rhsTRAIL (100 ng/mL). As shown in Figure 3, the Annexin-V–positive population was increased to 95% in the highly sensitive cell line (KOPM28), 72% in the moderately sensitive cell line (YAMN73), but only 13% in the resistant cell line (KOPM30), indicating that the TRAIL–induced cell death in Ph1-positive leukemia cell lines was caused by apoptosis.

To confirm that the apoptotic cell death induced by TRAIL was dependent on the activation of caspases, we performed the
Ph1-positive leukemia cells.

The activation of caspases was required for the TRAIL-induced cell death in Ph1-positive leukemia cells, and almost completely in KOPM28, indicating that the activation of caspases was required for the TRAIL-induced cell death in Ph1-positive leukemia cells.

Expression of TRAIL receptors on Ph1-positive leukemia cell lines

To investigate whether the sensitivity of Ph1-positive leukemia cells to TRAIL depends on the expression of TRAIL receptors, we analyzed the cell-surface expression of DR4, DR5, DcR1, and DcR2 by flow cytometry. The specificity of each mAb is shown against BHK cell lines transfected with respective TRAIL receptor cDNAs (Figure 5A).

Representative cytofluorographic data on highly sensitive (KOPM28, KOPM30), moderately sensitive (KOPN72bi), and resistant (KOPM53) cell lines are shown in Figure 5B, and the RFI in each cell line is summarized in Table 2. Among 4 TRAIL sensitive cell lines expressed DR4 and/or DR5 at significant levels. In contrast, the TRAIL-resistant cell lines except for K562 showed undetectable or low levels of DR4 and DR5. These results suggested that the sensitivity of Ph1-positive leukemia cells to TRAIL is mostly correlated with the cell-surface expression levels of DR4 and DR5.

Cytotoxic effects of TRAIL and FasL against primary Ph1-positive leukemia cells

To verify the antileukemic effects of TRAIL and FasL against primary leukemia cells, leukemic blasts from 10 Ph1-positive ALL cases and 2 CML-BC cases were tested. Each sample was cultured for 24 hours in the absence or presence of 100 ng/mL of rhsFasL or rhsTRAIL in combination with a neutralizing anti-TRAIL mAb, RIK-2, and the viability was determined by the trypan blue exclusion assay. As summarized in Table 3, viability of the cells was significantly reduced by the addition of TRAIL in 6 of 10 Ph1-positive ALL cases, while only 1 case was moderately sensitive to FasL. The specific activity of TRAIL was demonstrated by the significant recovery of viability with the RIK-2 treatment. Induction of apoptosis by TRAIL was also confirmed in leukemic blasts from patients 8 and 9 by Annexin-V binding on flow cytometry (Figure 6A). Importantly, primary leukemia cells from patient 3 were sensitive to TRAIL, while the cell line (KOPN57bi)
established from this patient was resistant to TRAIL (Figure 2A and Table 2). Leukemic blasts from patients 4 and 8, the origin of TRAIL-sensitive YAMN91 and YAMN73, respectively, were sensitive to TRAIL, while those from patient 2, the origin of TRAIL-resistant KOPN30bi, were resistant. Regarding TRAIL sensitivity in leukemic blasts from CML-BC, only one case (patient 11) was evaluated and showed resistance.

Next, the cell-surface expression of DR4 and DR5 was analyzed by flow cytometry as indicated in Figure 6B and Table 3. In Ph1-positive ALL cases, the expression of DR4/DR5 was detectable on TRAIL-sensitive leukemia cells from patients 4, 8, and 9, but almost undetectable on TRAIL-resistant leukemia cells from patients 1, 2, and 5. In the CML-BC case (patient 12), both DR4 and DR5 were clearly detectable, although its TRAIL-sensitivity could not be evaluated because of excessive spontaneous cell death in control culture.

Expression of molecules consisting of death-inducing signaling complex (DISC)

Ligation of death receptors by FasL^{44} and TRAIL^{45,46} triggers a series of protein-protein interactions that leads to assembly of a DISC. Thus, expression levels of the molecules consisting of DISC are critical determinants for sensitivity. It is known that Fas and DR4/DR5 recruit FADD^{47,48} and caspase-8^{49,50} into DISC, and that FLIP^{51} acts as a negative regulator of caspase-8.

We therefore performed Western blot analysis of these molecules in Ph1-positive leukemia cell lines (Figure 7). FADD, caspase-8, and FLIP were almost ubiquitously expressed in Ph1-positive leukemia cell lines irrespective of their differential sensitivities to FasL and TRAIL. These observations indicated that an absence of FADD and/or caspase-8 or an excessive expression of FLIP could not be a mechanism for resistance to TRAIL and FasL.

Modulation of TRAIL sensitivity by NF-κB inhibitors

It has been reported that NF-κB is constitutively activated by BCR-ABL in Ph1-positive leukemias^{52} and that NF-κB could modulate TRAIL-induced cell death^{53,53} We attempted to examine whether the NF-κB inhibitors could modulate the TRAIL sensitivity of Ph1-positive leukemia cells. For this purpose, 10 leukemia cell lines (6 sensitive and 4 resistant) were preincubated for 3 hours with 2.5 μM proteasome inhibitor LLnL^{54} which is known to inhibit the activation of NF-κB by blocking the degradation of the IκB inhibitory protein, followed by 42-hour exposure to lower
concentrations of rhsTRAIL (10 ng/mL for sensitive and 50 ng/mL for resistant cell lines), and then the 3H-thymidine uptake was assessed for the last 6 hours (Figure 8A). As expected from a considerable role of NF-κB in the BCR-ABL-mediated transformation, the treatment with LLnL alone moderately repressed the 3H-thymidine uptake in most of Ph1-positive leukemia cell lines. In the TRAIL-sensitive cell line KOPM28, for instance, the treatment with either TRAIL or LLnL reduced the 3H-thymidine uptake to approximately 70% or 50%, respectively, but the treatment with both TRAIL and LLnL almost completely abrogated the 3H-thymidine uptake. Similar results were obtained in all TRAIL-sensitive cell lines examined. In contrast, LLnL did not enhance the proapoptotic activity of TRAIL in all TRAIL-resistant cell lines, including K562, which considerably expressed DR5. These results suggested that the inhibition of NF-κB activation by LLnL either augmented the TRAIL sensitivity or synergistically acted with TRAIL in the process of apoptosis induction, but could not convert the TRAIL-resistant cells to be TRAIL sensitive.

A similar experiment was also performed in KOPM28 using another NF-κB–specific inhibitor, SN50, which blocks the nuclear translocation of NF-κB. As shown in Figure 8B, SN50

Table 3. Sensitivity to FasL/TRAIL and TRIL receptors expression in primary Ph1-positive leukemia cells

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age</th>
<th>Derived cell line</th>
<th>Onset/Relapse</th>
<th>Sample</th>
<th>Type of BCR-ABL</th>
<th>Viability after 24-h culture*</th>
<th>Expression of TRAIL receptors, RFI ‡</th>
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<tbody>
<tr>
<td>Ph1-ALL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control, %</td>
<td>Fast-L, %</td>
<td>TRAIL, %</td>
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<td>1</td>
<td>M</td>
<td>7</td>
<td>—</td>
<td>Onset</td>
<td>BM</td>
<td>p190</td>
<td>89 ± 2</td>
<td>91 ± 1</td>
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<tr>
<td>2</td>
<td>M</td>
<td>8</td>
<td>KOPN30 bi</td>
<td>Onset</td>
<td>PB</td>
<td>p190</td>
<td>75 ± 4</td>
<td>71 ± 6</td>
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<td>3</td>
<td>M</td>
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<td>KOPN57 bi</td>
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<td>84 ± 1</td>
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<td>4</td>
<td>M</td>
<td>4</td>
<td>YAMN91</td>
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<td>80 ± 4</td>
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<td>5</td>
<td>F</td>
<td>12</td>
<td>—</td>
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<td>85 ± 1</td>
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<td>16</td>
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<td>M</td>
<td>9</td>
<td>—</td>
<td>Relapse</td>
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<td>8</td>
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<td>YAMN73</td>
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<td>PB</td>
<td>p203i</td>
<td>87 ± 1</td>
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<td>9</td>
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<td>Relapse</td>
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<td>92 ± 2</td>
<td>87 ± 3</td>
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<tr>
<td>12</td>
<td>F</td>
<td>11</td>
<td>—</td>
<td>—</td>
<td>PB</td>
<td>p210</td>
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</table>

M indicates male; F, female; BM, bone marrow; PB, peripheral blood; ND, not determined; NA, not available; and —, not applicable.

*Data are shown as mean ± SE.
†Significant reduction in viability (P < .01, t-test).
‡RFI was determined by the ratio of mean fluorescence intensity for specific staining to that for control staining.
§Significant increase in viability (P < .01, t-test) in comparison with TRAIL treatment alone.
∥p203 corresponds to the Abl exon 2-spliced BCR-ABL protein.
#Sensitivity could not be evaluated because of complete spontaneous cell death in control culture.
but not its mutant control peptide SN50M, reduced the $^3$H-thymidine uptake to nearly 60% by itself and enhanced sensitivity to TRAIL, further substantiating the modulation of TRAIL sensitivity by NF-κB.

Comparison of antileukemic effects of TRAIL and imatinib mesylate

Imatinib mesylate is a specific inhibitor of BCR-ABL tyrosine kinase activity and shows a potent cytotoxic effect on Ph1-positive leukemias. In a clinical trial, imatinib mesylate was reported to be very effective in CML patients who were resistant to the IFN-α therapy.

Thus, we compared the cytotoxic effects of TRAIL and imatinib mesylate against 12 Ph1-positive cell lines (Figure 9). All these cell lines were established from the patients who had not been treated with imatinib mesylate and were not selected by imatinib mesylate treatment in vitro. All myeloid cell lines (KOPM28, KOPM30, KOPM53, and K562; closed symbols) showed high sensitivity to imatinib mesylate (percent inhibition, > 60%). In contrast, only 1 of 8 lymphoid cell lines (KOPN57bi) was highly sensitive, and 4 lymphoid cell lines (Nalm1, KOPN66bi, YAMN73, and YAMN91) were naturally resistant to imatinib mesylate (percent inhibition, < 40%). Two myeloid cell lines (KOPM28 and KOPM30) showed high sensitivity to both TRAIL and imatinib mesylate. The 4 lymphoid cell lines resistant to imatinib mesylate were sensitive to TRAIL, while 5 cell lines (myeloid 2; lymphoid 3) were resistant to TRAIL but sensitive to imatinib mesylate. Most importantly, none of 12 cell lines were resistant to both TRAIL and imatinib mesylate. These results suggested that TRAIL and imatinib mesylate could be used complementarily to eliminate Ph1-positive leukemia cells.

Discussion

In the present study, to explore the possible contribution of FasL and TRAIL to the immune-mediated antileukemic effects against Ph1-positive leukemia cells, we first investigated whether FasL or TRAIL could induce cell death in 12 Ph1-positive leukemia cell lines. Consistent with a previous report, most of the Ph1-positive cell lines were resistant to FasL. In contrast, 2 of 5 CML-BC–derived and 5 of 7 Ph1-AL–derived cell lines were highly or moderately sensitive to TRAIL-induced apoptotic cell death. Similar results were obtained with primary Ph1-positive ALL cells. Consistent with our data, Plasilova et al recently reported that the growth of CML progenitors in chronic phase as well as in accelerated or blastic phases was significantly suppressed by recombinant TRAIL.

We next analyzed the cell-surface expression of TRAIL receptors to understand the differences in TRAIL sensitivity among the Ph1-positive leukemia cell lines. All TRAIL-sensitive cell lines and primary cells expressed the death-inducing receptors DR4 and/or DR5 on their surface, whereas the TRAIL-resistant cell lines, except for K562, and primary cells expressed neither DR4 nor DR5. In addition, none of these cell lines expressed DcR1 and DcR2, which are thought to act as decoy receptors. These results suggested that the sensitivity of Ph1-positive leukemia cells to TRAIL is primarily determined by the cell-surface expression of DR4 and/or DR5.

In addition to the receptor expression, regulators in the death-signaling pathway would be critical for the determination of sensitivity to FasL and TRAIL. We demonstrated that caspase-8,

(100 μg/mL), but not its mutant control peptide SN50M, reduced the $^3$H-thymidine uptake to nearly 60% by itself and enhanced sensitivity to TRAIL, further substantiating the modulation of TRAIL sensitivity by NF-κB.

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In addition to the receptor expression, regulators in the death-signaling pathway would be critical for the determination of sensitivity to FasL and TRAIL. We demonstrated that caspase-8,
FADD, and FLIP were ubiquitously expressed in Ph1-positive leukemia cell lines irrespective of their differential sensitivities to FasL and TRAIL, suggesting that these molecules are not critically involved in the determination of sensitivity to FasL and TRAIL at least in their expression levels. We observed a marked discrepancy between sensitivity to TRAIL and FasL in several Ph1-positive cell lines despite expression of both death receptors. In particular, KOPM28 expressing both DR5 and Fas at highest levels showed a high sensitivity to TRAIL but not to FasL. Recently, a similar discrepancy has also been documented in some solid tumors.58,59 In addition, distinct intracellular signaling pathways in TRAIL- and FasL-mediated apoptosis have been reported.60 Thus, KOPM28 would serve as a useful subject to explore distinct intracellular signaling via TRAIL and FasL in further studies.

It has been demonstrated that the binding of TRAIL to DR4 and DR5 as well as DcR2 induced the NF-κB activation,63,64 and that the increased NF-κB activity protects tumor cells from various proapoptotic stimuli.62 Therefore, we examined whether the NF-κB activity could modulate the sensitivity to TRAIL in Ph1-positive leukemia cells. The pretreatment with NF-κB inhibitors, LLnL and SN50, enhanced sensitivity to TRAIL in the TRAIL-resistant cell lines, suggesting that the DR4/DR5-mediated or BCR-ABL-mediated NF-κB activation plays a substantial role in protecting Ph1-positive leukemia cells from TRAIL-induced cell death. Of importance, in TRAIL-resistant cell lines not expressing DR4 or DR5, NF-κB inhibitors could not overcame their TRAIL resistance. Since a proteasome inhibitor that inhibits NF-κB activation (PS-341) has already been in phase 3 clinical trials against multiple myelomas,62,63 the combination with PS-341 would be a promising way to enhance the TRAIL-mediated elimination of Ph1-positive leukemia cells in a clinical setting.

We previously demonstrated that IFN-α up-regulated the cell-surface expression of TRAIL, on TCR/CD3-stimulated T cells in vitro, which mediated cytotoxicity against TRAIL-resistant renal cell carcinomas.13 In addition, Wen et al reported that antileukemic agents including Ara-C increased the expression levels of DR5 on leukemia cell lines.64 Moreover, it has been reported that a combination of Ara-C and IFN-α increased the rate of remission and prolonged survival in CML patients.65 Therefore, it may be feasible to expect that Ara-C enhances the DR5 expression in CML progenitors and IFN-α induces the TRAIL expression on CTLs in CML patients, leading to elimination of Ph1-positive leukemias through the TRAIL/DR5 interaction. To address this possibility, the correlation between the in vitro susceptibility of CML progenitors to TRAIL and the clinical responses to AraC and/or IFN-α therapy have to be determined.

Allo-SCT is a potentially curative therapy against CML and Ph1-positive ALL, and its effectiveness is thought to be achieved mainly by the immune-mediated GVL effect. We herein showed that TRAIL could efficiently induce apoptosis in Ph1-positive AL-derived cell lines as well as CML–BC–derived cell lines. In this regard, endogenously expressed TRAIL on CTLs or NK cells may be involved in the GVL effect. To address this hypothesis, the correlation between the in vitro TRAIL sensitivity of leukemia cells and the leukemia-free survival after allo-SCT in CML and Ph1-positive ALL patients has to be determined in future study.

A significant number of CML patients cannot tolerate the IFN-α therapy because of lethargy, malaise, anorexia, depression, and autoimmune-like syndromes. The GVL effect after allo-SCT is frequently associated with life-threatening graft-versus-host disease. In contrast, at least in mice and nonhuman primates, administration of rhsTRAIL did not exhibit a serious toxicity.66,67 Thus, rhsTRAIL could be a novel therapeutic agent for CML and Ph1-positive ALL patients as a safer alternative.

Finally, we investigated the correlation between TRAIL sensitivity and imatinib mesylate sensitivity. Imatinib mesylate exerted a potent antileukemic effect against Ph1-positive cells not only in patients with CML in chronic phase who failed to the IFN-α therapy,56 but also in patients with CML in advanced phase or Ph1-positive ALL.56,61 Importantly, we demonstrated that imatinib mesylate efficiently repressed most of the TRAIL–resistant cell lines, while TRAIL repressed most of the imatinib mesylate-resistant cell lines. This suggests a potential clinical utility of TRAIL particularly for patients with imatinib mesylate-resistant CML and Ph1-positive ALL.

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25. Interferon alpha-2a as compared with conven


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