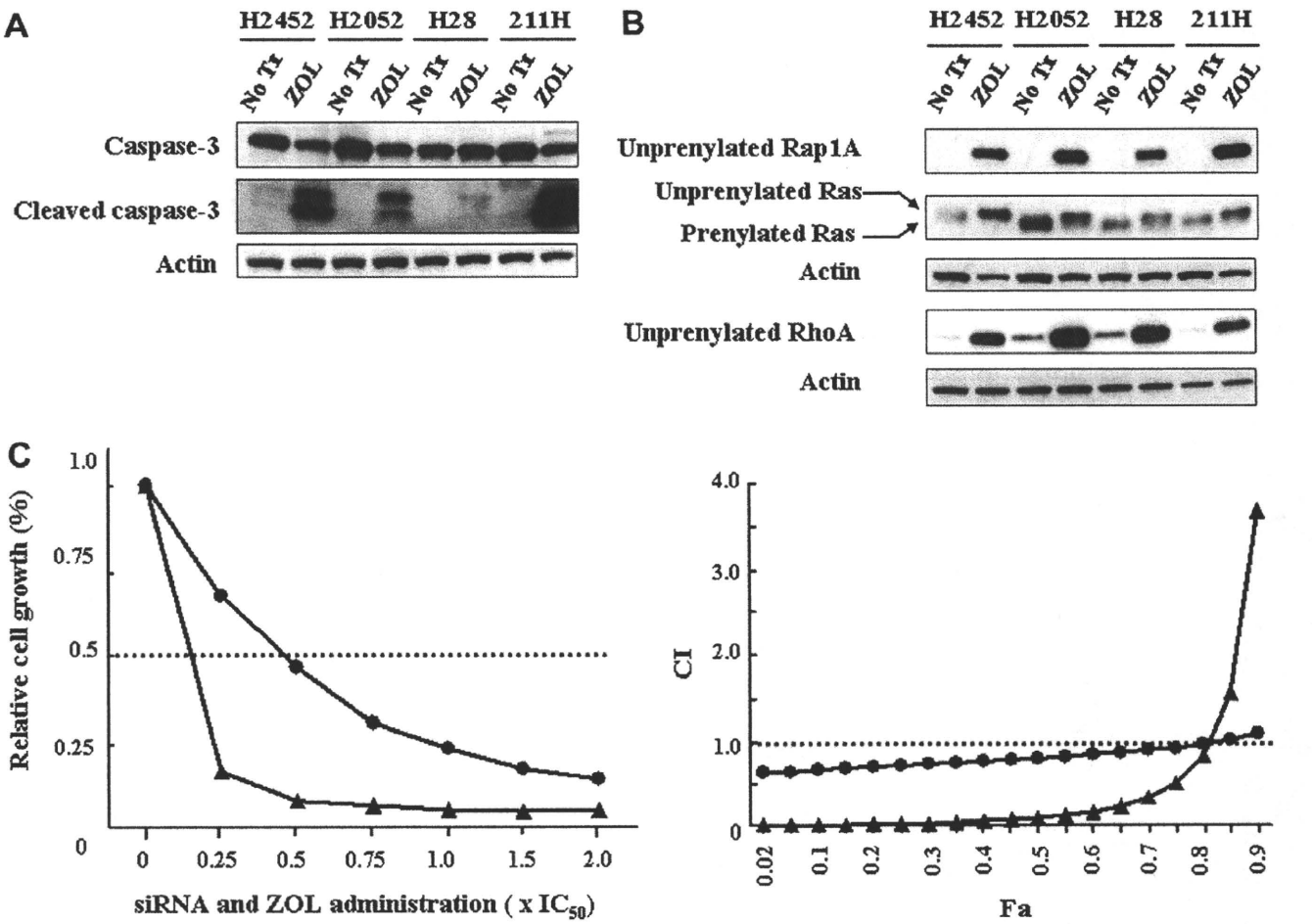

Fig. 5. Induction of apoptosis in MM cells by ZOL treatment (A and B) and combined effects of chimeric PLK-1 siRNA with ZOL. (A) Cleavage of caspase-3 by ZOL treatment. Four MM cell lines were incubated with ZOL at 50 μM for 72 h. Whole cell lysates were obtained and immunoblotting was performed as described in Section 2. (B) Evaluation of the inhibition of small GTPase protein prenylation by ZOL treatment (50 μM). Immunoblotting and immunoprecipitation were performed as described in Section 2. (C) Evaluation of the combined effects of DNA-chimeric PLK-1 siRNA and ZOL treatment on H2452 (filled circles) and H28 (filled triangles) mesothelioma cell lines. Cells were incubated for 72 h with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the IC_{50}) of each agent or both in combination using the constant ratio design followed by the modified MTT assay as described in Section 2. The IC_{50} values of PLK-1 siRNA for H2452 cells and H28 cells were 1.6 nM and 38.7 nM, respectively, and those of ZOL were 11.4 μM and 58.1 μM, respectively. Left panel: The killing curves of the concurrent administration of PLK-1 siRNA and ZOL. Right panel: Combination index (CI)-fraction affected (Fa) plots. Combination indexes were determined with the nonlinear regression program CalcuSyn.

ss counterparts; however, ds nucleic acids, including siRNAs, are still degraded and must be protected from endogenous nucleases in the bloodstream. Degradation can be avoided by the use of a suitable delivery system or by the nuclease-resistant chemical modification of the siRNA [10]. One approach involves the modification of the 2′-position of the ribose of the siRNA. Sugar modifications such as 2′-*O*-methylation, 2′-*O*-methoxyethylation, and 2′-fluoro-2′-deoxynucleoside modification can improve nuclease resistance [41,42]. Another approach involves the replacement of certain siRNA ribonucleotides with their deoxyribonucleotide counterparts.

We generated a DNA-chimeric siRNA, and substituted six basepairs from the 5′ end of the guide strand with their deoxyribonucleotide counterparts. This DNA-chimeric siRNA was more stable in human serum than the non-chimeric siRNA and showed resistance to endogenous nucleases. Moreover, the chimeric siRNA against PLK-1 decreased PLK-1 expression almost as effectively as the non-chimeric siRNA, and inhibited the proliferation of MM cells through the induction of apoptosis by cleaving caspase-3. These anti-neoplastic effects in the present study are consistent with our previous reports [19,20]. PLK-1 is overexpressed in MM cells compared to normal fibroblasts and, therefore, PLK-1 is a novel target that inhibits the proliferation of MM cells. Ui-Tei et al. [13] also demonstrated that DNA-chimeric siRNAs, in which the eight ribonucleotides from the 5′ end of the guide strand were substituted with deoxyribonucleotides, effectively induced gene-silencing without exerting the off-target effects. Our findings, taken together with the Ui-Tei's report, demonstrate that DNA-chimeric siRNAs result in safe and more effective gene-silencing and that the DNA-chimeric siRNA is much suitable for an *in vivo* administration. Further studies are warranted to evaluate the therapeutic potential of the DNA-chimeric siRNA *in vivo*.

ZOL, a third-generation BP, inhibits the activity of farnesyl diphosphate synthase in the mevalonate pathway, resulting in inhibition of the prenylation of small GTPases

[24,25]. The prenylated small GTPases including Ras, Rap-1, and Rho proteins transduce signals downstream for cell functions such as cell proliferation, adhesion, and migration [43–45], while the inhibition of these small GTPase prenylation by BPs results in the suppression of cancer progression [26–30,46–48]. In the present study, we have revealed that ZOL also inhibits the proliferation of MM cells. Treatment of MM cells with ZOL inhibited the prenylation of Rap-1A, Ras, and RhoA proteins, and induced apoptosis by cleaving capsase-3. Other investigators have also reported that ZOL inhibits the proliferation of MM cells [49,50]. Interestingly, one report demonstrated that Ca^{2+} regulates the growth inhibitory effects of BPs on MM cells [49]. Calcification is a well-known feature in MM [51,52], and BPs accumulate rapidly in bone [24,25]. We have demonstrated previously that ZOL has anti-tumor effects against osteosarcoma [29,46]. These findings collectively suggest that malignancies with ossifying features, including MM and breast cancers [48], are suitable candidates for ZOL treatment.

PLK-1 regulates cell division at several points during the mitotic phase, and RhoA is also implicated in the regulation of cytokinesis. Prenylated Rho proteins are delivered to the plasma membrane and are concentrated at the cleavage furrow of the cell during the M phase of cell cycle [53,54]. PLK-1 regulates the local activation of RhoA to promote cytokinesis [38,39] and, therefore, we hypothesized that ZOL treatment would augment the cytotoxic efficacy of the PLK-1 siRNA in MM cells. The combined ZOL and PLK-1 siRNA treatment showed synergistic effects at both low (Fa 0.5) and high (Fa 0.8) concentrations.

In conclusion, a DNA-chimeric PLK-1 siRNA inhibited cellular proliferation and induced apoptosis in MM cells, and a combination of PLK-1 siRNA and ZOL treatment revealed synergistic inhibitory effects on MM cells. The observation reported in the present study indicates that PLK-1 is a novel target for the treatment of MM and that the DNA-chimeric siRNA against PLK-1 combined with ZOL treatment would be an attractive strategy in the fight against this aggressive disease.

Conflicts of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2010.02.008.

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Rakicidin A effectively induces apoptosis in hypoxia adapted Bcr-Abl positive leukemic cells

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Treatment with Abl tyrosine kinase inhibitors (TKI) drastically improves the prognosis of chronic myelogenous leukemia (CML) patients. However, quiescent CML cells are insensitive to TKI and can lead to relapse of the disease. Thus, research is needed to elucidate the properties of these quiescent CML cells, including their microenvironment, in order to effectively target them. Hypoxia is known to be a common feature of solid tumors that contributes to therapeutic resistance. Leukemic cells are also able to survive and proliferate in severely hypoxic environments. The hypoxic conditions in the bone marrow (BM) allow leukemic cells that reside there to become insensitive to cell death stimuli. To target leukemic cells in hypoxic conditions, we focused on the hypoxia-selective cytotoxin, Rakicidin A. A previous report showed that Rakicidin A, a natural product produced by the *Micromonospora* strain, induced hypoxia-selective cytotoxicity in solid tumors. Here, we describe Rakicidin A-induced cell death in hypoxia-adapted (HA)-CML cells with stem cell-like characteristics. Interestingly, apoptosis was induced via caspase-dependent and -independent pathways. In addition, treatment with Rakicidin A in combination with the TKI, imatinib, resulted in synergistic cytotoxicity against HA-CML cells. In conclusion, Rakicidin A is a promising compound for targeting TKI-resistant quiescent CML stem cells in the hypoxic BM environment. (*Cancer Sci* 2011; 102: 591–596)

In solid tumors, oxygen supply is frequently insufficient to adequately support rapid cell proliferation and angiogenesis, which leads to the development of hypoxic regions within the tumor.[1,2] These hypoxic regions are associated with altered cell metabolism, changes in cell cycling and increased resistance to cell death stimuli, and are often associated with a poor prognosis in cancer patients.[3–5] Therefore, the development of cytotoxic compounds that are effective in hypoxic conditions might overcome the therapeutic resistance of cancer cells in hypoxic micro environments.

Bone marrow (BM) is known to have low levels of oxygen, and it has been suggested that the hematopoietic stem cells in the BM niche are protected from DNA damage induced by reactive oxygen species (ROS).[6,7] Chronic myelogenous leukemia (CML) is a disorder of hematopoietic stem cells caused by the constitutive activation of Bcr-Abl tyrosine kinase.[8] Treatment of CML has been markedly improved by the development of Abl tyrosine kinase inhibitors (TKI), but the complete eradication of CML cells may not be possible with TKI alone because they are largely ineffective against quiescent CML cells.[9–12] Although it has not yet been confirmed, CML stem cells could reside in the BM niche because they exhibit characteristics similar to normal hematopoietic stem cells. Previously, we found that CML cells engrafted in the BM survived and proliferated in the severely hypoxic environment; furthermore, these hypoxia-adapted leukemic cells acquired a more primitive phenotype.[13]

Recently, Yamazaki *et al.* screened 20 000 cultured broths of microorganisms and isolated Rakicidin A, a highly hypoxia-selective cytotoxic compound from *Micromonospora* strain ML99-43F1. Rakicidin A showed significant selective cell-killing effects in solid cancer cell lines.[14] In this study, Rakicidin A inhibited the proliferation of TKI-resistant, hypoxia-adapted CML (HA-CML) cells by inducing apoptosis. These findings indicate that hypoxia-selective therapy may be a novel strategy for the treatment of CML, especially Abl TKI-resistant quiescent CML cells.

Materials and Methods

Cell lines and reagents. The human CML derived cell line K562 was obtained from the American Type Culture Collection (Manassas, VA, USA) and KCL22 cells were kindly provided by Dr Tadashi Nagai (Jichi Medical School, Tochigi, Japan). The CML cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (Vitromex, Miami, FL, USA) at 37°C in a humidified atmosphere of 20% O_2, 5% CO_2 and 75% N_2. K562-HA and KCL22-HA cells were cultured continuously in 1.0% O_2 (7.2 mmHg), 5% CO_2 and 94% N_2.[13] Rakicidin A was synthesized and purified as described previously.[14] Imatinib and dasatinib were purchased from a pharmacy. zVAD was purchased from the Peptide Institute (Osaka, Japan). Imatinib, dasatinib, Rakicidin A and zVAD were dissolved in dimethyl sulfoxide and stored at −20°C until required for use. zVAD was used at 50 μM. The cells were preincubated with zVAD for 90 min.

Cell death. Cell viability was measured by the incorporation of propidium iodide (PI). Cells were seeded in a flat-bottomed 48-well plate at a concentration of 1.5×10^5 cells in 300 μL medium in each well and incubated with various concentrations of imatinib, dasatinib or Rakicidin A for 48 h. The mean of three measurements at each concentration was calculated. The IC_{50} values were obtained using the nonlinear regression program CalcuSyn (Biosoft, Cambridge, UK). We also evaluated the combined effects of concurrent Rakicidin A and imatinib treatment on both CML and HA-CML cells and their parental cells, and the results are presented as the combination index (CI). The CI is a method for quantifying drug cytotoxic synergism based on the mass-action law principle derived from enzyme kinetic models.[15,16] Cells were incubated for 48 h with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5 or 2.0 times the IC_{50}) of each agent or both in combination, and the cytotoxic effect

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was measured by PI staining. The CI was calculated as described previously,[17] and the fraction affected (Fa) was calculated at each dilution (i.e. an Fa of 0.25 would correspond to 75% viable cells). This method provides quantification of the synergism (CI <1), additive effect (CI = 1) and antagonistic effect (CI >1) at different dose and effect levels.

Colony-forming cell (CFC) assays. Bone marrow cells from two normal volunteers and CML patients in the chronic phase before imatinib treatment were purchased from AllCells Inc. (Emeryville, CA, USA). Thawed normal cells (2×10^4) and CML cells (1×10^4) were exposed to Rakicidin A, 0.25 μM, and 0.125 and 0.25 μM, respectively. A 0.1 mL volume of these cells was added to 1 mL standard methylcellulose culture medium (MethoCult 04435; StemCell Technologies, Vancouver, BC, Canada) and cultured for 14 days in 20% O_2 or 1% O_2. The numbers of CFC were counted under inverted microscopy (Olympus, Tokyo, Japan). Two dishes per treatment were evaluated.

Apoptosis and cell cycle analysis. The mitochondrial transmembrane potential ($\Delta\psi$m) was determined by staining with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR, USA), as described previously.[18] For analysis of DNA content, the cells were fixed with ice-cold 70% ethanol and then incubated with propidium iodide (PI), as described previously.[19] The percentage of cells that incorporated PI, the $\Delta\psi$m and the number of cells in the $subG_1$ phase of the cell cycle were determined using Facs Diva software (Becton Dickinson, San Jose, CA, USA) and ModiFit-LT software (Becton Dickinson). DNA fragmentation was analysed with the Flow-TACS Apoptosis Detection kit (Trevigen, Gaithersburg, MD, USA).

Morphologic evaluation by light microscopy. K562-HA cells were treated with 0.25 μM Rakicidin A for 12 h, and then subjected to cytospin and stained using a Diff-Quick kit (International Reagents, Kobe, Japan) before examination by light microscopy.

Measurement of caspase activity. Caspase-3 activities in untreated cells or cells treated with 0.25 μM Rakicidin A were measured using a Caspase Fluorometric Protease assay kit (MBL, Tokyo, Japan), as described previously.[18,20]

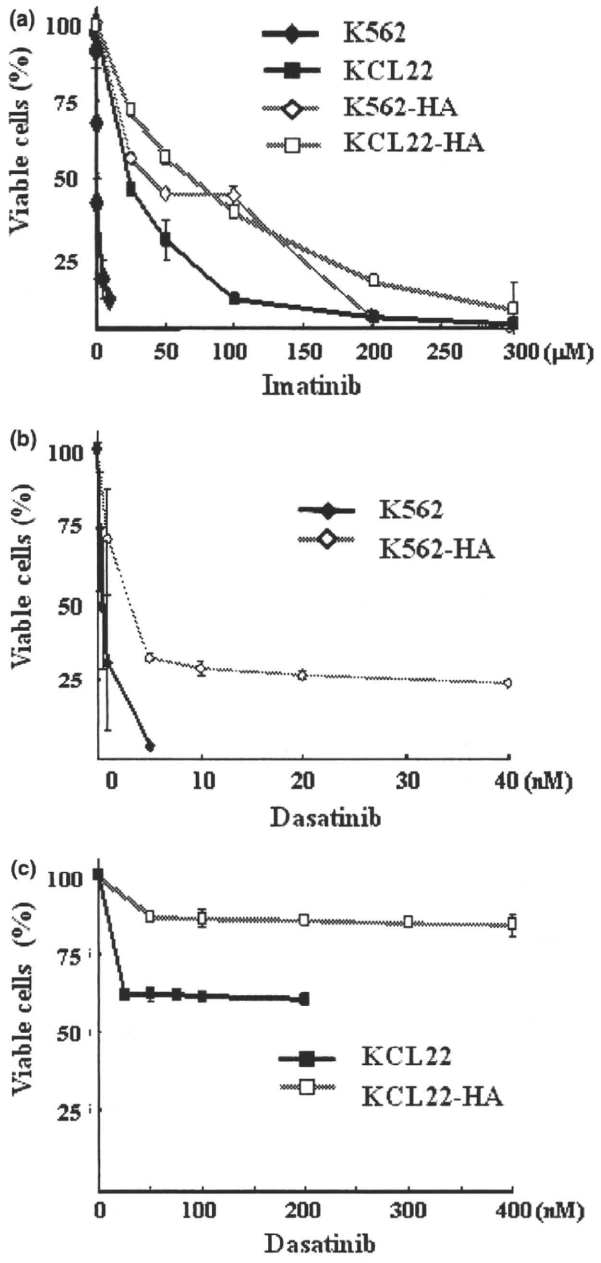

Fig. 1. Tyrosine kinase inhibitors (TKI) resistance induced by hypoxia-adapted chronic myelogenous leukemia (CML) cells *in vitro*. The CML cell lines K562 and KCL22 were subjected to continuous culture in 1.0% oxygen, and hypoxia-adapted subclones of K562-HA and KCL22-HA were selected. These hypoxia-adapted cells were maintained in suspension in low-oxygen conditions for more than 6 months. The cytotoxic effects of the indicated concentrations of imatinib against (a) parental K562 (closed diamond), parental KCL22 (closed square), K562-HA (open diamond) and KCL22-HA (open square) are shown. The cytotoxic effects of dasatinib against (b) parental K562 (closed diamond) and K562-HA (open diamond), and (c) parental KCL22 (closed square) and KCL22-HA (open square) are shown.

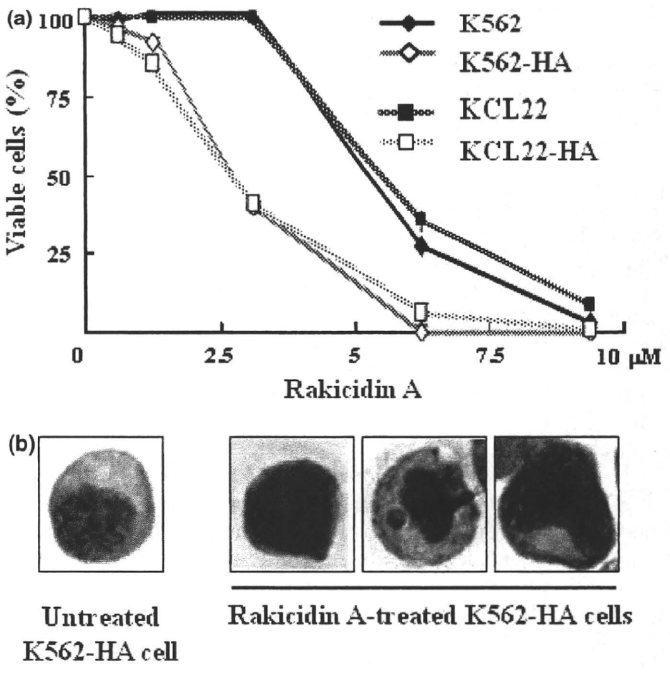

Fig. 2. Hypoxia-selective cell death inducing effects of Rakicidin A. Effect of Rakicidin A on hypoxia-adapted chronic myelogenous leukemia (HA-CML) cells. *In vitro* cytotoxic effects of Rakicidin A against (a) parental K562 (closed diamond), parental KCL22 (closed square), K562/HA (open diamond) and KCL22/HA (open square). (b) Light microscopy analysis of K562-HA cells 12 h after treatment with Rakicidin A (0.25 μM). Morphology of untreated healthy K562-HA (left) and apoptotic cells (right) showed shrinkage, nuclear condensation and fragmentation. Original magnification, ×400.

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Results

Hypoxia adaptation induced TKI resistance in CML cells. To confirm the effect of TKI on hypoxia-adaptation in CML cells,[13] sensitivity to imatinib and dasatinib was investigated in HA-CML cells. The IC_{50} values of imatinib in K562, K562-HA, KCL22 and KCL22-HA cells were 1.36, 41.5, 25.9 and 57.6 μM, respectively. The K562-HA and KCL22-HA cells were highly resistant to imatinib compared to the parental K562 and KCL22 cells, respectively (Fig. 1a). The anti-proliferative effect of dasatinib, which has higher affinity for Abl than imatinib, was also examined in the HA-CML cells and their parental cells. The IC_{50} values of dasatinib for K562 and K562-HA were 0.514 and 2.7 nM, respectively. The IC_{50} values of dasatinib in KCL22 and KCL22-HA cells could not be evaluated. The K562-HA cells were less sensitive than the K562 cells; however, both KCL22-HA and KCL22 cells were less sensitive to dasatinib (Fig. 1b). These results indicated that the K562-HA and KCL22-HA cells were resistant to TKI.

Rakicidin A-induced apoptosis in HA-CML cells. Recently, Yamazaki *et al.*[14] screened 20 000 cultured broths of microorganisms and isolated Rakicidin A, a highly hypoxia-selective cytotoxic compound from *Micromonospora* strain ML99-43F1. The inhibitory effects of Rakicidin A on HA-CML or their parental CML cells was investigated, and the IC_{50} values of Rakicidin A in K562, K562-HA, KCL22 and KCL22-HA cells were 5.24, 1.55, 5.45 and 1.64 μM, respectively. Rakicidin A elicited an approximately fivefold increase in the cytotoxic effects in HA-CML cells compared with the parental cells (Fig. 2a). Examination by light microscopy showed that Rakicidin A treatment induced cell shrinkage, nuclear condensation and fragmentation, which are typical in cells undergoing apoptosis (Fig. 2b). Therefore, Annexin-V staining and mitochondrial outer membrane permeabilization with $DiOC_6$ was used to determine the mechanism of cell death induced by Rakicidin A. The percentage of cells undergoing apoptosis was greater in Rakicidin A-treated K562-HA and KCL22-HA cells than in parental K562 and KCL22 cells (Fig. 3a).

Next, the effects of Rakicidin A on the cell cycle were examined. In both K562-HA and KCL22-HA cells, the number of cells in the subG1 fraction was increased (Fig. 3b), and DNA fragmentation was detected in all phases of the cell cycle in the K562-HA cells (Fig. S1). These findings indicated that Rakicidin A induced apoptotic cell death in HA-CML cells independent of the cell cycle.

Effects of Rakicidin A in normal hematopoietic progenitors and primary CML cells. The effect of Rakicidin A on normal hematopoietic progenitors and primary CML cells was then investigated. When normal progenitor cells were treated with 0.25 μM

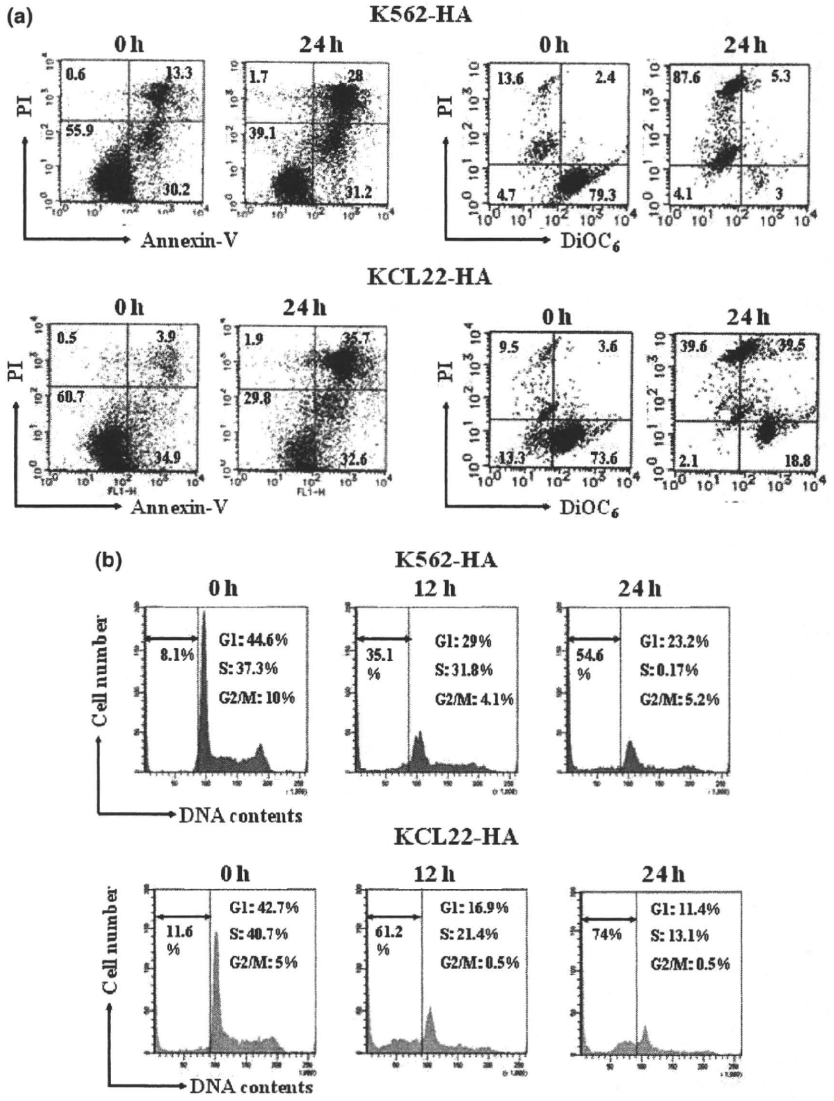

Fig. 3. Induction of apoptosis by Rakicidin A. Flow cytometric analysis of K562-HA (upper panels) and KCL22-HA (lower panels) cells treated with 0.25 μM Rakicidin A for the indicated periods of time. (a) Cells were simultaneously stained with Annexin-V to determine the expression of phosphatidylserine or stained with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) to determine the mitochondrial transmembrane potential, and propidium iodide (PI) to determine the cell viability. (b) Flow cytometric analysis of K562-HA and KCL22-HA cells using PI was performed after 12 and 24 h of Rakicidin A treatment (0.25 μM). The numbers inside each histogram indicate the percentage of the sub G1 fraction. K562-HA (upper panels) and KCL22-HA (lower panels) DNA fragmentation was detected Rakicidin A (0.25 μM) treatment using incorporation of biotinylated nucleotides into the DNA by TdT at the indicated time.

Rakicidin A at 20% O_2 or 1% O_2, the CFC were 113% ± 8% of the untreated control in 20% O_2 and 36% ± 12% of untreated control in 1% O_2. The CFC of primary CML cells were treated with 0.125 and 0.25 μM Rakicidin A at 20% O_2 or 1% O_2, the CFC were 92% ± 23% of untreated control and 93% ± 21% of untreated control for each Rakicidin A concentrations in 20% O_2, and 45% ± 1% and 6% ± 3% of control in 1% O_2 for each Rakicidin A concentrations in 1% O_2, respectively (Fig. 4). These findings indicated that Rakicidin A selectively inhibited colony formation in cells derived from CML patients under hypoxic conditions.

Activation of caspase-3 by Rakicidin A. To investigate the mechanism of Rakicidin A-induced apoptosis, the effect of Rakicidin A on the $\Delta\psi m$ was investigated. In the HA-CML cells, treatment with Rakicidin A decreased the $\Delta\psi m$, suggesting that Rakicidin A activated the mitochondria-mediated apoptosis pathway in these cells. Additionally, fluorometric analysis of caspase activity in the HA-CML cells demonstrated that Rakicidin A activated caspase-3 (Fig. S2). Therefore, we next examined whether inhibiting caspase-3 activity prevented Rakicidin A from inducing cell death in the HA-CML cells. Although caspase-3 activity was effectively blocked by pre-treatment with zVAD (Fig. S2), cell death was still induced, although it was significantly delayed (Fig. 5). These findings suggested that Rakicidin A-induced apoptosis in HA-CML cells is mediated only partially via the mitochondria-mediated pathway and that an alternative mitochondria-independent pathway may exist.

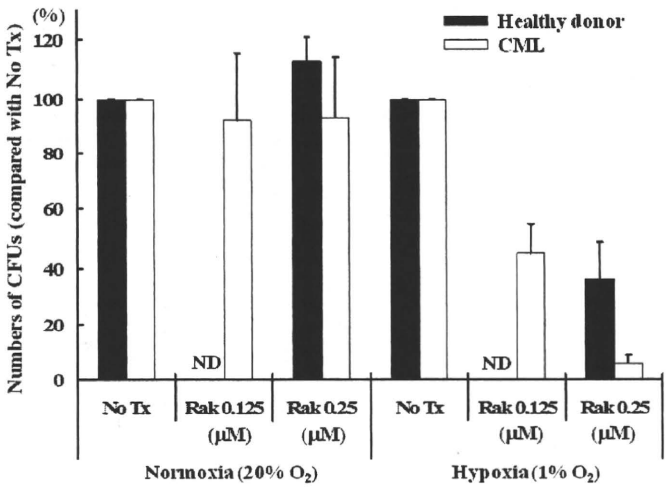

Fig. 4. The effects of Rakicidin A (Rak) in normal hematopoietic progenitors and primary chronic myelogenous leukemia (CML) cells. The effects of Rak on normal hematopoietic progenitors and primary CML cells was investigated using a standard methylcellulose culture assay as described in the Materials and Methods. The rates of inhibitory effects on the colony-forming unit (CFU) in normal progenitor (black bars) and primary CML cells (white bars) at each indicated concentration of Rakicidin A compared with the number of colony-forming cells without Rakicidin A were examined using a colony assay. ND, not done.

Effect of combined treatment with Rakicidin A and imatinib on HA-CML cells. Next, the effect of treatment with both Rakicidin A and imatinib was evaluated in the HA-CML cells. Combination indexes (CI) were calculated according to the method of Chou using CalcuSyn for Windows software as described elsewhere.[15,16,21] This method provides quantitation of synergism (CI <1) and antagonism (CI >1) at different doses and effect levels. The combination of imatinib and Rakicidin A induced a synergistic effect on cell death in both HA-CML cell lines (Fig. 6a–d). In the parental K562 and KCL22 cells, combined treatment is antagonistic rather than synergistic (Fig. 6c–f). These observations suggest that concomitant treatment with Rakicidin A and imatinib selectively augmented growth inhibition of leukemic cells under hypoxic conditions, such as those found in the BM.

Discussion

The prognosis of CML patients has been drastically improved by the use of TKI; however, the success of treatment is still plagued by problems that lead to CML relapse, such as resistance to TKI.[11,22] The TKI are able to kill actively proliferating CML cells, whereas resting CML cells are largely resistant to treatment.[23] The CML stem cells are rare and divide less frequently, and thus exist primarily as quiescent cells. Therefore, CML stem cells are resistant to TKI and represent a reservoir for relapse. It is speculated that leukemic stem cells are kept in a quiescent state in the BM niche microenvironment. Recently, it was revealed that leukemic stem cells that reside within the endosteal region exhibit relatively high resistance to anticancer drugs.[24] In particular, the

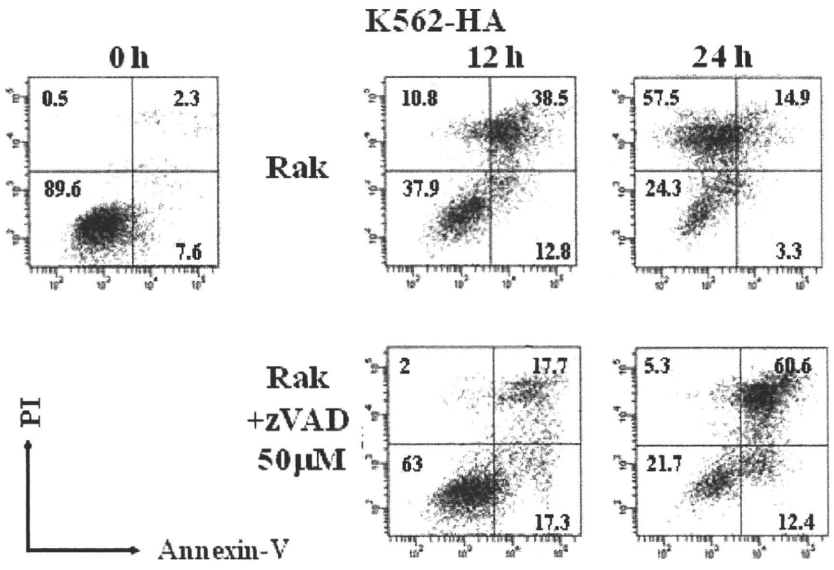

Fig. 5. The Rakicidin A inducing apoptosis delayed effect of pan-caspase inihibitor, zVAD. Effects of pre-treatment with or without 50 μM zVAD on K562-HA cell death induced by 0.25 μM Rakicidin A at the indicated periods. zVAD did not prevent cell death by Rakicidin A treatment, but showed a delay in Rakicidin A-inducing apoptosis (lower panels). PI, propidium iodide.

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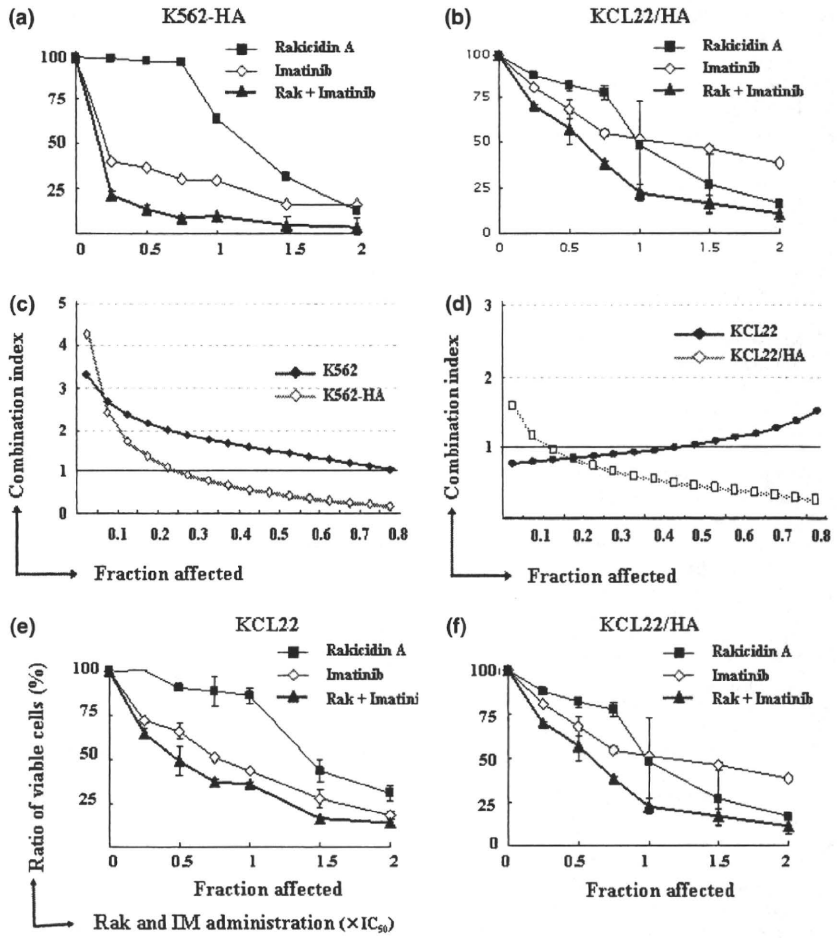

Fig. 6. The combined effects of imatinib amd Rakicidin A on K562 and K562-HA. Evaluation of the combined effects of imatinib and Rakicidin A on K562-HA (a) and KCL22-HA (b). Cells were incubated for 48 h with six concentrations (0.25, 0.5, 0.75, 1, 1.5 or 2 times the IC_{50}) of each agent or both in combination. The IC_{50} of Imatinib and Rakicidin A were previously described in Figure 1(a–c). The killing curves of the concurrent administration of imatinib and Rakicidin A are shown. (c,d) The combination index (CI)-fraction affected (Fa) plots are shown. The combination index was determined with the nonlinear regression program CalcuSyn. The combined effects of imatinib and Rakicidin A on K562 (e) and KCL22 (f) were also evaluated as described above.

BM endosteum is recognized as a severely hypoxic region. Hypoxia maintains both normal and leukemic stem cells in the resting phase.[6,25] Previously, we investigated the microenvironment of leukemic cells in the BM and confirmed that leukemic cells survived under severely hypoxic conditions, resulting in resistance to TKI and other anticancer agents (Fig. 1).[13] Additionally, hypoxia inhibits Bcr-Abl phosphorylation and fosters the establishment of a more primitive population of CML cells, which might explain why those cells are less sensitive to TKI.[13,26,27] Therefore, the development of a novel therapeutic approach for targeting hypoxic cells in the BM may lead to a cure for CML patients.

Although the target molecule of Rakicidin A has not been identified, Rakicidin A exhibits approximately 17.5-fold more cytotoxicity in solid tumor cells in a hypoxic environment compared with cells in normoxic conditions.[14] The cytotoxic effect of Rakicidin A was also observed under transient hypoxic conditions, but Rakicidin A exhibited the highest degree of cytotoxicity in CML cells incubated under long term hypoxic conditions (Fig. 2a). It is believed that CML stem cells reside in the juxta endosteum lesion or niche, which allows the leukemic cells to evade cell-death stimuli.[28,29] Hypoxia is one of the important factors that constitute the BM niche.[30] Thus, the hypoxia-specific cytotoxicity induced by Rakicidin A may be able to eradicate leukemic stem cells.

We demonstrated that Rakicidin A inhibited the proliferation of HA-CML cells by inducing apoptosis. As shown in Figure 4a, Rakicidin A decreased the mitochondrial transmembrane potential in HA-CML cells, indicating that Rakicidin A induces apoptosis through the intrinsic pathway. However, treatment with zVAD, a pan-caspase inhibitor that blocks the activation of caspase-3, delayed but did not prevent apoptosis in K562-HA cells. The delayed apoptotic response was also observed in UV-irradiated Hela cells, due to suppressed caspase-3 activity and reduced activity of the caspases downstream of cytochrome *c* release.[31] These different responses to apoptotic stimuli may depend on cell-type specific apoptosis signalling pathways. It can be speculated that Rakicidin A also activates caspase-independent apoptosis pathways in HA-CML cells, such as apoptosis inducing factor (AIF) or Calpain.[32,33] Further investigation is necessary to clarify this issue.

Novel hypoxia-sensitizers including tirapazamine, a reducing agent, and HIF-1 inhibitor, including gene therapy, have recently been developed.[34–38] Moreover, several anticancer agents inhibit the proliferation of cancer cells by producing reactive oxygen species.[39,40] However, Rakicidin A does not act as a reductant because Rakicidin A has neither nitric oxide (NO) residues nor quinones in its structure. In addition, Rakicidin does not increase the transcriptional activity of HIF-1.[14] Interestingly, a previous study reported that Rakicidin A failed to induce apoptosis in solid cancer cells.[14] Further investigation is required to clarify the cytotoxic mechanisms of Rakicidin A in different cancer cell types.

In conclusion, Rakicidin A effectively induced apoptosis in HA-CML cells with primitive characteristics. Additionally, combined treatment with Rakicidin A and imatinib exhibited synergistic cytotoxic effects in HA-CML cells. These observations suggest that Rakicidin A may be a useful agent for the eradication of CML stem cells.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effects of Rakicidin A on K562-HA cells.

Fig. S2. The effects of Caspase-3 activation with or without Rakicidin A and/or zVAD on K562-HA cells.

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Glyoxalase-I is a novel target against Bcr-Abl$^+$ leukemic cells acquiring stem-like characteristics in a hypoxic environment

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Abl tyrosine kinase inhibitors (TKIs) such as imatinib and dasatinib are ineffective against Bcr-Abl$^+$ leukemic stem cells. Thus, the identification of novel agents that are effective in eradicating quiescent Bcr-Abl$^+$ stem cells is needed to cure leukemias caused by Bcr-Abl$^+$ cells. Human Bcr-Abl$^+$ cells engrafted in the bone marrow of immunodeficient mice survive under severe hypoxia. We generated two hypoxia-adapted (HA)-Bcr-Abl$^+$ sublines by selection in long-term hypoxic cultures (1.0% O_2). Interestingly, HA-Bcr-Abl$^+$ cells exhibited stem cell-like characteristics, including more cells in a dormant, increase of side population fraction, higher β-catenin expression, resistance to Abl TKIs, and a higher transplantation efficiency. Compared with the respective parental cells, HA-Bcr-Abl$^+$ cells had higher levels of protein and higher enzyme activity of glyoxalase-I (Glo-I), an enzyme that detoxifies methylglyoxal, a cytotoxic by-product of glycolysis. In contrast to Abl TKIs, Glo-I inhibitors were much more effective in killing HA-Bcr-Abl$^+$ cells both *in vitro* and *in vivo*. These findings indicate that Glo-I is a novel molecular target for treatment of Bcr-Abl$^+$ leukemias, and, in particular, Abl TKI-resistant quiescent Bcr-Abl$^+$ leukemic cells that have acquired stem-like characteristics in the process of adapting to a hypoxic environment.
Cell Death and Differentiation (2010) **0,** 000–000. doi:10.1038/cdd.2010.6

Chronic myeloid leukemia (CML) is a disorder of hematopoietic stem cells caused by the constitutive activation of the Bcr-Abl tyrosine kinase.[1] Treatment of CML has been drastically improved by the development of imatinib mesylate, an Abl tyrosine kinase inhibitor (TKI).[2,3] However, imatinib resistance is frequently observed, especially in patients with advanced-stage disease.[4] Second-generation Abl TKIs, such as dasatinib,[5] nilotinib[6] and INNO-406 (formerly NS-187),[7–9] potently overcome most imatinib resistance mechanisms.[10] However, whether TKI alone can kill all the cancerous cells, which is a prerequisite for curing CML, is in doubt because TKIs are much less effective against quiescent CML stem cells.[11,12]

Bone marrow (BM) is a hypoxic tissue, particularly at the epiphysis, which is distant from the BM arterial blood supply (Supplementary Figure 1).[13] In addition, leukemic cells are more hypoxic than normal cells in the BM because of cell crowding, due to accelerated cell growth, as well as the anemia that commonly accompanies the progression of leukemia.[14,15] The oxygen supply is frequently inadequate for the level of oxygen consumption in the microenvironment of rapidly proliferating cancer cells.[16] Although not identified conclusively until today, Bcr-Abl$^+$ CML stem cells are in a quiescent state in the niche. In addition, human primary leukemic cells expressing CD34 inoculated into immunodeficient mice initially populate the hypoxic epiphysial region.[17] Thus, it is likely that quiescent leukemic cells predominantly reside in and survive in a hypoxic BM environment.

Most cancer cells that have adapted to hypoxia are resistant to a variety of cell death stimuli.[18,19] A shift in energy production from aerobic to anaerobic respiration causes a number of dramatic changes in cell phenotype, including the accumulation of hypoxia-specific by-products, alterations in the cell cycle, and resistance to chemotherapeutic drugs. Given these observations, we hypothesized that adaptation to hypoxia is one of the causes of minimal residual disease in patients treated with Abl TKIs. The molecular mechanisms of adaptation to hypoxia may provide new targets for cancer-specific therapies that are effective against cells in hypoxic microenvironments.[20,21]

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Keywords: hypoxia; leukemia; stem cell; Glo-I; Abl tyrosine kinase

Abbreviations: TKI, tyrosine kinase inhibitor; CML, chronic myeloid leukemia; BM, bone marrow; HA, hypoxia-adapted; Glo-I, glyoxalase-I; NOG, NOD/SCID/γ_c^{null}; BBGC, *S-p*-bromobenzyl glutathione cyclopentyl diester; COTC, 2-crotonyloxymethyl-4,5,6-trihydroxycylohex-2-enone; $\Delta\psi$m, mitochondrial transmembrane potential; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; ECL, enhanced chemiluminescence; PMSF, phenylmethylsulfonyl fluoride; PI, propidium iodide; PB, peripheral blood

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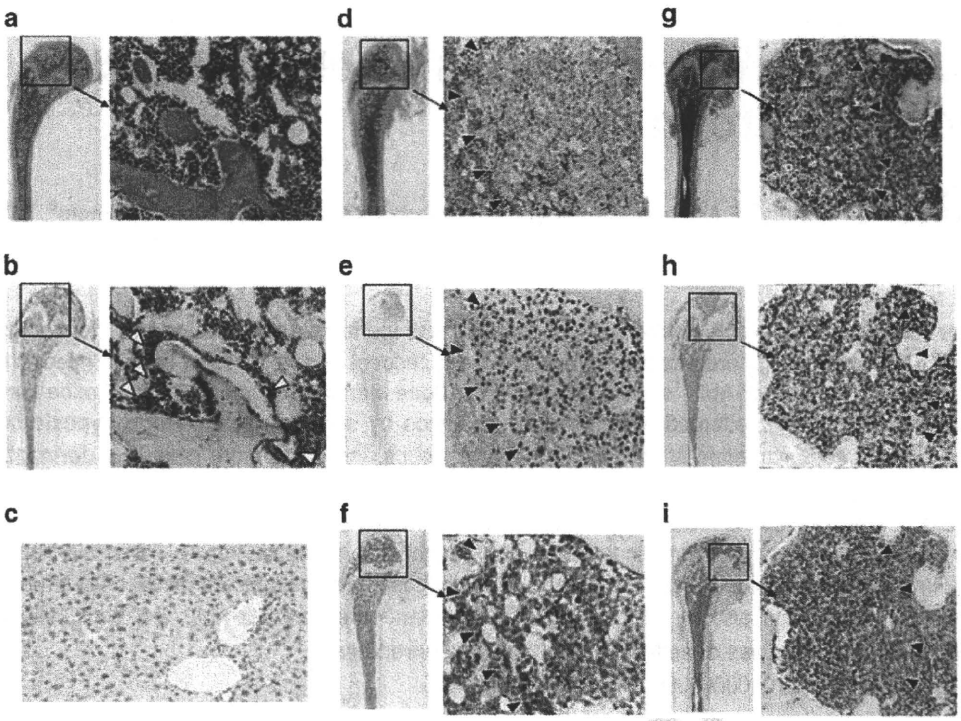

Figure 1 Engraftment of human Bcr-Abl$^+$ leukemic cells in mice. The femur of a non-engrafted mouse was stained with (**a**) hematoxylin–eosin (H&E) and (**b**) anti-pimonidazole (Ab), and the (**c**) liver was stained with anti-pimonidazole. In (**a**), the bone marrow (BM) was populated by normal mouse hematopoietic cells, and in (**b**), only a small population of normal cells was positive for pimonidazole (open triangles, Δ) along the endosteum. In (**c**), no hepatic cells were positive for pimonidazole. The femur of a K562-engrafted mouse was stained with (**d**) H&E, (**e**) antihuman Ki-67 or (**f**) anti-pimonidazole. In (**d**), the epiphysis of the bone was populated by engrafted chronic myeloid leukemia (CML) cells (closed triangles, ▲). In (**e**), the area of Ki-67-positive cells (closed triangles) was in good agreement with the area of engrafted CML cells in (**d**). Most of the engrafted CML cells were positive for Ki-67, but a few cells were negative. In (**f**), most of the engrafted CML cells (encircled by closed triangles, ▲) were positive for pimonidazole. The femur of a mouse engrafted with primary Bcr-Abl$^+$ leukemic cells from a Ph$^+$ acute lymphoblastic leukemia (Ph$^+$ ALL) patient was stained with (**g**) H&E, (**h**) antihuman Ki-67 or (**i**) anti-pimonidazole

Results

Bcr-Abl$^+$ cells in the BM survive in hypoxic conditions. Four NOD/SCID/γ_c^{null} (NOG) mice were inoculated with 1.0×10^6 K562 cells, a human cell line established from a Bcr-Abl$^+$ CML patient. Mice were killed 35 days after transplantation and examined for engraftment. Viable K562 engraftments in the BM were identified in three of the four mice. In the mouse with failed engraftment (Figure 1a), only a small population of normal cells were positive for pimonidazole, which specifically accumulates in hypoxic cells ($<1.3\%$ O_2 concentration) along the endosteum (Figure 1b). Liver cells from this were also negative for pimonidazole (Figure 1c). The transplanted K562 cells initially populated the epiphysis in recipient NOG mice. The engrafted cells were easily distinguished from normal mouse hematopoietic cells by their larger size and prominent nuclei (Figure 1d). Immunohistochemical staining with an antibody specific for human Ki-67, which is expressed in actively cycling but not quiescent cells (G_0) (Figure 1e),[22] also confirmed that K562 cells were successfully engrafted. The area of Ki-67-positive staining was in good agreement with the engraftment area estimated by cell morphology (Figures 1d and e). Although most of the engrafted cells were positive for Ki-67, a few cells were not (Supplementary Figure 2), suggesting that some engrafted K562 cells may have entered a quiescent G_0 state.[22] The majority of engrafted K562 cells were also positively labeled by pimonidazole (Figure 1f). Next, we engrafted NOD/SCID mice with primary Bcr-Abl$^+$ cells from a Ph$^+$ acute lymphoblastic leukemia (Ph$^+$ ALL) patient. Engrafted primary Bcr-Abl$^+$ cells (Figure 1g) were very similar to engrafted K562 cells (Figures 1e and f) in Ki-67 expression and pimonidazole staining (Figures 1h and i). These results indicate that both the engrafted Bcr-Abl$^+$ cell line K562 and the primary leukemic cells survive in the severely hypoxic conditions of the BM.

HA-CML cell lines. To generate hypoxia-adapted (HA)-CML cells, four CML-derived cell lines, K562, KCL22, BV173 and MYL, were continuously cultured under hypoxic conditions (1.0% O_2). Most cells were arrested in the G_1 phase of the cell cycle 2 days after transfer to hypoxic conditions as shown by an increase in the percentage of sub-G_1 cells in all four cell lines. Most of these cells underwent apoptosis within 7 days, and none of the BV173 or MYL cells survived more than 7 days (Supplementary Figure 3). In contrast, a small fraction of K562 and KCL22 cells survived in 1.0% O_2 for more than 7 days. We isolated these HA sublines of K562 and KCL22 (termed K562/HA and KCL22/HA, respectively), and these cells continued to proliferate under 1.0% O_2 for more than a year (Figures 2a and b). The

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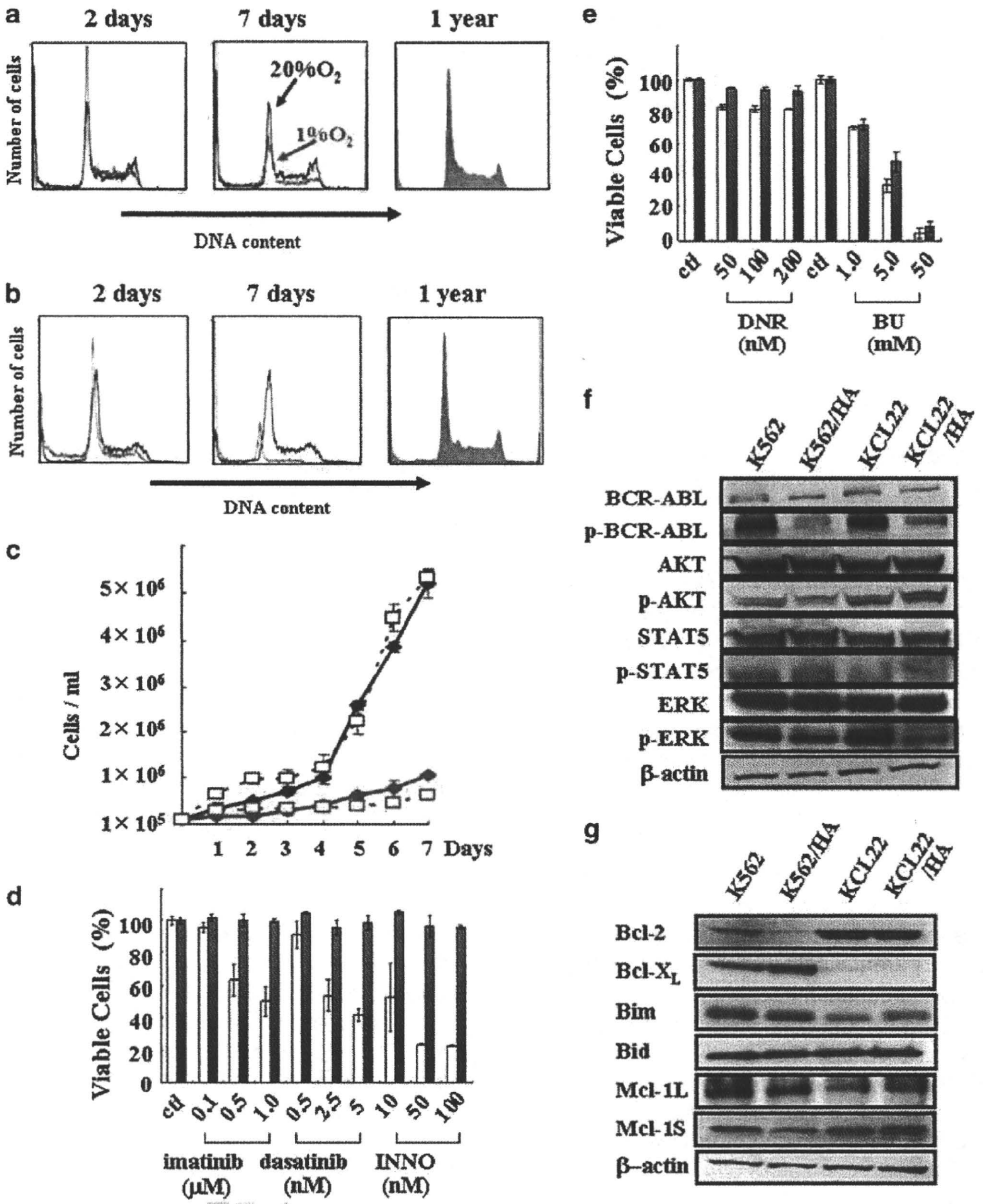

Figure 2 Characteristics of hypoxia-adapted chronic myeloid leukemia (HA-CML) cells. DNA histograms of (**a**) K562 and (**b**) KCL22 cells incubated in 1.0% O_2 (red) and 20% O_2 (blue) for 2 days, 7 days and 1 year. (**c**) Growth of K562 (blue, solid line), K562/HA (red, solid line), KCL22 (blue, broken line) and KCL22/HA (red, broken line) cells. K562 and KCL22 cells were cultured in 20% O_2, and K562/HA and KCL22/HA cells were cultured in 1.0% O_2. Antiproliferative effects of the indicated concentrations of (**d**) Abl tyrosine kinase inhibitors (TKIs) and (**e**) alkylating agents on parental K562 (white column) and K562/HA (black column) cells. (**f**) Protein expression and phosphorylation of Bcr-Abl and related kinases in parental CML cell lines and the corresponding HA subclones. (**g**) Protein levels of the indicated anti- and proapoptotic molecules in parental CML cell lines and the respective HA subclones

growth rate of both HA-CML cell lines *in vitro* was slower than that of the corresponding parental cells. Although the cell cycle distribution of both the HA cell lines was similar to that of the parental cell lines after 1 year (Figures 2a and b), the growth of the HA-CML cells was still much slower than their respective parental cell lines (Figure 2c).

We next examined the cytotoxic effects of the Abl TKIs, including imatinib, dasatinib and INNO-406, on K562, K562/HA, KCL22 and KCL22/HA cells. K562/HA cells were highly resistant to all Abl TKIs examined, compared with the parental K562 cells (Figure 2d, Table 1). As the parental KCL22 cells are intrinsically resistant to imatinib and INNO-406, we examined the antiproliferative effects of dasatinib, which has higher affinity for Abl than the other Abl TKIs, in KCL22 and KCL22/HA cells. KCL22/HA cells were approximately 50-fold less sensitive to dasatinib than the parental KCL22 cells (Table 1). Both K562/HA and KCL22/HA cells were less sensitive to the alkylating agents daunorubicin and busulfan than the respective parent cells (Figure 2e, Table 1). These results indicate that the K562/HA and KCL22/HA cells acquired resistance to a wide range of antileukemia agents during adaptation to hypoxia.

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Table 1 IC_{50} scores of tyrosine kinase inhibitors, alkylating agents and Glo-I inhibitors for human CML cell lines and their hypoxia-adapted (HA) subclones

	K562	K562/HA	KCL22	KCL22/HA
ABL TKIs				
Imatinib (nM)	900	7400	—	—
Dasatinib (nM)	3.6	8.9	46.2	2264
INNO-406 (nM)	10.7	142.6	—	—
Alkylating agents				
Daunorubicin (nM)	—	—	105	954.3
Busulfan (mM)	2.4	4.5	2.2	3.0
Glo-I inhibitor				
BBGC (μM)	21.6	5.7	40.7	12.6
COTC (μM)	45.9	16.8	29.6	17.9
m-GFN (μM)	>300	230.2	>300	174.2

BBGC, *S*-*p*-bromobenzyl glutathione cyclopentyl diester; CML, chronic myeloid leukemia; COTC, 2-crotonyloxymethyl-4,5,6-trihydroxycylohex-2-enone; Glo-I, glyoxalase-I; TKI, tyrosine kinase inhibitor.

The levels of phosphorylation of Bcr-Abl and its downstream effector Erk were reduced in K562/HA and KCL22/HA cells under hypoxic conditions (1.0% O_2), compared with levels in the parental cells cultured in normoxic conditions. The levels of phosphorylated Akt (p-Akt) and Stat5 (p-Stat5) were similar in both HA and parental cells (Figure 2f). We examined the levels of apoptosis-related proteins such as Bcl-2, Bcl-xL, Bim, Bad, Mcl-1L and Mcl-1S. The levels of Bcl-2 were lower in K562/HA cells than in K562 cells, whereas the levels of Bcl-xL were higher. Bim and Bid interact with Bcl-2 and Bcl-xL to induce apoptosis. The levels of Bim and Bid in K562/HA cells were not different from those in the parental cells. The level of the antiapoptotic Mcl-1L was the same in K562/HA and parental cells, whereas the level of the proapoptotic Mcl-1S decreased in K562/HA cells (Figure 2g). In contrast to K562 and K562/HA cells, there were no differences between KCL22 and KCL22/HA cells (Figure 2g). These findings suggest that adaptation to hypoxia may have some impact on the mechanisms for executing apoptosis, although further investigation will be required to confirm this hypothesis.

Glo-I in HA-CML cells. We examined the ATP levels in parental and HA-CML cells. The amounts of ATP in K562/HA and KCL22/HA cells were 73.0 and 93.2%, respectively, of the levels in their respective parental cell lines under normoxia (Figure 3a). In addition, both HA cell lines exhibited high levels of glucose consumption and lactate production compared with their respective parental cells (Supplementary Figure 4). Normally, one molecule of glucose produces approximately 34–36 ATPs by aerobic respiration, but only 2 ATPs by glycolysis (Figure 3b). Our results suggest that anaerobic glycolysis generates sufficient ATP for survival of HA-CML cells in hypoxic conditions.

When cells preferentially use glycolysis for energy production, glycolysis-specific cytotoxic by-products, such as methylglyoxal, accumulate intracellularly. As glyoxalase-I (Glo-I) protects cells and promotes cell survival by detoxifying methylglyoxal (Figure 3b), we examined Glo-I in HA-CML cells. Both K562/HA and KCL22/HA cells had higher Glo-I protein levels (Figure 3c) and enzymatic activity (Figure 3d) than the parental cells. Furthermore, the Glo-I protein level increased markedly in the parental K562 cells after cultivation in 1.0% O_2 for 28 days (Figure 3e). In addition, we examined the Glo-I protein levels in primary Bcr-Abl$^+$ cells from CML and Ph$^+$ ALL patients. Both samples of primary Bcr-Abl$^+$ cells possessed higher Glo-I protein levels than normal BM or PB cells (Supplementary Figure 5). These findings indicate that Glo-I expression was induced not only in the artificially generated HA-CML cell lines but also in primary Bcr-Abl$^+$ cells, suggesting that primary leukemic cells may be adapted to hypoxia *in vivo*.

High Glo-I expression is sustained in HA-CML cells after 6 months in normoxia. The HA cells returned to normoxic culture conditions revert to the parental cell proliferation rate after 5 days (Supplementary Figure 6a), and the fraction of cells in G_0 decreases within 48 h after return to normoxic conditions (Supplementary Figure 6b). Interestingly, the high level of Glo-1 expression developed by HA-CML cells was sustained after 6 months in culture under normoxic conditions (Supplementary Figure 6c).

Engraftment of HA-CML cells in NOG mice. We established stable subclones of K562 and K562/HA cells (K562$^{Luc\text{-}EGFP}$ and K562/HA$^{Luc\text{-}EGFP}$, respectively) that coexpressed luciferase and enhanced green fluorescent protein (EGFP). When we examined the bioluminescence of the cells, K562$^{Luc\text{-}EGFP}$ cells produced approximately 10 times more bioluminescence than did K562/HA$^{Luc\text{-}EGFP}$ cells (Figure 4a). As the strength of luciferase bioluminescence depends on the availability of ATP and oxygen, we hypothesized that these factors may have a role in the reduced bioluminescence of K562/HA$^{Luc\text{-}EGFP}$ cells. Engraftment and proliferation of K562$^{Luc\text{-}EGFP}$ and K562/HA$^{Luc\text{-}EGFP}$ cells in NOG mice were monitored using *in vivo* imaging (Figure 4a). Despite the lower level of bioluminescence in K562/HA$^{Luc\text{-}EGFP}$ cells in culture, there were no significant differences in total photon emission between K562$^{Luc\text{-}EGFP}$- and K562/HA$^{Luc\text{-}EGFP}$-engrafted mice before day 34. After day 34, the total photon emission of K562/HA$^{Luc\text{-}EGFP}$-engrafted mice increased much more sharply than that of K562$^{Luc\text{-}EGFP}$ mice (Figure 4b). In addition, K562/HA$^{Luc\text{-}EGFP}$-transplanted mice died significantly earlier than did K562$^{Luc\text{-}EGFP}$ mice (Figure 4c). These results indicate that K562/HA$^{Luc\text{-}EGFP}$ cells engrafted more efficiently in NOG mice than did the parental K562$^{Luc\text{-}EGFP}$ cells.

To investigate why K562/HA$^{Luc\text{-}EGFP}$ cells engrafted more efficiently, we examined the cell cycle distribution of K562 and K562/HA cells. The percentages of cells in G_0 in K562 and K562/HA cells were $0.87 + 0.58$ and $4.9 + 2.1$%, respectively, indicating that the K562/HA grafts included more quiescent cells than did the parental line (Figures 4d and e). Next, we examined the expression of c-kit, Tie-2, CXCR4, Notch, N-cadherin, VLA-4, LFA-1 and CD44, all of which have been reported to indicate stemness.[23] There was no difference in the expression levels of these proteins in K562/HA or KCL22/HA cells and the respective parental cell lines (data not shown). Interestingly, both HA cell lines expressed higher levels of β-catenin, which is thought to be important for

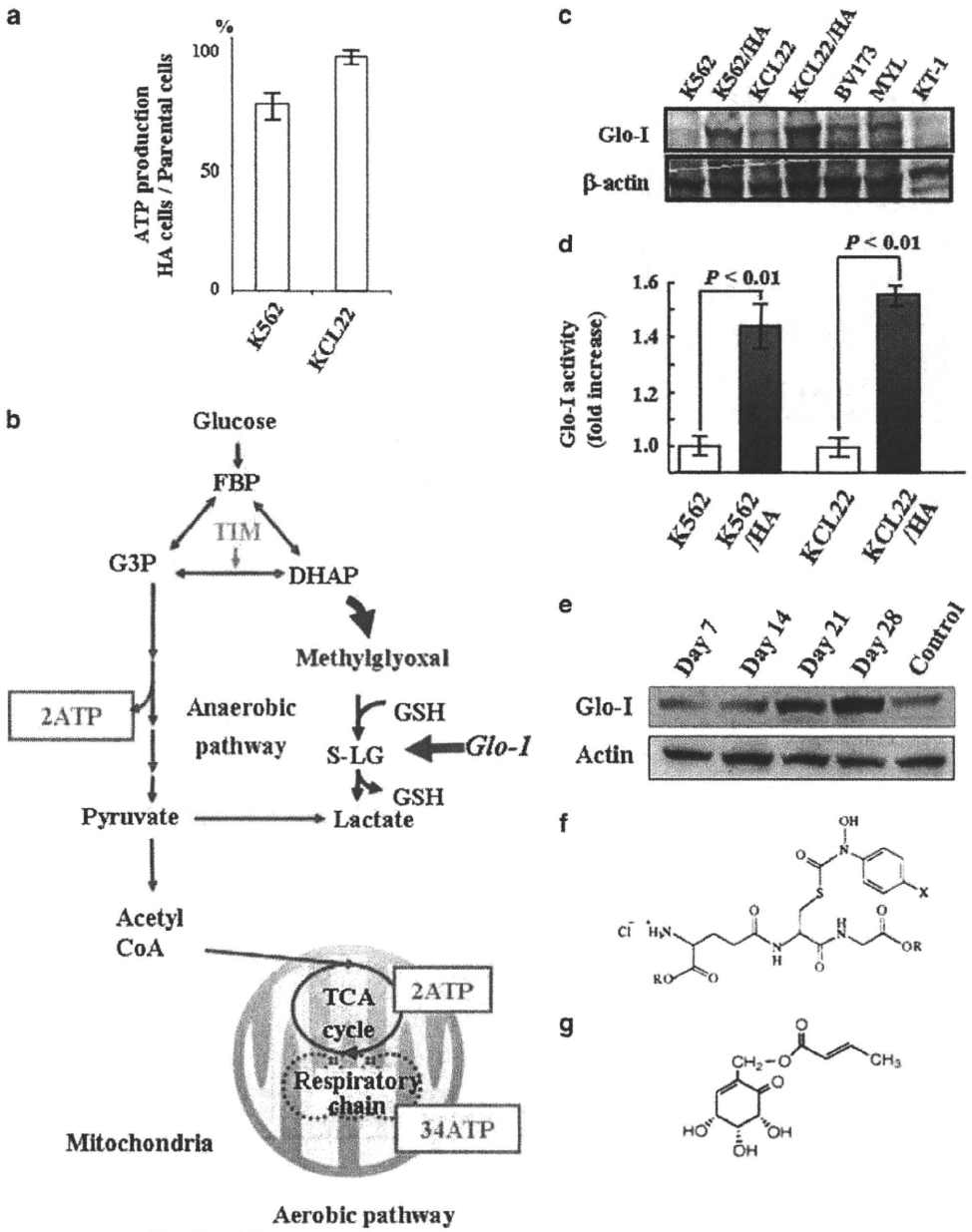

Figure 3 The influences of adaptation to hypoxia on ATP production and glyoxalase-I (Glo-I) activity. (**a**) ATP production is expressed as the ratio of production in hypoxia-adapted (HA) cells relative to parental chronic myeloid leukemia (CML) cells. (**b**) Schematic illustration of aerobic and anaerobic ATP production pathways. Methylglyoxal accumulates in cells that use the anaerobic pathway, and Glo-I functions to detoxify methylglyoxal. (**c**) Glo-I protein levels in parental CML cell lines and the respective HA subclones. (**d**) Glo-I activity in parental CML cell lines and the respective HA subclones. (**e**) Glo-I protein levels under hypoxic conditions. Glo-I expression was clearly evident 21 days after initiation of hypoxia (1.0% O_2). Chemical structures of *S-p*-bromobenzyl glutathione cyclopentyl diester (BBGC) (**f**) and 2-crotonyloxymethyl-4,5,6-trihydroxycylohex-2-enone (COTC) (**g**)

production of CML stem cells (Figure 4f).[24] In addition, the K562/HA cell line contained more cells with the side population marker for cancer stem cells (Figure 4g).[25] These findings suggest that the adaptation to hypoxia induces putative stem/progenitor cell-like characteristics in Bcr-Abl$^+$ cells.

Effect of Glo-I inhibitors on HA-CML cells. *S-p*-bromobenzyl glutathione cyclopentyl diester (BBGC) (Figure 3f) is a specific cell-permeable inhibitor of Glo-I,[26] and 2-crotonyloxymethyl-4,5,6-trihydroxycylohex-2-enone (COTC) (Figure 3g) is an inhibitor of Glo-I and glutathione.[27] Recently, methyl-gerfelin (Supplementary Figure 7a) was also identified as a Glo-I inhibitor.[28] Although HA-CML cells were resistant to the cytotoxic effects of Abl TKIs and alkylating agents (Figures 2d and e, Table 1), BBGC, COTC and methyl-gerfelin were more strongly cytotoxic in K562/HA and KCL22/HA cells than in the parental cell lines (Figures 5a–d, Supplementary Figures 7b and c).

To determine the mechanism of cell death induced by BBGC, we examined Annexin V staining (Supplementary

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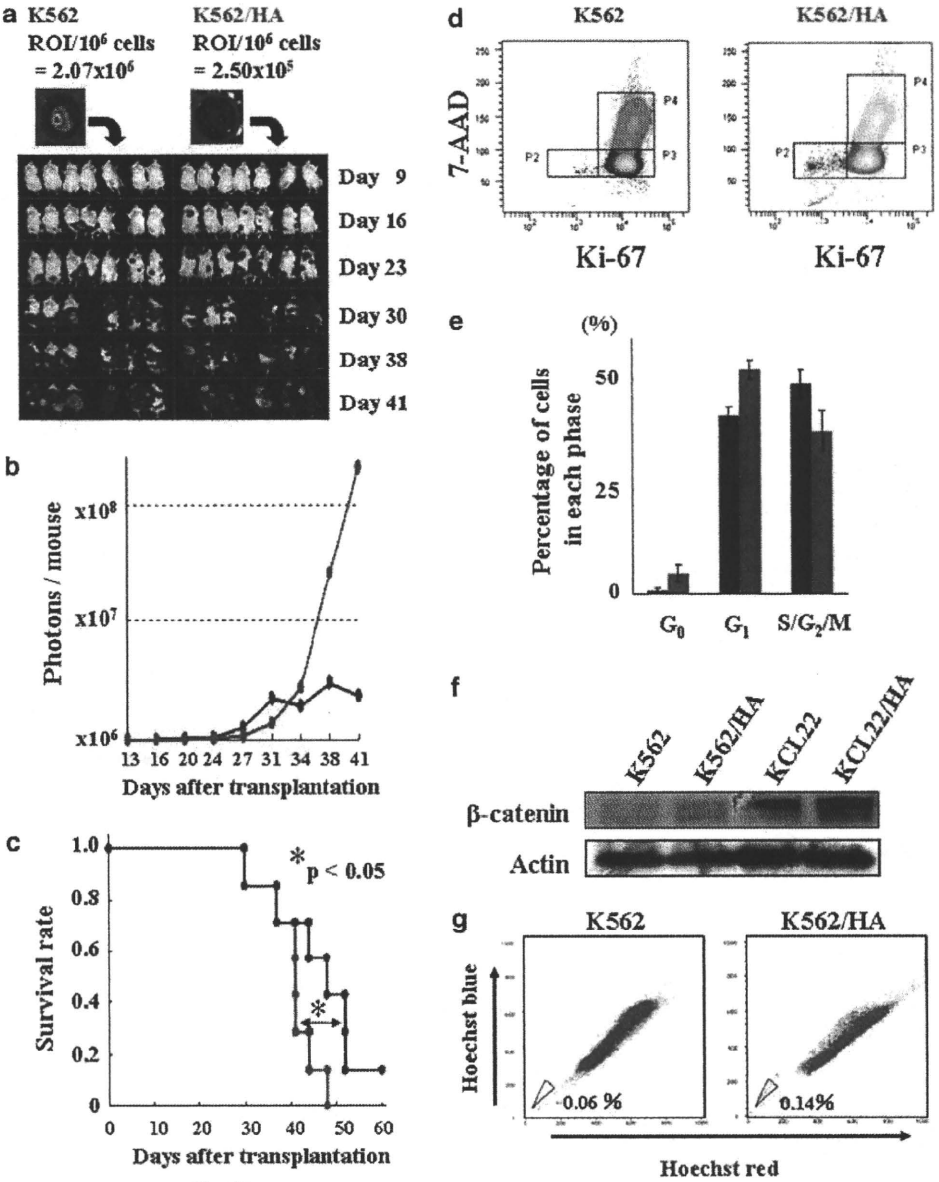

Figure 4 K562/hypoxia-adapted (HA) cells engraft more efficiently in NOD/SCID/γ_c^{null} (NOG) mice. (**a**) Whole animal experiments using NOG mice inoculated with K562$^{Luc\text{-}EGFP}$ or K562/HA$^{Luc\text{-}EGFP}$ cells. Engraftment was monitored by *in vivo* imaging. (**b**) Total photon emission from mice inoculated with K562$^{Luc\text{-}EGFP}$ (blue) or K562/HA$^{Luc\text{-}EGFP}$ (red) cells. (**c**) Survival rates of mice inoculated with K562$^{Luc\text{-}EGFP}$ (blue) or K562/HA$^{Luc\text{-}EGFP}$ (red) cells. (**d**) Cell cycle distributions of K562 and K562/HA determined by double staining with Ki-67 and 7-AAD. (**e**) The percentages of cells in each phase of the cell cycle in K562 (blue) and K562/HA (red), (**f**) β-catenin protein expression levels in K562, K562/HA, KCL22 and KCL22/HA cells. (**g**) Number of side population cells in K562 and K562/HA cells

Figures 8a–d) and mitochondrial outer membrane permeabilization (Supplementary Figures 8e–h). The proportion of cells undergoing apoptosis was greater in the BBGC- and COTC-treated K562/HA and KCL22/HA cells than in the parental cells (Supplementary Figures 9a–d). These results indicate that HA-CML cells were dependent on Glo-I activity for survival under hypoxic conditions, whereas the parental cells, cultured in normoxic conditions, were not dependent on Glo-I activity.

To evaluate the *in vivo* antileukemic activity of BBGC, we treated NOG mice inoculated with K562$^{Luc\text{-}EGFP}$ or K562/HA$^{Luc\text{-}EGFP}$ cells with BBGC. BBGC had no apparent effect on the survival of K562$^{Luc\text{-}EGFP}$-engrafted mice (Figure 5e). However, it significantly prolonged the survival of K562/HA$^{Luc\text{-}EGFP}$-transplanted mice (Figure 5f). The body weight of BBGC-treated mice did not decrease during the course of treatment (data not shown). These findings indicate that BBGC has potent antileukemic effects *in vivo*, and preferentially targets CML cells with higher Glo-I activity, with minimal associated toxicity.

Discussion

Abl TKIs can induce apoptosis only in actively proliferating Bcr-Abl$^+$ cells, making these drugs much less active against CML stem cells, which are predominantly in a quiescent

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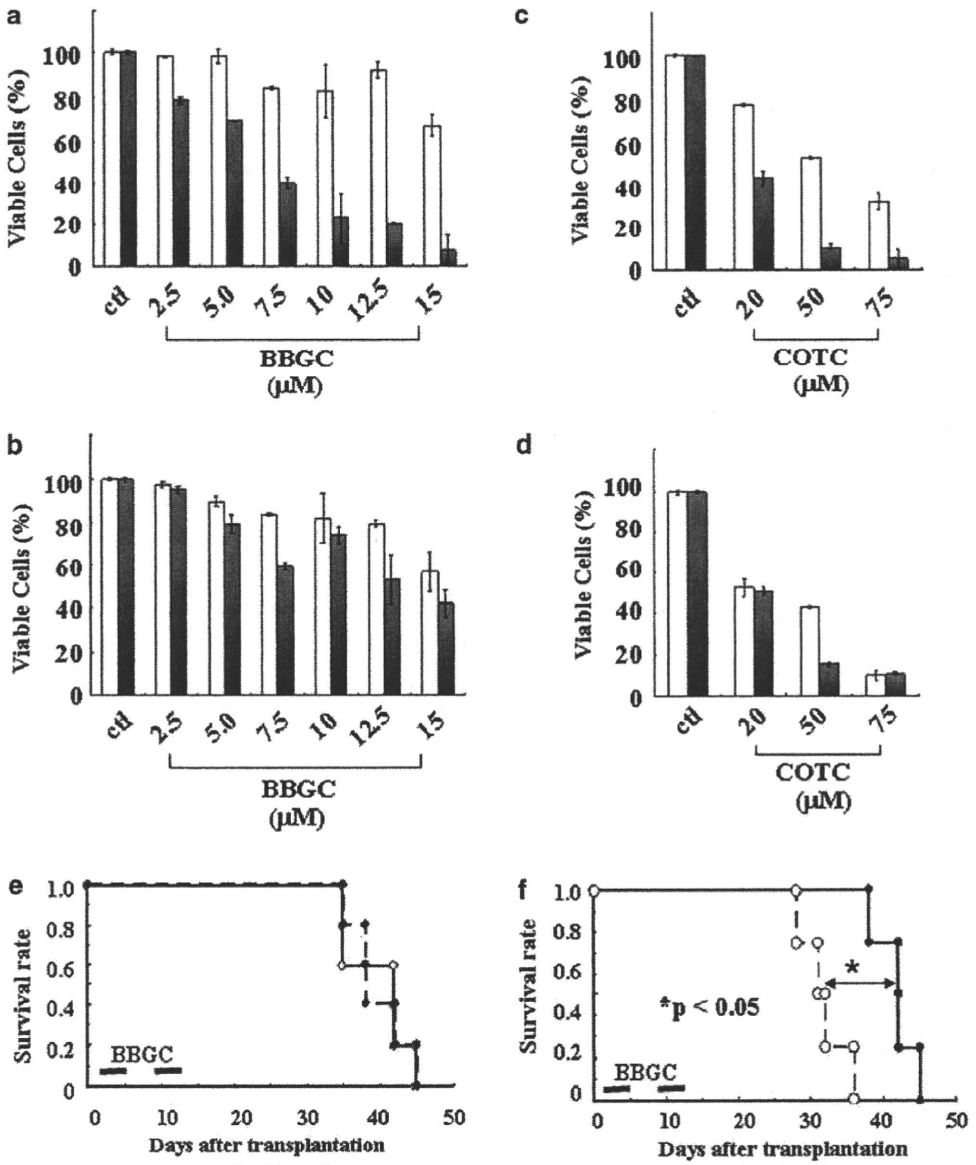

Figure 5 Effect of glyoxalase-I (Glo-I) inhibitors on hypoxia-adapted chronic myeloid leukemia (HA-CML) cells. *In vitro* cytotoxic effects of (**a**) *S-p*-bromobenzyl glutathione cyclopentyl diester (BBGC) and (**c**) 2-crotonyloxymethyl-4,5,6-trihydroxycylohex-2-enone (COTC) on parental K562 (white column) and K562/HA (black column) cells. *In vitro* cytotoxic effects of (**b**) BBGC and (**d**) COTC on parental KCL22 (white column) and KCL22/HA (black column) cells. *In vivo* effects of BBGC on transplanted (**e**) parental K562 and (**f**) K562/HA cells in NOD/SCID/γ_c^{null} (NOG) mice. Vehicle-treated mice, ○; BBGC-treated mice, ●

state.[10,11,29] Hypoxia favors the self-renewal of normal hematopoietic stem cells,[30] and resistance to hypoxia is one of the defining features of leukemic stem cells.[31] Therefore, it may be more important to search for new antileukemic agents that target quiescent CML stem cells residing in the hypoxic BM milieu. To this end, we have established two HA-CML sublines (Figures 2a–c). Previously, investigators have studied the role of hypoxia in leukemia using relatively short-term assays.[30–32] Our results suggest that transient hypoxia may not adequately mimic the physiological environment of CML cells (Figure 3e). Furthermore, in the mouse CML xenograft model, the oxygen concentration of engrafted leukemic cells in the BM was <10 mm Hg ($\sim 1.3\%$ O_2) (Figure 1f). Interestingly, the HA-CML cells exhibited characteristics similar to CML stem cells, including greater numbers of cells in a dormant, side population fraction, higher β-catenin expression, resistance to Abl TKIs and higher transplantation efficiency (Figures 2d, e and 4, Supplementary Figures 8 and 9).

The level of Bcr-Abl phosphorylation was lower in HA-CML cells, which may explain why those cells are less sensitive to Abl TKIs (Figure 2f). Giuntoli *et al.*[32] have also reported that the hypoxic selection of CML cells resulted in decreased cell sensitivity to imatinib and activation of Bcr-Abl-independent survival signaling pathways. Erk, a downstream effector of Bcr-Abl, was also less phosphorylated in HA-CML cells compared with parental cells, whereas the levels of p-Akt and p-Stat5 were similar (Figure 2f). These observations suggest that alternative mechanisms of activation exist for these signaling molecules in HA-CML cells.[33] In addition to

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the phosphorylation status of Bcr-Abl, the sensitivity to antileukemic agents depends on the balance of pro- and antiapoptotic molecules.[8] Akt pro-survival effects have been reported to be dependent on the first step in glycolysis.[34] However, Glo-1 induction in HA-CML cells was not directly controlled by Akt because Akt phosphorylation was unchanged in both HA cell lines (Figure 2f). The adaptation to hypoxia may also alter the status of Bcl-2 family members, because the expression levels of several proteins were altered in K562/HA cells. However, there were no changes in KCL22/HA cells. As the parental KCL22 cells are intrinsically resistant to imatinib and exhibit very high Bcl-2 expression, alterations in Bcl-2 family proteins may not be obvious. Further experiments will be required to clarify the hypoxia-induced changes.

We tried to identify a specific target in HA-CML cells that could be inhibited by small molecules with therapeutic potential. The dependence on glycolysis-mediated ATP production for uncontrolled cellular growth under limited O_2 conditions is a hallmark of malignant cells (Figure 3b).[35,36] Reduced ATP production (Figure 3a), as well as increased glucose consumption and lactate production (Supplementary Figure 4) in HA-CML cells, suggested that there was preferential utilization of glycolysis for ATP production in these cells. We focused on the components of glycolysis to identify leukemia cell targets that would circumvent drug resistance acquired through adaptation to hypoxia.[37,38] Glo-I is an enzyme that detoxifies methylglyoxal, a cytotoxic α-oxoaldehyde side product of glycolysis. Accumulation of methylglyoxal damages cells through multi-base DNA deletions and base-pair substitutions. The overexpression of Glo-I induces drug resistance to alkylating agents in leukemia as well as other solid tumors.[26,39] Glo-I activity was elevated in both K562/HA and KCL22/HA cells (Figures 3c and d), possibly because of their increased dependence on glycolysis during adaptation to hypoxia. Both K562/HA and KCL22/HA cells were more sensitive to cell killing by Glo-I inhibitors, indicating that these cells are indeed dependent on Glo-I activity for survival under hypoxic conditions (Figures 5a–d, Supplementary Figures 7b, c, 8 and 9).

All the Glo-I inhibitors examined were effective for killing HA-CML cells *in vitro*. We selected BBGC for *in vivo* analysis because BBGC was the most potent compound *in vitro*, and because BBGC has previously been used against lung cancer *in vivo*.[40] As engrafted K562 cells survive in the hypoxic environment of the BM (Figure 1f), we expected that BBGC would be effective against the parental K562$^{Luc\text{-}EGFP}$ cells, even if Glo-I activity in these cells was low. However, BBGC had no effect on the survival of K562$^{Luc\text{-}EGFP}$-engrafted mice (Figure 5e). This result may be due to the time differences between Glo-I induction by hypoxia and administration of BBGC. BBGC was administered from day 1 to day 11 after transplantation, whereas, in *in vitro* cultures, Glo-I was not induced until 21 days after the initiation of hypoxia (Figure 3e). Intriguingly, BBGC significantly prolonged the survival of K562/HA$^{Luc\text{-}EGFP}$-engrafted mice compared with untreated mice (Figure 5b). Thus, BBGC may be a promising therapeutic agent for use against CML cells with high Glo-I activity, which also more frequently accompanies the quiescent status. As the Abl inhibitor imatinib is currently the drug of choice for CML treatment, we examined the combined effects of BBGC with imatinib *in vitro*. BBGC augmented the effects of imatinib in killing CML cells *in vitro* (Supplementary Figure 10).

In conclusion, the survival of engrafted leukemic cells in the BM under severe hypoxia depends on the induction of Glo-I activity, and adaptation to hypoxia seems to result in the acquisition of Abl TKI resistance in CML cells. Glo-I inhibitors were much more effective against HA-CML cells than parental cells both *in vitro* and *in vivo*. These findings indicate the importance of the hypoxic environment for maintaining quiescent CML cells, and suggest that Glo-I is a novel target for CML treatment.

Materials and Methods

Reagents and cell lines. The Glo-I inhibitors, BBGC, COTC and methylgerfelin, were synthesized and purified as previously described.[26–28] The K562 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The other CML-derived cell lines (KCL22, BV173 and MYL) were kindly provided by Dr Tadashi Nagai (Jichi Medical School, Tochigi, Japan), Dr Oliver G Ottmann (Frankfurt University, Frankfurt, Germany) and Dr Hideo Tanaka (Hiroshima University, Hiroshima, Japan), respectively. The CML cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (Vitromex, Vilshofen, Germany) at 37°C in a humidified atmosphere of 20% O_2, 5% CO_2 and 75% N_2. CML cells were subjected to continuous culture in 1.0% O_2 (7.2 mm Hg), 5% CO_2 and 94% N_2, and HA subclones of K562 (K562/HA) and KCL22 (KCL22/HA) were selected and maintained in suspension in low O_2 conditions for more than 6 months. Parental K562 and K562/HA cells were cotransfected with pGL3, a control luciferase (Luc) reporter vector (Promega, Madison, WI, USA) and pCAG.*egfp.neo* using the Nucleofector Kit V, protocol T-03 (Amaxa AG, Cologne, Germany). Stable transfectants (K562$^{Luc\text{-}EGFP}$ and K562/HA$^{Luc\text{-}EGFP}$) were selected by culturing in medium containing G418 (1 mg/ml, Sigma Aldrich, Tokyo, Japan) and isolated by agarose gel cloning assays. Human primary Bcr-Abl$^+$ leukemic cells were obtained from patients with informed consent, according to the Declaration of Helsinki.

***In vivo* engraftment of CML cells and histological analysis.** Animal studies were performed in accordance with the guidelines of the Institutional Review Board for animal studies at Kyoto University. To evaluate the oxygen status of engrafted leukemic cells, 1.0×10^6 cells were injected into sublethally irradiated (2 Gy) male NOG or NOD/SCID mice at 6–8 weeks of age. At 35 days (NOG mice) or 50 days (NOD/SCID mice) post-inoculation, the mice were injected intraperitoneally with pimonidazole hydrochloride (Pimo, Chemicon, Temecula, CA, USA) (60 mg/kg), and 60 min later, the animals were killed and the femur and liver removed. Tissues were subjected to hematoxylin–eosin staining and immunohistochemical analysis using anti-Pimo (Chemicon) and antihuman Ki67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, and examined by microscopy. *In vivo*, leukemia cell proliferation was detected by monitoring luciferase expression using an *in vivo* imaging system (Xenogen, Berkeley, CA, USA), as previously described.[41] For survival analysis, time of death was determined either by spontaneous death, or by date of euthanasia due to pain or suffering, according to established criteria. We also confirmed the oxygen concentration of primary Bcr-Abl$^+$ leukemic cells that were obtained from a Ph$^+$ ALL patient and engrafted in the BM of NOD/SCID mice. Primary Bcr-Abl$^+$ leukemic cells (1×10^6) were transplanted into four NOD/SCID mice, which were killed 50 days after transplantation, and the BM sections were stained as above.

Cell death. Cell viability was measured by incorporation of propidium iodide (PI). Mitochondrial transmembrane potential ($\Delta\psi m$) was determined by staining with 3,3′-dihexyloxacarbocyanine iodide (Molecular Probes, Eugene, OR, USA), as previously described.[9] For analysis of DNA content, cells were fixed with ice-cold 70% ethanol and then incubated with PI as previously described.[42] The percentage of cells that incorporated PI, the $\Delta\psi m$, and the percentage of cells in sub-G_1 of the cell cycle were determined by FACS using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Western blot analysis. Proteins were separated by SDS-PAGE and then electroblotted onto a Hybond-PDVF membrane (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated with 5% (wt/vol) nonfat dry milk in

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phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 (Sigma, Saint Louis, MO, USA). Antibodies specific for Akt (#9272, 60 kDa), Erk1/2 (#9102, 42, 44 kDa), phospho (p)-Akt (#9271, 60 kDa), p-Erk1/2 (#9101, 42, 44 kDa) and p-Stat5 (##9351, 90 kDa, Cell Signaling Technologies, Beverly, MA, USA); for Bcl-2 (clone 100, #05–729, 26 kDa) and p-tyrosine (#05-321, Upstate, Lake Placid, NY, USA); as well as for Stat5 (#sc-835, 92 kDa) and c-Abl (#sc-23, 120 kDa, Santa Cruz Biotechnology); Bcl-X_L (#AAM-080, 26 kDa, Stressgen, Victoria, British Columbia, Canada); Glo-I (#H00002739-A01, 28 kDa, Novus Bio, Littleton, CO, USA); β-actin (#A2066, 42 kDa, Sigma); Mcl-1L (38 kDa) and Mcl-1S (30 kDa) (#LS-C43163, Life Span Biosciences, Seattle, WA, USA); and for β-catenin (#610153, 92 kDa, BD Biosciences, San Diego, CA, USA) were used as indicated. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies using enhanced chemiluminescence (ECL Advance, Amersham Biosciences).

Measurement of Glo-I activity. Cells were lysed in PBS containing 1 mM phenylmethylsulfonyl fluoride by freezing and thawing, followed by sonication. The lysates were centrifuged at 12 000 $\times$ *g* for 20 min and the supernatant was used as the cytosolic fraction. The Glo-I assay was performed in 0.1 M sodium phosphate (pH 7.0), 7.9 mM methylglyoxal (Sigma), 1 mM GSH and 14.6 mM $MgSO_4$ at 25 °C. An increase in absorbance at 240 nm because of the formation of *S*-D-lactoyl-glutathione was measured using a temperature-controlled spectrophotometer (Beckman Coulter$_\lambda$ DU640).

ATP assay. ATP levels were measured using an ATP assay kit (TOYO INK, Tokyo, Japan), according to the manufacturer's instructions.

Measurement of glucose consumption and lactate production. Cells were suspended in fresh culture medium. After 6 h, the cells were collected by centrifugation and resuspended in 5 ml of RPMI at a density of 2×10^5cells/ml. Cells were incubated for 24 h, and the culture medium was collected for measurement. Glucose levels were determined using a glucose assay kit (GO, Sigma). Glucose consumption was determined from the difference in glucose concentration compared with the starting medium. Lactate levels were determined using a lactate assay (F-kit L-lactate, JK International).

Determination of quiescent cells. Numbers of quiescent leukemic cells (in G_0 phase) were determined by double staining with Ki-67 and 7-AAD as previously described.[43] Briefly, 1×10^6 K562 or K562/HA cells were fixed in ice-cold 70% EtOH for at least 12 h, and then resuspended in 100 μl PBS. Cells were stained with 20 μl Ki-67 antibody (BD Biosciences) and incubated for 30 min at RT. Subsequently, 20 μl 7-AAD (BD Biosciences) was added, and the cells were resuspended in 500 μl PBS with 1% FBS and analyzed by FACS. Three independent analyses were performed.

BBGC treatment of CML mice. NOG mice 6–8 weeks of age were sublethally irradiated (2 Gy) and inoculated with 1.0×10^6 K562/HA$^{Luc\text{-}EGFP}$ or K562/HA$^{Luc\text{-}EGFP}$ cells by intravenous tail vein injection. Therapeutic treatments (seven mice per group) were started 1 day (day 1) after transplantation. To prepare the BBGC solution, BBGC dissolved in cremophor EL/DMSO (1 : 1) was diluted to 10 mg/ml using DMSO and PBS. Within each group, half of the mice were administered EL/DMSO only (vehicle controls), and the remainder were administered 10 mg/kg BBGC on days 1 through 4, and days 8 through 11 after transplantation. For survival analysis, the time of death was determined either by spontaneous death or by date of euthanasia due to pain or suffering, according to established criteria.

Statistical analysis. Survival curves were drawn using the Kaplan–Meier method and compared using the log-rank test. *P*-values were derived from two-sided tests and a *P*-value < 0.05 was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Modified ELISPOT assay may predict T-cell hyporesponsiveness to non-inherited maternal antigens

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ELISPOT assay, noninherited maternal antigens, immunological tolerance

SUMMARY

Clinical reports have suggested the existence of immunological tolerance to noninherited maternal antigens (NIMA) in human leukocyte antigen (HLA) mismatched allogeneic stem cell transplantation (allo-SCT). We studied the T-cell reactivity using IFN-γ enzyme-linked immunospot (ELISPOT) assay in three HLA fully matched allo-SCT cases and one healthy volunteer family case. In HLA fully matched allo-SCT cases, ELISPOT assay could detect the hyporesponsiveness of T cells from donors to the B cells from recipients. Moreover, ELISPOT assay showed that the T cells from an individual responded to B cell from his mother significantly weakly than those from an unrelated HLA-haploidentical individual. These observations suggest that our IFN-γ ELISPOT assay-based method may predict the presence of immunological tolerance to NIMA.

INTRODUCTION

Highly transfused renal transplant candidates develop anti-HLA antibodies significantly less frequently against their noninherited maternal HLA antigens (NIMA) than noninherited paternal HLA antigens (NIPA), suggesting the existence of feto-maternal immunological tolerance (Class *et al.*, 1988). Based on this hypothesis, Burlingham *et al.* (1998) showed the superior graft survival rate in NIMA- to NIPA-mis-

