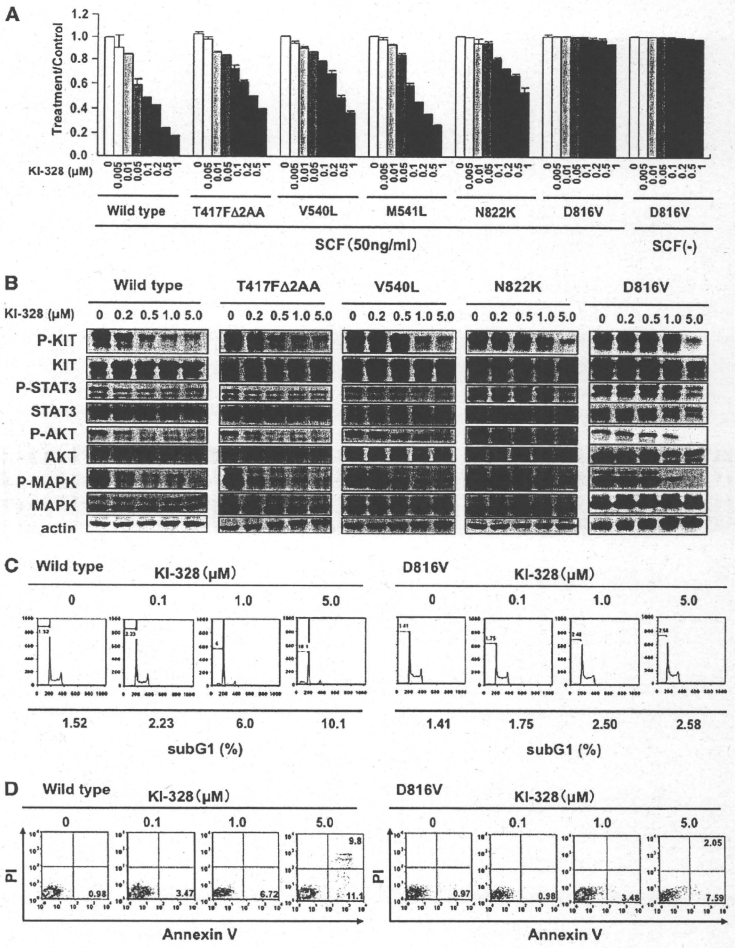


Fig. 3 Inhibitory effects of KI-328 on proliferation and KIT-mediated signals. Since Wt- and mutant-KIT, except for D816V-KIT-expressing 32D cells require SCF for proliferation, the inhibitory effects of KI-328 are evaluated in the presence of 50 ng/mL SCF. **a** KI-328 potently inhibits the growth of Wt- and mutant-KIT-expressing cells, while it has little potency against D816V-KIT-expressing cells. **b** Consistent with the growth inhibitory effects, KI-328 reduces the phosphorylation levels of KIT and downstream molecules at a concentration over the GI_{50} value; however, more than 2 μM of KI-328 is required for the dephosphorylation of constitutively activated D816V-KIT. **c** Wt- and D816V-KIT-expressing 32D cells were treated with increasing concentrations of KI-328 for 24 h. After treatment, Wt-KIT-expressing cells show an increase in the percentage of G_1 cells and a reciprocal reduction in the percentage of the S phase, while D816V-KIT-expressing cells do not. **d** An apparent increase of apoptotic cells is observed at a concentration of over the GI_{50} value in Wt-KIT-expressing cells, while it is observed at over 2 μM KI-328 in D816V-KIT-expressing cells

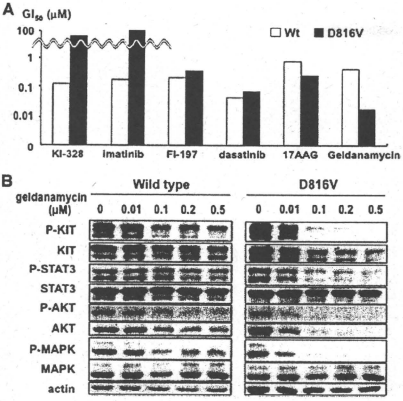


Fig. 4 Comparison of inhibitory effects of potent KIT inhibitors against Wt- and D816V-KIT. **a** Growth inhibitory effect of each inhibitor on Wt- and D816V-KIT-expressing 32D cells is shown. Selective KIT inhibitors, KI-328 and imatinib, have little potency against D816V-KIT. Multi-kinase inhibitors, KI-197 and dasatinib, have the same potency against Wt- and D816V-KIT; however, HSP90 inhibitors, 17-AAG and geldanamycin, are more potent against D816V-KIT than Wt-KIT. **b** Geldanamycin more sensitively reduces the phosphorylated D816V-KIT as well as its downstream molecules than SCF-stimulated Wt-KIT. Furthermore, it reduces the total amount of D816V-KIT proteins more sensitively than that of Wt-KIT protein

3.5 Cell cycle and apoptosis-inducing effects of KI-328

After treatment with increasing concentrations of KI-328 for 24 h, Wt-, T417FΔ2AA-, V540L-, M541L- and N822K-KIT-expressing cells exhibited an increase in the percentages of sub-G_1 cells. Simultaneously, reciprocal reduction in the percentage of cells in the S/G_2 phase was observed (Fig. 3c). In addition, an apparent increase of apoptotic cells was also observed at over the GI_{50} value of KI-328 in each cell (Fig. 3d). These results indicated that the dephosphorylation of KIT by KI-328 could induce cell cycle arrest and eventually cause apoptosis at concentrations over the GI_{50} value against Wt-, T417FΔ2AA-, V540L-, M541L- and N822K-KIT-expressing cells; however, the increase of sub-G1 apoptotic cells was observed at over 2 μM KI-328 in D816V-KIT-expressing cells.

3.6 Comparison of sensitivities against D816V-KIT among potent KIT inhibitors

Although KI-328 has selective potency against Wt-, T417FΔ2AA-, V540L-, M541L- and N822K-KIT, its potency against D816V-KIT is limited. To examine whether this is a result of the characteristic structure of KI-328 itself or selectivity against KIT, we compared the sensitivities against Wt- and D816V-KIT among several potent KIT inhibitors. As shown in Fig. 4a, the growth inhibitory effects of imatinib, which reportedly has relatively KIT-selective potency, on D816V-KIT-expressing cells was apparently lower than that of SCF-stimulated Wt-KIT-expressing cells. In contrast, FI-197 and dasatinib, which are known to be multi-kinase inhibitors, inhibited the growth of Wt- and D816V-KIT-expressing cells at the same GI_{50} values. These results suggested that increasing KIT selectivity might reduce the potency against D816V-KIT; however, even multi-kinase inhibitors did not show higher potency against D816V-KIT than Wt-KIT. We therefore looked for other compounds that are more potent against D816V-KIT, and found that HSP90 inhibitors, 17-AAG and geldanamycin more sensitively inhibited the growth of D816V-KIT-expressing cells (GI_{50} values were 0.243 and 0.018 μM, respectively) than that of Wt-KIT-expressing cells (GI_{50} values were 0.726 and 0.384 μM, respectively) (Fig. 4a). Western blot analysis showed that HSP90 inhibitors more sensitively reduced phosphorylation level of D816V-KIT as well as its downstream molecules than that of SCF-stimulated Wt-KIT. Notably, HSP90 inhibitors more sensitively reduced the total amount of D816V-KIT protein than that of Wt-KIT protein, indicating that the reduced phosphorylation level of D816V-KIT mainly reflected the degradation of D816V-KIT protein (Fig. 4b).

3.7 HSP90 inhibitor has selective and sensitive potency against D816V-KIT

As shown in Fig. 4b, D816V-KIT protein seemed to be dependent on HSP90. We further examined how the stability of each mutant KIT protein was dependent on HSP90. HSP90 was precipitated from each Wt- and mutant-KIT-expressing 32D cell after SCF stimulation, and subjected to immunoblotting with the anti-KIT antibody. D816V-KIT was most strongly co-precipitated with HSP90, followed by N822K- and V540L-KIT. T417FΔ2AA-KIT was weakly co-precipitated with HSP90, although Wt-KIT was not. The reciprocal experiment revealed the same result, and the interactions between mutant KITs and HSP90 were clearly abolished by treatment with 17-AGG (Fig. 5a). In parallel with the extent of dependence on HSP90, 17-AAG reduced phosphorylation levels of mutant KIT as well as the downstream molecules (Fig. 5b). In contrast to the dramatic reduction of phosphorylation levels of mutant KIT, the apparent reduction of each mutant KIT protein was not observed after the 17-AAG treatment. These results suggested that HSP90 may play an important role in stabilizing

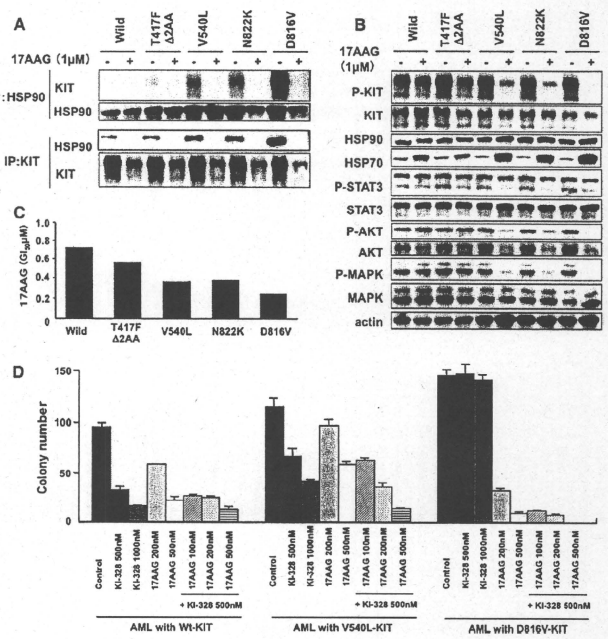


Fig. 5 HSP90 inhibitor has selective and sensitive potency against D816V-KIT. **a** Immunoprecipitation analysis reveals that D816V-KIT is the strongest substrate of HSP90 among mutant KITs. **b** In parallel with the extent of dependence on HSP90, 17-AAG reduces the total amount of each mutant KIT, resulting in the reduced phosphorylation levels of KIT and downstream molecules. **c** Growth inhibitory effects of 17-AAG on Wt- and mutant-KIT-expressing cells reflect the dependence of each protein on HSP90. **d** Growth inhibitory effects of KI-328 and 17-AAG on human AML cells with Wt-, V540L- and D816V-KIT were evaluated in semi-solid medium. 17-AAG potently inhibits the colony formation of AML cells with D816V-KIT. Combination of KI-328 and 17-AAG shows an additive inhibitory effect on KIT-expressing human AML cells

the active form of mutant KIT proteins, particularly D816V-, N822K- and V540L-KIT. Therefore, the disruption of this interaction by 17-AAG may lead to the reduction of their phosphorylation levels before the apparent degradation of the total amount KIT proteins. Furthermore, the growth inhibitory effects of 17-AAG on Wt- and mutant-KIT-expressing cells also reflected the extent of dependence of each KIT protein on HSP90 (Fig. 5c). Finally, we evaluated the growth inhibitory effects of KI-328 and 17-AAG on Wt-, V540L- and D816V-KIT-expressing human primary AML cells in the semi-solid medium (Fig. 5d). Consistent with the results in KIT-expressing 32D cells, KI-328 dose dependently inhibited the colony formation of Wt- and V540L-KIT-expressing AML cells, but not of D816V-KIT-expressing AML cells. 17-AAG more potently inhibited the colony formation of D816V-KIT-expressing AML cells than that of Wt- or V540L-KIT-expressing AML cells. Furthermore, a combination of KI-328 and 17-AAG showed an additive inhibitory effect on D816V-KIT-expressing AML cells.

4 Discussion

In this study, we evaluated the sensitivity and selectivity of a novel KIT inhibitor, KI-328, in consideration of its potency against several types of mutation, which are recurrently identified in AML cells. KI-328 showed potent and selective inhibitory activity against KIT kinase by in vitro kinase assays. This kinase inhibition profile was also confirmed by the cellular system as potent and selective growth inhibition against mutant-KIT-expressing 32D cells and human leukemia cell line Kasumi-1. It was further demonstrated that the growth inhibitory effect of KI-328 was correlated with the reduced phosphorylation levels of activated KIT kinases, as well as STAT3, AKT and MAPK. In addition, cell cycle analysis revealed that growth inhibition was induced by G_1 arrest over the concentration of each GI_{50} value, resulting in apoptosis.

On the other hand, KI-328 has little potency against constitutively active D816V-KIT kinase. Although several small molecules have been demonstrated to have potency

against Wt- and mutant-KIT kinases, their inhibitory effects highly depend on the mutation type. Since the D816V mutation stabilizes the activation loop of KIT in the active conformation, the binding of inhibitors with selective affinity for the open configuration of the kinase domain is precluded [23]. Imatinib has relatively selective potency against KIT kinase as well as ABL kinase, while it cannot inhibit the activity of D816V-KIT, interfering with their binding to the enzymatic pocket [18, 35]. In contrast, it has been reported that dasatinib has the same potency against D816V-KIT as Wt- and the other mutant KITs. Since dasatinib can bind to the ATP-binding site of BCR-ABL, irrespective of the conformation of the activation loop, its broad potency against mutant KIT, including D816V, is consistent with the structural model of dasatinib to BCR-ABL [36]. In this study, we also evaluated the potency of FI-197, which is a derivative of KI-328. FI-197 has potency against a variety of tyrosine kinases, including KIT, while its potency against KIT is lower than KI-328. Although the binding affinity of KI-328 and FI-197 to KIT in active and inactive forms has not been clarified, these results collectively suggested that increasing the selectivity against KIT kinase might reduce the binding affinity against KIT in the active form.

The D816V KIT mutation has been identified in a majority of neoplastic mast cells [37]. In AML, this mutation was also frequently identified in CBF-AML. Furthermore, the retrospective clinical study suggested that the D816V mutation might be more strongly implicated in the prognosis of patients with CBF-AML than other types of KIT mutations [16]. To date, several agents with potency against KIT have been approved for clinical use; however, most have little potency against D816V-KIT. Multi-kinase inhibitors, such as dasatinib and FI-197, have the same potency against D816V-KIT as Wt- and the other mutant KITs, while D816V-KIT-selective agents have not been developed. Since KIT is expressed on hematopoietic stem/progenitor cells, higher sensitivity against KIT may increase the risk of severe bone marrow suppression in clinical use; therefore, it is necessary to establish other strategies for selectively inhibiting D816V-KIT.

HSPs are molecular chaperones, and regulate protein folding to ensure correct conformation and translocation and to avoid protein degradation [38, 39]. HSPs are increased in a variety of cancers and hematological malignancies. Since many oncogenic proteins have been demonstrated to be client proteins of HSP90, HSP90 inhibitors act as promising anticancer agents [40–43]; however, there are several problems, such as adverse effects and a narrow range of therapeutic concentration, which remain to be resolved for the clinical use. We here demonstrated that D816V-KIT is the strongest substrate of HSP90 among mutant KIT proteins. In addition, HSP90 inhibitors suppressed the growth of the D816V-KIT-expressing cells at a concentration at which SCF-dependent growth of Wt-KIT-expressing cells was not affected. In our preliminary analysis in the liquid culture system, the combination of KI-328 with 17-AAG or geldanamycin showed additive inhibitory effects on the growth of D816V-KIT-expressing cells. Since the high selectivity and sensitivity of KIT inhibitors might not necessarily resolve the resistance to D816V-KIT, combination therapy with KIT and HSP90 inhibitors would be an ideal strategy for the treatment of D816V-KIT-carrying malignancies; however, further analysis is required to clarify the efficacy and safety of this combination therapy in vivo.

Acknowledgments We would like to thank Ms. Manami Kira for secretarial assistance. This study was supported by Grants-in-Aid from the National Institute of Biomedical Innovation, the Ministry of Health, Labor and Welfare, the Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology and the Global COE Program "Integrated Functional Molecular Medicine for Neuronal and Neoplastic Disorders" Japan.

References

1. Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. 1987;6:3341–51.
2. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell. 1991;65:1143–52.
3. Broudy VC. Stem cell factor and hematopoiesis. Blood. 1997; 90:1345–64.
4. Mol CD, Lim KB, Sridhar V, Zou H, Chien EY, Sang BC, Nowakowski J, Kassel DB, Cronin CN, McRee DE. Structure of a c-kit product complex reveals the basis for kinase transactivation. J Biol Chem. 2003;278:31461–4.
5. Ikeda H, Kanakura Y, Tamaki T, Kuriu A, Kitayama H, Ishikawa J, Kanayama Y, Yonezawa T, Tarui S, Griffin JD. Expression and functional role of the proto-oncogene c-kit in acute myeloblastic leukemia cells. Blood. 1991;78:2962–8.
6. Furitsu T, Tsujimura T, Tono T, Ikeda H, Kitayama H, Koshimizu U, Sugahara H, Butterfield JH, Ashman LK, Kanayama Y, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. J Clin Invest. 1993;92:1736–44.
7. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science. 1998;279:577–80.
8. Lennartsson J, Jelacic T, Linnekin D, Shivakrupa R. Normal and oncogenic forms of the receptor tyrosine kinase kit. Stem Cells. 2005;23:16–43.
9. Roskoski R Jr. Structure and regulation of Kit protein-tyrosine kinase—the stem cell factor receptor. Biochem Biophys Res Commun. 2005;338:1307–15.
10. Beghini A, Peterlongo P, Ripamonti CB, Larizza L, Cairoli R, Morra E, Mecucci C. C-kit mutations in core binding factor leukemias. Blood. 2000;95:726–7.

11. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002;2:502–13.
12. Ishikawa Y, Kiyoi H, Tsujimura A, Miyawaki S, Miyazaki Y, Kuriyama K, Tomonaga M, Naoe T. Comprehensive analysis of cooperative gene mutations between class I and class II in de novo acute myeloid leukemia. Eur J Haematol. 2009;83:90–8.
13. Frohling S, Scholl C, Gilliland DG, Levine RL. Genetics of myeloid malignancies: pathogenetic and clinical implications. J Clin Oncol. 2005;23:6285–95.
14. Schnittger S, Kohl TM, Haferlach T, Kern W, Hiddemann W, Spiekermann K, Schoch C. KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. Blood. 2006;107:1791–9.
15. Cairoli R, Beghini A, Grillo G, Nadali G, Elice F, Ripamonti CB, Colapietro P, Nichelatti M, Pezzetti L, Lunghi M, et al. Prognostic impact of c-KIT mutations in core binding factor leukemias: an Italian retrospective study. Blood. 2006;107:3463–8.
16. Paschka P, Marcucci G, Ruppert AS, Mrozek K, Chen H, Kittles RA, Vukosavljevic T, Perrotti D, Vardiman JW, Carroll AJ, et al. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a Cancer and Leukemia Group B Study. J Clin Oncol. 2006;24:3904–11.
17. Heinrich MC, Blanke CD, Druker BJ, Corless CL. Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies. J Clin Oncol. 2002; 20:1692–703.
18. Ma Y, Zeng S, Metcalfe DD, Akin C, Dimitrijevic S, Butterfield JH, McMahon G, Longley BJ. The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors: kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations. Blood. 2002;99:1741–4.
19. Corbin AS, Griswold IJ, La Rosee P, Yee KW, Heinrich MC, Reimer CL, Druker BJ, Deininger MW. Sensitivity of oncogenic KIT mutants to the kinase inhibitors MLN518 and PD180970. Blood. 2004;104:3754–7.
20. Schittenhelm MM, Shiraga S, Schroeder A, Corbin AS, Griffith D, Lee FY, Bokemeyer C, Deininger MW, Druker BJ, Heinrich MC. Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies. Cancer Res. 2006;66:473–81.
21. Gotlib J, Berube C, Growney JD, Chen CC, George TI, Williams C, Kajiguchi T, Ruan J, Lilleberg SL, Durocher JA, et al. Activity of the tyrosine kinase inhibitor PKC412 in a patient with mast cell leukemia with the D816V KIT mutation. Blood. 2005; 106:2865–70.
22. Pan J, Quintas-Cardama A, Kantarjian HM, Akin C, Manshouri T, Lamb P, Cortes JE, Tefferi A, Giles FJ, Verstovsek S. EXEL-0862, a novel tyrosine kinase inhibitor, induces apoptosis in vitro and ex vivo in human mast cells expressing the KIT D816V mutation. Blood. 2007;109:315–22.
23. Gajiwala KS, Wu JC, Christensen J, Deshmukh GD, Diehl W, DiNitto JP, English JM, Greig MJ, He YA, Jacques SL, et al. KIT kinase mutants show unique mechanisms of drug resistance to imatinib and sunitinib in gastrointestinal stromal tumor patients. Proc Natl Acad Sci USA. 2009;106:1542–7.
24. Dubreuil P, Letard S, Ciufolini M, Gros L, Humbert M, Casteran N, Borge L, Hajem B, Lermet A, Sippl W, et al. Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. PLoS One. 2009;4:e7258.
25. Abrams TJ, Lee LB, Murray LJ, Pryer NK, Cherrington JM. SU11248 inhibits KIT and platelet-derived growth factor receptor beta in preclinical models of human small cell lung cancer. Mol Cancer Ther. 2003;2:471–8.
26. Wardelmann E, Thomas N, Merkelbach-Bruse S, Pauls K, Speidel N, Buttner R, Bihl H, Leutner CC, Heinicke T, Hohenberger P. Acquired resistance to imatinib in gastrointestinal stromal tumours caused by multiple KIT mutations. Lancet Oncol. 2005;6:249–51.
27. Fumo G, Akin C, Metcalfe DD, Neckers L. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is effective in down-regulating mutated, constitutively activated KIT protein in human mast cells. Blood. 2004;103:1078–84.
28. Bauer S, Yu LK, Demetri GD, Fletcher JA. Heat shock protein 90 inhibition in imatinib-resistant gastrointestinal stromal tumor. Cancer Res. 2006;66:9153–61.
29. Ishida H, Isami S, Matsumura T, Umehara H, Yamashita Y, Kajita J, Fuse E, Kiyoi H, Naoe T, Akinaga S, et al. Novel and orally active 5-(1,3,4-oxadiazol-2-yl)pyrimidine derivatives as selective FLT3 inhibitors. Bioorg Med Chem Lett. 2008; 18:5472–7.
30. Toki T, Kanezaki R, Adachi S, Fujino H, Xu G, Sato T, Suzuki K, Tauchi H, Endo M, Ito E. The key role of stem cell factor/KIT signaling in the proliferation of blast cells from Down syndrome-related leukemia. Leukemia. 2009;23:95–103.
31. Foster R, Byrnes E, Meldrum C, Griffith R, Ross G, Upjohn E, Braue A, Scott R, Varigos G, Ferrao P, et al. Association of paediatric mastocytosis with a polymorphism resulting in an amino acid substitution (M541L) in the transmembrane domain of c-KIT. Br J Dermatol. 2008;159:1160–9.
32. Kiyoi H, Shiotsu Y, Ozeki K, Yamaji S, Kosugi H, Umehara H, Shimizu M, Arai H, Ishii K, Akinaga S, et al. A novel FLT3 inhibitor FI-700 selectively suppresses the growth of leukemia cells with FLT3 mutations. Clin Cancer Res. 2007;13:4575–82.
33. Shiotsu Y, Kiyoi H, Ishikawa Y, Tanizaki R, Shimizu M, Umehara H, Ishii K, Mori Y, Ozeki K, Minami Y, et al. KW-2449, a novel multikinase inhibitor, suppresses the growth of leukemia cells with FLT3 mutations or T315I-mutated BCR/ABL translocation. Blood. 2009;114:1607–17.
34. Minami Y, Kiyoi H, Yamamoto Y, Yamamoto K, Ueda R, Saito H, Naoe T. Selective apoptosis of tandemly duplicated FLT3-transformed leukemia cells by Hsp90 inhibitors. Leukemia. 2002;16:1535–40.
35. Frost MJ, Ferrao PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKit is more sensitive to imatinib (STI571) compared with wild-type c-kit whereas the kinase domain mutant D816VKit is resistant. Mol Cancer Ther. 2002;1:1115–24.
36. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science. 2004;305:399–401.
37. Orfao A, Garcia-Montero AC, Sanchez L, Escribano L. Recent advances in the understanding of mastocytosis: the role of KIT mutations. Br J Haematol. 2007;138:12–30.
38. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. Nat Rev Cancer. 2005;5:761–72.
39. Wandinger SK, Richter K, Buchner J. The Hsp90 chaperone machinery. J Biol Chem. 2008;283:18473–7.
40. Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. Cancer Cell. 2003;3:213–7.
41. Mahalingam D, Swords R, Carew JS, Nawrocki ST, Bhalla K, Giles FJ. Targeting HSP90 for cancer therapy. Br J Cancer. 2009;100:1523–9.
42. Banerji U. Heat shock protein 90 as a drug target: some like it hot. Clin Cancer Res. 2009;15:9–14.
43. Zhao M, Kiyoi H, Yamamoto Y, Ito M, Towatari M, Omura S, Kitamura T, Ueda R, Saito H, Naoe T. In vivo treatment of mutant FLT3-transformed murine leukemia with a tyrosine kinase inhibitor. Leukemia. 2000;14:374–8.

Bone Marrow Transplantation (2010) 45, 1665–1667

www.nature.com/bmt

LETTER TO THE EDITOR

Successful engraftment of a second transplant from unrelated cord blood identifying acceptable HLA Ag mismatches as treatment for primary graft failure possibly mediated by anti-HLA Abs after 'mega-dose' haploidentical PBSC transplantation

Bone Marrow Transplantation (2010) **45**, 1665–1667; doi:10.1038/bmt.2010.30; published online 1 March 2010

Patients with preformed Abs are at increased risk of rejection after solid organ transplantation;[1] however, the function of Ab-mediated rejection in hematopoietic SCT is still not completely understood. The use of sensitive solid-phase Ab detection assays, such as LABScreen (One Lambda, Canoga Park, CA, USA), which are based on flow cytometry, has enhanced our ability to detect and identify donor-specific anti-HLA Abs (DSA). LABScreen Single Antigen (One Lambda) had single recombinant HLA class I and class II Ag on each bead.

This report describes a patient with anti-HLA Abs who achieved successful engraftment after HLA-mismatched cord blood transplantation (CBT) due to primary graft failure after 'mega-dose' PBSC transplantation. Table 1 shows the anti-HLA Abs identified before treatment, and the HLA of the patient and the donors. We identify the epitopes with unique amino-acid positions according to the epitope analysis that was reported by Maruya *et al.*[2]

A 40-year-old female developed AML (FAB: M1), which was refractory to two courses of standard induction therapy. There was no available HLA-identical donor in her family or from the Japan Marrow Donor Program. Also, no cord blood with HLA Ags was not reactive to her Abs, which were thought to have been produced during pregnancy. She received PBSC transplantation from a haploidentical child, despite the presence of high-level Abs against the donor-specific Ag (A*2402 and B*5201) in the host-versus-graft reaction. She also displayed strong reactions against many non-donor-specific Ags, which were divided into two groups according to the presence or absence of the epitopes common to A*2402 and B*5201.

After obtaining informed consent from both the patient and donor, the patient received plasmapheresis (on day −16 and −15), rituximab (375 mg/m^2 on day −14) and polyclonal intravenous Ig (i.v. Ig; 0.4 g/kg on day −8, −7 and −6) for reducing Ab levels. 'Mega-dose' transplant of CD34$^+$ stem cells (14.6×10^6 per kg) was conducted following a conditioning regimen that included of TBI of 12 Gy in four fractions from −10 to −7 and 60 mg/kg CY once daily i.v. on day −4 and −3 (total dose 120 mg/kg), in addition to cytarabine 2 g/m^2 twice daily i.v. on day −6 and −5 (total four doses). Tacrolimus (TAC), short-term MTX and methylprednisolone were given for GVHD prophylaxis.

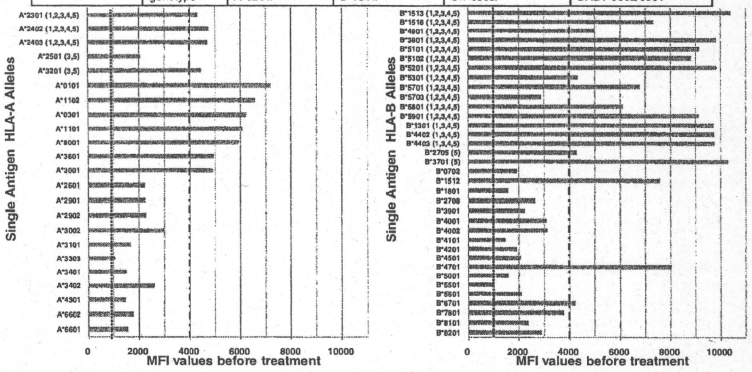
Figure 1 shows the clinical course. Peripheral cytopenia persisted, and primary engraftment failure was diagnosed based on severe BM hypoplasia on day 21. Despite the treatment for reducing Abs, the levels of anti-A*2402, anti-B*5201 and non-DSA sharing epitopes common to A*2402 and B*5201 remained very high. In contrast, the levels of Abs without those epitopes, such as A*2601, decreased rapidly.

CBT was performed from the two HLA-Ag-mismatched (A26, B46) cord blood graft 32 days after PBSC transplantation, because the Ab level against A*2601, which was present in the cord blood, was observed to have significantly decreased. The preparative regimen consisted of fludarabine 30 mg/m^2 once daily i.v. on day −5 to −2 (total dose 120 mg/m^2) and L-phenyl-alanine mustard 50 mg/m^2 once daily i.v. on day −3 and −2 (total two doses). Prophylaxis for GVHD was TAC and short-term MTX. She received a cord blood infusion containing 3.36×10^7 nucleated cells per kg. Primary engraftment with neutrophils $> 0.5 \times 10^9$ per l and complete donor chimerism by sex chromosome were achieved on day 25 after CBT. She has since remained in CR for 11 months after CBT without any GVHD even after the discontinuation of TAC. The Ab levels against most of the Ag including A*2601 were not detectable after engraftment. On the contrary, anti-A*2402, anti-B*5201 and non-DSA sharing epitopes common to A*2402 and B*5201 increased and remained high.

The mechanism for the rejection of PBSCs in this case has not yet been clearly elucidated. A marked increase of anti-A*2402 and anti-B*5201 levels, however, suggested that the most likely mechanism could be the host-mediated humoral response. Sensitization to HLA Ags increases the risk of graft failure in Allo-SCT.[3,4] Plasmapheresis, i.v. Ig, and rituximab are commonly used in solid organ transplantation for the abrogation of allo-Ab-mediated rejection.[5,6] Indeed, these were sufficient in our case to reduce the levels of the Abs not sharing epitopes with A*2402 and B*5201. However, these had no more effect than to hold the increase in anti-A*2402 and anti-B*5201 levels down to within the specified time. These procedures failed to cause the apoptosis of plasma cells *in vitro*, thereby preventing allo-Ab production.[7] Donor platelet transfusion is another procedure to eliminate DSA.[8] However, it could not be conducted in this case because the patient had experienced severe nonhemolytic transfusion reactions to random pooled plts. In addition to allo-Ab-mediated rejection,

Table 1 Anti-HLA Abs before treatment, donors and recipient HLA

PBSCT donor HLA	phenotype	A2/24	B75/52	Cw9/Cw12	DR8/9
	genotype	A*0201/2402	B*1511/5201	Cw*0303/1202	DRB1*0803/0901
CBT donor HLA	phenotype	A2/26	B46/-	Cw1/-	DR8/9
	genotype	A*0207/2601	B*4601/-	Cw*0102/-	DRB1*0803/0901
Recipient HLA	phenotype	A2/-	B75/-	Cw9/-	DR8.2/9
	genotype	A*0201/-	B*1511/-	Cw*0303/-	DRB1*0802/0901



Donor and recipient HLA were identified by high-resolution typing. The results have been reported as the mean fluorescence intensity (MFI) and the cutoff value was 1000 MFI. In this case, fluorescence values of the negative control beads were below 100 and the ratio of positive control to negative control beads was over 100. Some sample data did not fit these conditions. The sera were cleaned, using Adsorb Out to reduce the background fluorescence. Abs against HLA-C and DR were not detected.

Numbers (1–5) indicate epitopes common to A*2402 and B*5201. Unique position and amino acid is denoted by a three-digit number that pinpoints the position on the HLA molecule. This is followed by a single letter that stands for a particular amino acid.

(1), 73T, 76E and 77N; (2), 76E, 77N and 80I; (3), 79R and 80I; (4), 80I and 90A; (5), 82L and 83R.

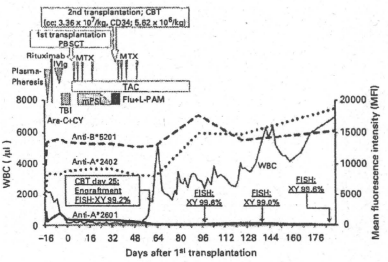


Figure 1 Clinical course: CBT, cord blood transplantation; i.v. Ig, polyclonal intravenous immunoglobulin; TAC, tacrolimus; mPSL, methylprednisolone; Flu, fludarabine.

there is no denying that the absence of a killer Ig-like receptor 2DL ligand mismatch may have contributed to the graft failure observed in this case. This is because killer Ig-like receptor ligand incompatibility in the graft-versus-host direction has been suggested to reduce graft failure risk in haploidentical hematopoietic SCT.

Although anti-HLA Ab remained after the PBSC transplantation, the reduced anti-A*2601 level allowed for the successful engraftment after HLA-mismatched CBT. Kataoka *et al.*[9] also reported two cases of CBT similar to ours.

The levels of only anti-A*2402, anti-B*5201 and non-DSA sharing their common epitopes remained high, which suggests that epitope sharing between unrelated HLA molecules was the mechanism underlying DSA and non-DSA generation. In renal transplantation, epitope matching could be better than Ag matching for avoiding the development of Abs,[10] whereas in hematopoietic SCT epitope matching could not improve transplant

outcome. In addition, epitope matching remains difficult because epitopes are often not in linear sequences. However, mismatching at the immunogenic amino-acid positions has been reported to not always result in Ab production, and the differences between producer and nonproducer were due to the efficiency of HLA class II alleles in presenting the mismatched alleles. This finding indicates that full epitope matching is therefore not required, and the hematopoietic SCT outcome might thus improve.[2]

The persistence of the anti-donor immune response suggests the existence of the residual host hematopoietic cell. Therefore, monitoring of anti-HLA Ab serves as a sensitive method in detection of minimal residual disease.

Identifying acceptable HLA Ag mismatches might be useful to achieve engraftment after HLA-mismatched SCT for allosensitized patients. In addition, this case suggests that epitope matching might be more effective than Ag matching.

Conflict of interest

The authors declare no conflict of interest.

T Hoshino[1], T Sakura[1], K Miyawaki[1], N Hatsumi[1], S Takada[1], E Maruya[2], H Saji[2] and S Miyawaki[1]

[1]*Leukemia Research Center, Saiseikai Maebashi Hospital, Maebashi, Gunma, Japan and*

[2]*HLA Laboratory, NPO, Kyoto, Kyoto, Japan*

E-mail: takumihoshino4249@yahoo.co.jp

References

1 Terasaki PI. Humoral theory of transplantation. *Am J Transplant* 2003; 3: 665–673.

2 Maruya E, Sasaki N, El-Awar N, Akaza T, Saji H, Terasaki PI *et al.* Immunogenic HLA class I epitopes identified by humoral response to pregnancies. *Clin Transpl* 2008, 215–227.

3 Anasetti C, Amos D, Beatty PG, Appelbaum FR, Bensinger W, Mickelson E *et al.* Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med* 1989; **320**: 197–204.

4 Ottinger HD, Rebmann V, Pfeiffer KA, Beelen DW, Kremens B, Grosse-Wilde H *et al.* Positive serum crossmatch as predictor for graft failure in HLA-mismatched allogeneic blood stem cell transplantation. *Transplantation* 2002; **73**: 1280–1285.

5 Jordan SC, Vo AA, Peng A, Toyoda M, Tyan D. Intravenous gammaglobulin (IVIG): a novel approach to improve transplant rates and outcomes in highly HLA-sensitized patients. *Am J Transplant* 2006; **6**: 459–466.

6 Vo AA, Lukovsky M, Toyoda M, Wang J, Reinsmoen NL, Lai CH *et al.* Rituximab and intravenous immune globulin for desensitization during renal transplantation. *N Engl J Med* 2008; **359**: 242–251.

7 Perry DK, Burns JM, Amiot BP, Gloor JM, Gores GJ, Stegall MD *et al.* Proteasome inhibition causes apoptosis of normal human plasma cells preventing alloantibody production. *Am J Transplant* 2009; **9**: 201–209.

8 Narimatsu H, Wake A, Miura Y, Tanaka H, Matsumura T, Taniguchi S *et al.* Successful engraftment in crossmatch-positive HLA-mismatched peripheral blood stem cell transplantation after depletion of antidonor cytotoxic HLA antibodies with rituximab and donor platelet infusion. *Bone Marrow Transplant* 2005; **36**: 555–556.

9 Kataoka K, Yamamoto G, Nannya Y, Yoshimi A, Okada S, Kurokawa M *et al.* Successful engraftment following HLA-mismatched cord blood transplantation for patients with anti-HLA Abs. *Bone Marrow Transplant* 2008; **42**: 129–130.

10 Thaunat O, Hanf W, Dubois V, McGregor B, Perrat G, Morelon E *et al.* Chronic humoral rejection mediated by anti-HLA-DP alloantibodies: Insights into the role of epitope sharing in donor-specific and non-donor specific alloantibodies generation. *Transpl Immunol* 2009; **20**: 209–211.

blood

2010 116: 2839-2846
Prepublished online Jul 13, 2010;
doi:10.1182/blood-2009-10-249219

The impact of anti-HLA antibodies on unrelated cord blood transplantations

Minoko Takanashi, Yoshiko Atsuta, Koki Fujiwara, Hideki Kodo, Shunro Kai, Hiroyuki Sato, Masatoshi Kohsaki, Hiroshi Azuma, Hidenori Tanaka, Atsuko Ogawa, Kazunori Nakajima and Shunichi Kato

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

The impact of anti-HLA antibodies on unrelated cord blood transplantations

Minoko Takanashi,[1] Yoshiko Atsuta,[2] Koki Fujiwara,[1] Hideki Kodo,[3] Shunro Kai,[4] Hiroyuki Sato,[5] Masatoshi Kohsaki,[6] Hiroshi Azuma,[7] Hidenori Tanaka,[1] Atsuko Ogawa,[1] Kazunori Nakajima,[1] and Shunichi Kato[8]

[1]Japanese Red Cross Tokyo Blood Center, Tokyo, Japan; [2]Department of HSCT Data Management, Nagoya University School of Medicine, Nagoya, Japan; [3]Tokyo Cord Blood Bank, Tokyo, Japan; [4]Department of Transfusion Medicine, Hyogo College of Medicine, Nishinomiya, Japan; [5]Japanese Red Cross Fukuoka Blood Center, Fukuoka, Japan; [6]Japanese Red Cross Osaka North Blood Center, Osaka, Japan; [7]Japanese Red Cross Hokkaido Blood Center, Sapporo, Japan; and [8]Department of Cell Transplantation & Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan

The majority of cord blood transplantations (CBTs) have human leukocyte antigen (HLA) disparities. We investigated the impact that patients' pretransplantation anti-HLA antibodies have on the outcome of CBTs. Testing for anti-HLA antibody and its specificity was performed retrospectively at the Japanese Red Cross Tokyo Blood Center with sensitive solid-phase antibody detection assays. Among 386 CBTs, which were first myeloablative stem cell transplantations for malignancies and used a single unit of cord blood, 89 tested positive. Among the antibody-positive group, the cord blood did not have the corresponding HLA type for the antibody in 69 cases (ab-positive), while 20 cases had specificity against the cord blood HLA (positive-vs-CB). Cumulative incidence of neutrophil recovery 60 days after transplantation was 83% (95% confidence interval [CI], 79%-87%) for the antibody-negative group (ab-negative), 73% (95% CI, 61%-82%) for ab-positive, but only 32% (95% CI, 13%-53%) for the positive-vs-CB ($P < .0001$, Gray test). With multivariate analysis, the ab-positive showed significantly lower neutrophil recovery than the ab-negative (relative risk [RR] = 0.69, 95% CI, 0.49-0.96, p = .027). The positive-vs-CB had significantly lower neutrophil recovery (RR = 0.23, 95% CI, 0.09-0.56, $P = .001$) and platelet recovery (RR = 0.31, 95% CI, 0.12-0.81, $P = .017$) than the ab-negative. Patients' pretransplantation anti-HLA antibodies should be tested and considered in the selection of cord blood. (*Blood.* 2010;116(15): 2839-2846)

Introduction

The number of unrelated cord blood transplantations (CBTs) has increased, mainly because cord blood (CB) is more readily available than bone marrow. CB can be collected without burden or risk to the donors, and successful outcomes have been reported with less stringent requirements for human leukocyte antigen (HLA) compatibility.[1-3] Comparable outcomes have been reported with analyses of unrelated bone marrow transplantations (BMTs) and CBTs, although there were lower neutrophil and platelet recoveries in CBTs.[4-7] Graft failure, with a high mortality rate, has been noted as a problem.[8,9]

The role of anti-HLA antibodies in graft rejection of organ transplantations has been analyzed extensively.[10,11] However, only a few studies have analyzed the significance of anti-HLA antibodies in stem cell transplantations,[12-14] in which the recipient's immune system is taken over by the donor's cells and for which a great effort is made to match the recipient and donor HLA types at the allele level.

We investigated the impact that patients' pretransplantation anti-HLA antibodies have on the outcome of CBTs, for which the majority have HLA mismatches. We previously reported that anti-HLA antibodies, when the specificity corresponded to a mismatched antigen, had a negative effect on engraftment of CBTs.[15] However, this finding was from a single cord blood bank study with limited samples. In this study, the number of cases is increased retrospectively, in cooperation with 7 of the public banks in Japan, with the intention of clarifying the significance of anti-HLA antibodies.

Methods

Patients

This study included patients with hematologic malignancies who received their first hematopoietic stem cell transplantation with a myeloablative conditioning regimen, using a single unit of CB from 1 of the 7 CB banks. All patients underwent CBT between 2001 and 2007. To be eligible for this study, patients' plasma/sera had to be available for analysis. Patients were excluded if they had not received conditioning, received reduced intensity conditioning or had not received graft-versus-host disease (GVHD) prophylaxis. The criteria were met by 386 patients, including the 153 cases analyzed in our previous report.[15] As a standard procedure, the CB banks confirmed the HLA types of the patients and CB units before shipping, with samples being stored frozen, having obtained written consent from the patients in accordance with the Declaration of Helsinki. The procedures of our CB bank were approved by the institutional review board of the Japanese Red Cross Blood Service. HLA matching of CB and patient was performed using the antigen levels for HLA-A, -B and -DR. Each CB bank collected recipients' clinical information at 100 days after transplantation. Patient information on survival, disease status and long-term complications was updated annually with follow-up questionnaires.

Antibody testing

Patients' plasma/sera samples stored in each CB bank were sent to the Japanese Red Cross Tokyo Blood Center, where the plasma samples were treated with thrombin. All samples were tested with FlowPRA (One Lambda) for class I (ie, HLA-A/B/C) and class II (ie, HLA-DR/DP/DQ) anti-HLA antibodies. Samples of 20 μL were incubated with HLA class I–coated and HLA class II–coated microspheres, respectively, for

Submitted October 19, 2009; accepted June 28, 2010. Prepublished online as *Blood* First Edition paper, July 13, 2010; DOI 10.1182/blood-2009-10-249219.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

30 minutes in the dark under gentle agitation. The specimens were then washed before being incubated with anti–human immunoglobulin G–conjugated fluorescein isothiocyanate in the same conditions as in the first incubation. Next, the samples were analyzed with a flow cytometer. When the histogram showed more than a 10% shift in the positive gate or multiple peaks, the samples were further tested for the specificity of the antibody using LABScreen PRA and Single Antigen (One Lambda).[16] Fluorescence was measured with the Luminex100 flow analyzer (Luminex) and the data were examined with an HLA software program (One Lambda). Generally, a median fluorescence intensity (MFI), adjusted for background signals, of 1000 or more was considered to be positive, and when there was a cross-reactive HLA antigen with an MFI of more than 800, it was also considered positive.

Definitions

A case was defined as being antibody-negative (ab-negative) when the screening test with FlowPRA was negative, or when the antibody specificity was not evident or was against the self-HLA type only. After identifying the specificity of the detected antibody, and when the designated specificity did not correspond to the recipient's own HLA, the case was defined as antibody-positive. The antibody-positive cases were further classified as either positive-vs-CB, when the specificity of the antibody corresponded to the mismatched antigen of the rejection direction for the donor-recipient HLA pair, or as ab-positive, when the mismatched donor antigen did not correspond to the antibody.

Neutrophil recovery was defined by an absolute neutrophil count of at least 500 cells/mm^3 for 3 consecutive days, by 60 days after transplantation, and platelet recovery was defined by a count of at least 20 000 platelets/mm^3 without transfusion support. An absence of neutrophil recovery by day 60 was defined as graft failure. Diagnosis and clinical grading of acute GVHD were performed according to the established criteria.[17] Relapse was defined as a recurrence of an underlying hematologic malignant disease. Treatment-related death was defined as death during continuous remission. Event-free survival (EFS) was defined as survival with engraftment in a state of continuous remission.

Statistical analysis

Descriptive statistical analysis was performed to assess variables that are related to patient, disease and transplant characteristics. The 2-sided χ^2 test was used for categorical variables, and the 2-sided Wilcoxon rank sum test was used for continuous variables. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of neutrophil and platelet recovery, acute GVHD, relapse and transplant-related mortality (TRM).[18] For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event; for GVHD, relapse and death without GVHD were the competing events; for relapse, death without relapse was the competing event; and, for TRM, relapse was the competing event. The Gray test was used for group comparisons of cumulative incidence.[19] Overall survival (OS) and EFS were calculated using the Kaplan-Meier method. The log-rank test was used for group comparisons of OS and EFS.

The association of anti-HLA antibodies with outcomes was evaluated by multivariate analyses, with the use of the Cox proportional-hazards regression to adjust for OS and EFS, and with the use of the Fine and Gray proportional-hazards model for subdistribution of a competing risk for other outcomes.[20] All models were adjusted for the variables that are known to be associated with outcome or for the variables differing in distribution between the groups ($P < .10$): patient's age at transplantation, patient's sex, donor-patient sex mismatch, donor-patient ABO mismatch, diagnosis, disease status at conditioning, the number of HLA mismatches by antigen level, the number of CD34 cells per patient weight, the type of prophylaxis against GVHD and the usage of granulocyte colony-stimulating factor (G-CSF). Variables with more than 2 categories were dichotomized for the final multivariate model. Variables were dichotomized as follows: patient age greater or less than 45 years at transplantation, recipient's sex, sex-mismatched donor-patient pair versus matched sex pair, donor-recipient ABO major mismatch versus others for ABO matching, myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) with multilineage dysplasia versus leukemia, lymphoproliferative disease (lymphoma or myeloma) versus leukemia, advanced versus standard risk of the disease, 2 loci HLA mismatches versus matched or 1 locus mismatch, CD34$^+$ cell dose $\leq 0.85 \times 10^5$/kg versus $> 0.85 \times 10^5$/kg, cyclosporine-based versus tacrolimus-based GVHD prophylaxis, GVHD prophylaxis including methotrexate (MTX) versus no MTX, and G-CSF usage versus no usage. Disease status at transplantation was categorized as standard risk for the first complete remission or the second complete remission of AML, the first complete remission of acute lymphoblastic leukemia (ALL), the first chronic phase of chronic myeloid leukemia (CML), refractory anemia of MDS or the first complete remission of lymphoproliferative disorders. No significant interactions were identified between each variable and anti-HLA antibody positivity. All P values were 2-sided.

Results

Anti-HLA antibodies

Of 386 cases tested, 89 (23.1%) were antibody-positive. Of the 89 antibody-positive cases, 69 were defined as ab-positive and 20 as positive-vs-CB. Among the 69 ab-positive cases, 45 had an antibody against an HLA class I antigen, 10 against an HLA class II, and 14 against both a class I and a class II. Among 20 positive-vs-CB cases, 15 had an antibody against an HLA class I antigen and 5 against an HLA class II.

Patient characteristics

The characteristics of the patients are shown in Table 1. There was a significant difference in recipients' age at transplantation, with the median age higher in the anti-HLA antibody-positive group than the antibody-negative group ($P < .0001$). The proportion of female patients in the anti-HLA antibody-positive group was larger than that in the negative group (75% vs 44%, $P < .0001$), and this resulted in a difference in the sex-matching proportion. In addition, the proportion of patients with MDS or AML with trilineage dysplasia after MDS was larger in the positive group (14% vs 6%). HLA-A, -B, and -DR (antigen level) were mismatched in the rejection direction in 88% of the cases. There were 46 pairs with 0 mismatches (6/6 HLAs matched), 157 pairs with 1 mismatch (5/6 HLAs matched), 182 pairs with 2 mismatches (4/6 HLAs matched), and 1 pair with 3 mismatches (3/6 HLAs matched). The CD34 dose was significantly lower in the anti-HLA antibody-positive group than the antibody-negative group ($P = .041$).

Outcomes

Effect of anti-HLA antibodies on hematologic recovery

Anti-HLA antibodies showed a significant effect on neutrophil and platelet recovery (Figure 1A-B). Compared with the ab-negative group, the unadjusted cumulative incidence of neutrophil recovery at day 60 was significantly lower in the ab-positive and positive-vs-CB groups (83% vs 73% and 32%; $P < .0001$). The unadjusted cumulative incidence of platelet recovery at 9 months was also significantly different for the ab-negative, ab-positive and positive-vs-CB groups, at 72%, 60% and 33%, respectively ($P = .0036$). There was no difference recognized between the effect of anti–class I antibodies and anti–class II antibodies (data not shown).

With multivariate analysis, adjusted for other recipient- and transplant-related variables, neutrophil recoveries in the ab-positive and positive-vs-CB groups were significantly affected compared with that in the ab-negative group (Hazard ratio [HR] = 0.69, 95% confidence interval [CI], 0.49-0.96, $P = .027$

Table 1. Characteristics of cord blood recipients for 297 patients with negative HLA antibody and 89 patients with positive HLA antibody

	Negative anti-HLA IgG		Positive anti-HLA IgG		
	N	%	N	%	*P*
Number of transplants	297		89		
Patient age at transplantation					< .0001
Median (range)	33	(0-69)	46	(0-68)	
Patient sex					< .0001
Male	166	(55.9)	22	(24.7)	
Female	131	(44.1)	67	(75.3)	
Sex matching					.001
Matched	146	(49.2)	46	(51.7)	
Male to female	66	(22.2)	33	(37.1)	
Female to male	85	(28.6)	10	(11.2)	
Disease					.002
ALL	109	(36.7)	18	(20.2)	
AML	122	(41.1)	47	(52.8)	
ATL	11	(3.7)	4	(4.5)	
MDS	17	(5.7)	12	(13.5)	
CML	9	(3.0)	6	(6.7)	
NHL	28	(9.4)	2	(2.2)	
MM	1	(0.3)	0	(0.0)	
Disease status*					.13
Standard	124	(41.8)	44	(49.4)	
Advanced	168	(56.6)	41	(46.1)	
Unknown	5	(1.7)	4	(4.5)	
Human leukocyte antigen matching (A, B, DR)					
No. of serologically mismatched loci					
Graft-versus-host disease direction					.27
0	34	(11.4)	17	(19.1)	
1	131	(44.1)	38	(42.7)	
2	131	(44.1)	34	(38.2)	
3	1	(0.3)	0	(0.0)	
Rejection direction					.75
0	36	(12.1)	10	(11.2)	
1	124	(41.8)	33	(37.1)	
2	136	(45.8)	46	(51.7)	
3	1	(0.3)	0	(0.0)	
ABO matching					.36
Matched	98	(33.0)	36	(40.4)	
Minor mismatch	71	(23.9)	17	(19.1)	
Major mismatch	128	(43.1)	35	(39.3)	
Unknown	0	(0.0)	1	(1.1)	
Number of nucleated cells infused, $\times 10^7$/kg					.61
Median (range)	2.58	(0.64-20.8)	2.56	(1.65-13.9)	
Number of CD34$^+$ cells infused, $\times 10^5$/kg					.041
Median (range)	0.88	(0.06-9.44)	0.80	(0.17-3.82)	
GVHD prophylaxis					.66
Cyclosporine based	174	(58.6)	48	(53.9)	
Tacrolimus based	111	(37.4)	38	(42.7)	
Other	12	(4.0)	3	(3.4)	
MTX for GVHD prophylaxis					.72
No	68	(22.9)	22	(24.7)	
Yes	229	(77.1)	67	(75.3)	
G-CSF					.71
No	20	(6.7)	7	(7.9)	
Yes	277	(93.3)	82	(92.1)	

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATL, adult T-cell leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; GVHD, graft-versus-host disease; MTX, methotrexate; and G-CSF, granulocyte colony-stimulating factor.

*Standard risk: 1st and 2nd complete remission of AML, 1st complete remission of ALL, 1st chronic phase of CML, refractory anemia of MDS, 1st complete remission of lymphoproliferative diseases.

for the ab-positive group, HR = 0.23, 95% CI, 0.09-0.56, $P = .001$ for the positive-vs-CB group). Neutrophil recovery was also significantly better for the ab-positive group than the positive-vs-CB group (HR = 0.31, 95% CI, 0.12-0.80, $P = .015$). Platelet recovery in the positive-vs-CB group was significantly affected compared with that in the ab-negative group (HR = 0.31, 95% CI, 0.12-0.81, $P = .017$), but not with that in the ab-positive group (HR = 0.35, 95% CI, 0.11-1.10, $P = .071$; Table 2). Of 84 patients defined as having graft failure, 2 recipients showed neutrophil recovery 60 days after transplantation (at day 65 [ab-negative] and

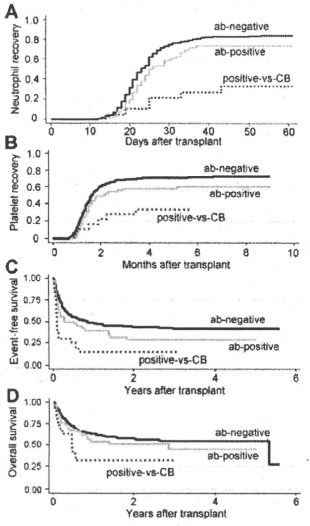


Figure 1. Cumulative incidence of neutrophil/platelet recovery and survival for 386 CBT cases. ab-negative indicates patient does not have anti-HLA antibody; ab-positive, patient has anti-HLA antibody but the CB does not have the corresponding antigen for the antibody specificity; and positive-vs-CB, patient has anti-HLA antibody and the CB has the corresponding antigen for the antibody specificity. The cumulative incidences of neutrophil recovery (A) for the ab-negative, ab-positive and positive-vs-CB groups were 83% (95% CI, 79%-87%), 73% (95% CI, 61%-82%), and 32% (95% CI, 13%-53%), respectively, at day 60 ($P < .0001$). The cumulative incidence for the ab-positive group was significantly lower than that of the ab-negative group ($P = .024$). The cumulative incidence for the positive-vs-CB group was significantly lower than that of the ab-positive group ($P = .005$). The cumulative incidences of platelet recovery (B) for the ab-negative, ab-positive and positive-vs-CB groups were 72% (95% CI, 67%-77%), 60% (95% CI, 47%-71%), and 33% (95% CI, 14%-55%), respectively, at 9 months ($P = .0036$). The differences between the ab-negative and ab-positive groups ($P = .05$) and the ab-positive and positive-vs-CB groups ($P = .062$) were not significant. For the Kaplan-Meier estimate of event-free survival (EFS; C), the impact of anti-HLA antibodies was significant ($P = .0001$), with EFS at 2 years for the ab-negative, ab-positive and positive-vs-CB groups being 43% (95% CI, 37%-49%), 29% (95% CI, 17%-41%), and 15% (95% CI, 4%-33%), respectively. The differences between the ab-negative and ab-positive groups ($P = .037$) and the ab-positive and positive-vs-CB groups ($P = .016$) were significant. For overall survival (OS; D), the impact of anti-HLA antibodies was significant ($P = .030$) with the OS at 2 years for the ab-negative, ab-positive and positive-vs-CB groups being 57% (95% CI, 51%-63%), 50% (95% CI, 36%-63%), and 31% (95% CI, 10%-55%), respectively. The differences between the ab-negative and ab-positive ($P = .25$) and the ab-positive and positive-vs-CB groups ($P = .13$) were not significant.

day 81 [positive-vs-CB]), 11 patients (13%) showed autologous recovery and 32 patients (38%) received a second transplantation.

Effect of anti-HLA antibodies on GVHD, relapse, and mortality

Anti-HLA antibodies did not have a significant effect on grade II-IV acute GVHD, relapse, or TRM. The EFS at 2 years after transplantation were 43%, 29%, and 15% for the ab-negative, ab-positive, and positive-vs-CB groups, respectively ($P = .0001$, log-rank test). The OS at 2 years after transplantation were 57%, 50%, and 31% for the ab-negative, ab-positive, and positive-vs-CB groups, respectively ($P = .030$, log-rank test; Figure 1C-D).

With multivariate analysis, adjusted for other recipient- and transplant-related variables, the positive-vs-CB group showed significantly inferior OS (HR = 2.33, 95% CI, 1.18-4.59, $P = .015$) and EFS (HR = 3.46, 95% CI, 2.01-5.96, $P < .001$) compared with the ab-negative group (Table 2). There was no significant increase in the risk of relapse (HR = 1.75, 95% CI, 0.73-4.21, $P = .21$) or TRM (HR = 2.06, 95% CI, 0.96-4.43, $P = .064$). The ab-positive group showed increased risks of relapse (HR = 1.98, 95% CI, 1.14-3.43, $P = .015$) and inferior EFS (HR = 1.53, 95% CI, 1.07-2.19, $P = .021$) compared with the ab-negative group, but there was no significant increase in the risk of TRM (HR = 0.94, 95% CI, 0.55-1.60, $P = .81$) or OS (HR = 1.33, 95% CI, 0.86-2.04, $P = .20$). No significant difference was shown in the risk of developing grade II-IV GVHD in the ab-positive and positive-vs-CB groups compared with that in the ab-negative group (HR = 0.76, 95% CI, 0.44-1.30, $P = .31$ for the ab-positive group, and HR = 0.49, 95% CI, 0.13-1.78, $P = .28$ for the positive-vs-CB group; Table 2).

A comparison of the positive-vs-CB group with the ab-positive group was also performed. The positive-vs-CB group showed increased risks of TRM (HR = 3.82, 95% CI, 1.37-10.71, $P = .0011$) and inferior EFS (HR = 2.30, 95% CI, 1.20-4.43, $P = .012$) compared with the ab-positive group (Table 2).

Risk modification according to CD34 cell dose

As the dichotomized CD34 dose was a significant factor in multivariate analysis of neutrophil recovery (RR 0.66, $P = .0004$) and platelet recovery (RR 0.73, $P = .015$), we proceeded to analyze the effect of anti-HLA antibodies in subgroups of CD34 cell dose. Cumulative incidences of neutrophil recovery at day 60 for the ab-negative, ab-positive and positive-vs-CB groups were 86%, 69%, and 32% ($P = .058$), respectively, for the higher CD34 cell dose group (CD34 cell dose more than the median, ie, $> 0.85 \times 10^5$/kg), and 80%, 77%, and 33% ($P = .0061$) for the lower CD34 cell dose group. Cumulative incidences of platelet recovery at 9 months for the ab-negative, ab-positive and positive-vs-CB groups were 77%, 64%, and 60% ($P = .36$), respectively, for the higher CD34 cell dose group and 67%, 56%, and 25% ($P = .020$) for the lower CD34 cell dose group (Figure 2).

Multivariate analysis showed that neutrophil recovery was significantly affected for the ab-positive in the higher CD34 cell dose group, and for all antibody categories in the lower CD34 cell dose group, compared with the ab-negative in the higher CD34 cell dose group. For platelet recovery, the ab-positive and positive-vs-CB in the lower CD34 cell dose group were significantly affected (Table 3).

Discussion

The short time needed to obtain a cord blood unit and the less rigorous HLA matching required have contributed to an increased number of CBTs. There are concerns, however, regarding the time taken for engraftment and the high rejection rates.[4-9] An accumulation of HLA-mismatched CBT cases and graft failure drew our attention to anti-HLA antibodies. Although there were early reports on engraftment in haploidentical BMTs,[12,21] the anti-HLA antibody test had not been routinely used unless a patient had become refractory to platelet transfusion, and such a condition was sometimes regarded to be a contraindication for CBT.[1]

Table 2. Results of multivariate analysis of outcomes in 386 cord blood transplantations

		Hazard ratio	95% CI	P
Neutrophil recovery	ab-negative	1.00		
	ab-positive	0.69	(0.49-0.96)	.027
	positive-vs-CB	0.23	(0.09-0.56)	.001
	positive-vs-CB vs ab-positive	0.31	(0.12-0.80)	.015
Platelet recovery	ab-negative	1.00		
	ab-positive	0.73	(0.49-1.07)	.11
	positive-vs-CB	0.31	(0.12-0.81)	.017
	positive-vs-CB vs ab-positive	0.35	(0.11-1.10)	.071
Grade II-IV acute GVHD	ab-negative	1.00		
	ab-positive	0.76	(0.44-1.30)	.31
	positive-vs-CB	0.49	(0.13-1.78)	.28
	positive-vs-CB vs ab-positive	0.79	(0.19-3.20)	.74
Relapse	ab-negative	1.00		
	ab-positive	1.98	(1.14-3.43)	.015
	positive-vs-CB	1.75	(0.73-4.21)	.21
	positive-vs-CB vs ab-positive	0.69	(0.24-1.97)	.48
Transplant-related mortality	ab-negative	1.00		
	ab-positive	0.94	(0.55-1.60)	.81
	positive-vs-CB	2.06	(0.96-4.43)	.064
	positive-vs-CB vs ab-positive	3.82	(1.37-10.71)	.0011
Treatment failure (EFS)	ab-negative	1.00		
	ab-positive	1.53	(1.07-2.19)	.021
	positive-vs-CB	3.46	(2.01-5.96)	< .001
	positive-vs-CB vs ab-positive	2.30	(1.20-4.43)	.012
Overall mortality (OS)	ab-negative	1.00		
	ab-positive	1.33	(0.86-2.04)	.20
	positive-vs-CB	2.33	(1.18-4.59)	.015
	positive-vs-CB vs ab-positive	1.99	(0.85-4.70)	.12

CI indicates confidence interval; GVHD, graft-versus-host disease; ab-negative, patient does not have anti-HLA antibody; ab-positive, patient has anti-HLA antibody but the CB does not have the corresponding antigen for the antibody specificity; and positive-vs-CB, patient has anti-HLA antibody and the CB has the corresponding antigen for the antibody specificity.

For neutrophil recovery, other significant variables were CD34+ cell dose $\leq 0.85 \times 10^5$/kg, no usage of G-CSF, advanced disease status and diagnosis of MDS or AML with multilineage dysplasia.

For platelet recovery, other significant variables were advanced disease status, CD34+ cell dose $\leq 0.85 \times 0^6$/kg and GVHD prophylaxis without MTX.

For acute GVHD of grade II-IV, other significant variables were diagnosis of lymphoproliferative disease and CD34+ cell dose $\leq 0.85 \times 10^5$/kg.

For relapse, other significant variables were advanced disease status and patient's sex being male.

For transplant-related mortality, other significant variables were patient age more than 45 years at transplantation, GVHD prophylaxis without MTX and cyclosporin-based GVHD prophylaxis compared with tacrolimus-based GVHD prophylaxis.

For treatment failure (as a reverse of event-free survival), other significant variables were advanced disease status, patient age more than 45 years at transplantation, diagnosis of lymphoproliferative disease and GVHD prophylaxis without MTX.

For overall mortality (as a reverse of overall survival), other significant variables were advanced disease status, patient age more than 45 years at transplantation and GVHD prophylaxis without MTX.

By comparing patients with positive and negative anti-HLA antibody test results, we found that the positive group included a higher ratio of female and MDS patients, suggesting allosensitization in less immunocompromised circumstances. In this analysis, 23.1% of our samples tested positive for an anti-HLA antibody, which is higher than the 15% reported previously with data from a single CB bank.[15] However, in this analysis, 2 of the participating CB banks chose samples with positive results from their own screening tests, and thus excluded some of the clearly negative samples. In addition, the test results by FlowPRA and using Luminex beads should be considered carefully. There is a report that describes the high positivity rates obtained by this test method as being due to natural antibodies,[22] and others regard this test system as too sensitive and lacking clinical relevance.[23] The discrepancy in positivity rates between laboratories (data not shown) led us to collect the samples in 1 laboratory for this analysis.

We have clearly shown that the patients' pretransplantation anti-HLA antibodies are a negative factor for engraftment, especially when the specificity corresponds to the donor antigens. In such cases, the probability of engraftment falls to only 38%. In this analysis there was an effect shown on neutrophil recovery in the ab-positive group as well as in the positive-vs-CB group. This is different from our preliminary report, in which the neutrophil recovery of the ab-positive group was similar to that of the ab-negative group.[15] This discrepancy might be due to limiting the subjects to recipients of myeloablative conditioning, and to the increase in the number of subjects for analyses containing a higher proportion of alloimmunized cases, together with the difference between the cumulative incidence statistical method used in this study, which considers competing risks, and the Kaplan-Meier method that was used in the previous study. In addition, there is a report that shows that priming to 1 alloantigen results in the elimination of donor bone marrow of a different alloantigen.[24]

We confirmed the importance of the CD34 cell dose in engraftment, in accordance with the report by Wagner et al that showed that the CD34 cell dose has a significant impact on neutrophil recovery, TRM and survival.[25] Earlier, Rubinstein et al[26] reported that myeloid engraftment was associated with the cell number of the unit together with the degree of HLA compatibility, and suggested a role for HLA alloimmunization in some graft failures. In this study with multivariate analysis, the effect of the

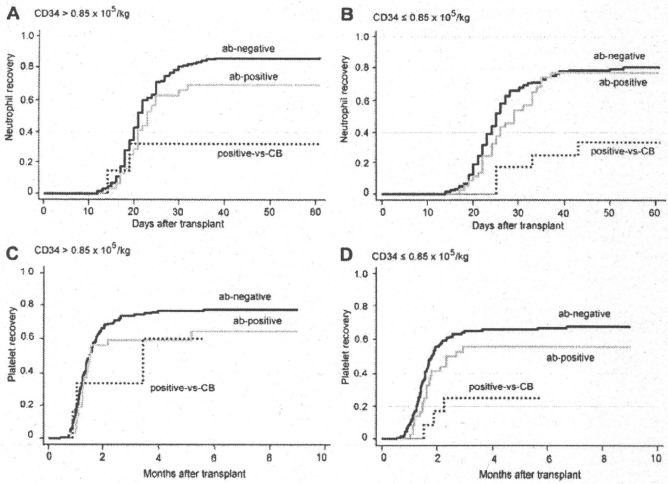


Figure 2. Cumulative incidence of neutrophil and platelet recovery in subgroups of CD34 cell dose. ab-negative indicates patient does not have anti-HLA antibody; ab-positive, patient has anti-HLA antibody but the CB does not have the corresponding antigen for the antibody specificity; and positive-vs-CB, patient has anti-HLA antibody and the CB has the corresponding antigen for the antibody specificity. Neutrophil (A) and platelet (C) recovery was not significantly affected by anti-HLA antibodies in the subgroup of CD34 cell dose $> 0.85 \times 10^5$/kg. In the subgroup of CD34 cell dose $\leq 0.85 \times 10^5$/kg, the anti-HLA antibodies showed a significant impact on neutrophil ($P = .0061$; B) and platelet recovery ($P = .020$; D).

antibody on neutrophil recovery was significant for ab-positive cases in the CD34 subgroup of more than the median cell dose. The hazard ratio for positive-vs-CB in the lower CD34 cell dose group was especially low.

Ottinger et al showed that the OS for their crossmatch positive cases was significantly lower than that for the crossmatch negative control group among those receiving a transplant at an early stage of the disease,[13] and also showed that graft failure is the dominant factor for low OS. In our study, patients with antibodies against the graft (positive-vs-CB) had a significantly higher graft failure rate, an approximately 4-fold increase, and had inferior OS and EFS compared with antibody-negative patients. They showed a tendency for increased TRM, yet the difference was marginal ($P = .064$). One possible reason for the TRM increase being marginal is the relatively high number of second transplantations. Among those who did not achieve neutrophil recovery, 38% received a second transplantation and 13% showed autologous recovery. The longer survival period under a highly immunosuppressed condition, provided by the second transplantation or autologous recovery, may have contributed to elevate the risk of relapse, which affected the OS. Another possible reason is the influence of other causes of TRM. Engraftment is important to control infection. However, infections often develop early. We have previously reported that in CB transplantations the median day of early infection was 8 days after transplantation, and bacterial infection in adults significantly affected mortality.[27]

Table 3. Multivariate analysis of anti-HLA antibody and CD34 cell dose

	CD34 cell dose	Anti-HLA antibody	Hazard ratio	(95%CI)	P
Neutrophil recovery	$> 0.85 \times 10^5$/kg	ab-negative	1		
		ab-positive	0.55	(0.31-0.98)	.042
		positive-vs-CB	0.27	(0.05-1.44)	.12
	$\leq 0.85 \times 10^5$/kg	ab-negative	0.59	(0.46-0.76)	< .0001
		ab-positive	0.52	(0.37-0.74)	.0002
		positive-vs-CB	0.17	(0.07-0.42)	.0001
Platelet recovery	$> 0.85 \times 10^5$/kg	ab-negative	1		
		ab-positive	0.78	(0.45-1.36)	.38
		positive-vs-CB	0.50	(0.09-2.92)	.44
	$\leq 0.85 \times 10^5$/kg	ab-negative	0.76	(0.57-1.01)	.057
		ab-positive	0.51	(0.32-0.82)	.0051
		positive-vs-CB	0.19	(0.06-0.61)	.0049

CI indicates confidence interval; ab-negative, patient does not have anti-HLA antibody; ab-positive, patient has anti-HLA antibody but the CB does not have the corresponding antigen for the antibody specificity; and positive-vs-CB, patient has anti-HLA antibody and the CB has the corresponding antigen for the antibody specificity.

For the long-term effect, we could not assess changes in the anti-HLA antibodies after the transplantation, as the CB banks do not have patients' blood samples taken after the CBT. In addition, the follow-up information does not include the effectiveness of the platelet transfusions from random donors.

Among variables used in this analysis, HLA disparity at the antigen level, 2 HLA mismatches versus matched or 1 mismatch, did not have a significant effect on the outcome. Previously, Rubinstein et al reported that an absence of HLA disparity was a significant factor for neutrophil recovery, together with cell dose.[26] HLA disparity has been reported by the Eurocord group to be significant in neutrophil and platelet recovery and in relapse after CBT in a study in which 65% of the patients were 15 years old or younger.[2] University of Minnesota researchers reported a significant effect of HLA disparity on survival but not on engraftment when the median age was 7.4 years,[25] but no clear association with it for adult cases.[3] One explanation for HLA disparity not having a significant effect in our study is that our cases consisted of pediatric and adult patients, and included a wide variety of GVHD prophylaxis, making the effect of HLA disparity unclear.

From the findings of our study, we simply suggest in clinical practice to determine the presence and specificity of any anti-HLA antibodies, and avoid the corresponding antigens when choosing a CB unit. There are already reports on successful engraftments after CBT by avoiding the corresponding antigens.[28,29] The quality of the CB unit also has to be considered, as cell dose is a significant factor for engraftment.[25,26] From the multivariate analysis shown in Table 3, it appears that the choice of a unit with an antigen(s) corresponding to the antibody specificity presents a higher risk of graft failure. As the test system is highly sensitive, it is possible that the result will show that a patient's antibodies have a wide range of specificity. For such an occurrence, instead of opting not to use CB for the transplantation, it would be beneficial for transplant centers to have access to confirmative tests for antibodies. A test method that targets antigens naturally expressed on the cell surface, such as immunocomplex capture fluorescence analysis[30] or a lymphocyte indirect immunofluorescence test,[31] or alternative crossmatch tests with HLA-typed cells could be helpful in clinical decision making.

We conclude that patients' pretransplantation anti-HLA antibodies should be identified for specificity and considered in the selection of cord blood units.

Acknowledgments

We thank all of the physicians and staff at the hospitals in Japan who collaborated in this study.

This work was supported in part by a Research Grant for Tissue Engineering (H17-014) and a Research Grant for Allergic Disease and Immunology (H20-015) from the Japanese Ministry of Health, Labor and Welfare.

Authorship

Contribution: M.T. designed the study, analyzed data, and wrote the paper; Y.A. performed statistical analysis and co-wrote the paper; K.F., H.T. and A.O. performed research and analyzed the data; H.K., S. Kai, H.S., M.K., H.A. and S. Kato submitted samples and extracted data; and K.N. reviewed and oversaw the research design.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Minoko Takanashi, MD, PhD, Japanese Red Cross Tokyo Blood Center, 2-1-67 Tatsumi, Koto-ku, Tokyo 135-8639, Japan; e-mail: mi-takanashi@tokyo.bc.jrc.or.jp.

References

1. Takahashi S, Ooi J, Tomonari A, et al. Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. *Blood.* 2007;109(3):1322-1330.
2. Gluckman E, Rocha V, Arcese W, et al. Factors associated with outcomes of unrelated cord blood transplant: guidelines for donor choice. *Exp Hematol.* 2004;32:397-407.
3. Brunstein CG, Barker JN, Weisdorf DJ, et al. Umbilical cord blood transplantation after nonmyeloablative conditioning: impact on transplantation outcomes in 110 adults with hematologic disease. *Blood.* 2007;110(8):3064-3070.
4. Rocha V, Labopin M, Sanz G, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med.* 2004;351:2276-2285.
5. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med.* 2004;351:2265-2275.
6. Takahashi S, Iseki T, Ooi J, et al. Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. *Blood.* 2004;104:3813-3820.
7. Atsuta Y, Suzuki R, Nagamura-Inoue T, et al. Japan Cord Blood Bank Network. Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. *Blood.* 2009;113(8):1631-1638.
8. Narimatsu H, Kami M, Miyakoshi S, et al. Graft failure following reduced-intensity cord blood transplantation for adult patients. *Br J Haematol.* 2006;132(1):36-41.
9. Mattsson J, Ringdén O, Storb R. Graft failure after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* 2008;14:165-170.
10. Süsal C, Döhler B, Opelz G. Presensitized kidney graft recipients with HLA class I and II antibodies are at increased risk of graft failure. A collaborative transplant study report. *Hum Immunol.* 2009; 70:569-573.
11. Everly MJ, Terasaki PI. Monitoring and treating posttransplant human leukocyte antigen antibodies. *Hum Immunol.* 2009;70:655-659.
12. Anasetti C, Amos D, Beatty PG, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med.* 1989;320:197-204.
13. Ottinger HD, Rebmann V, Pfeiffer KA, et al. Positive serum crossmatch as predictor for graft failure in HLA-mismatched allogeneic blood stem cell transplantation. *Transplantation.* 2002;73(8): 1280-1285.
14. Gutman JA, McKinney SK, Pereira S, et al. Prospective monitoring for alloimmunization in cord blood transplantation: "virtual crossmatch" can be used to demonstrate donor-directed antibodies. *Transplantation.* 2009;87(3):415-418.
15. Takanashi M, Fujiwara K, Tanaka H_1 et al. The impact of HLA antibodies on engraftment of unrelated cord blood transplants. *Transfusion.* 2008; 48:791-792.
16. Pei R, Wang G, Tarsitani C, et al. Simultaneous HLA class I and class II antibodies screening with flow cytometry. *Human Immunol.* 1998;59:313-322.
17. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant.* 1995;15:825-828.
18. Gooley TA, Leisenring W, Crowley J, et al. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med.* 1999;18:695-706.
19. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. *Ann Stat.* 1988;16:1141-1154.
20. Fine JP, Gray RJ. A proportional hazards model for subdistribution of a competing risk. *J Am Stat Assoc.* 1999;94:456-509.
21. Barge AJ, Johnson G, Witherspoon R, et al. Antibody-mediated marrow failure after allogeneic bone marrow transplantation. *Blood.* 1989;74(5): 1477-1480.
22. Morales-Buenrostro LE, Terasaki PI, Marino-Vázquez LA, et al. "Natural" human leukocyte antigen antibodies found in no alloimmunized healthy males. *Transplantation.* 2008;86:1111-1115.
23. Claas FHJ, Doxiadis IIN. Human leukocyte antigen antibody detection and kidney allocation within Eurotransplant. *Human Immunology.* 2009; 70:636-639.
24. Taylor PA, Ehrhardt MJ, Roforth MM, et al. Preformed antibody, not primed T cells, is the initial and major barrier to bone marrow engraftment in allosensitized recipients. *Blood.* 2007;109(3): 1307-1315.
25. Wagner JE, Barker JN, DeFor TE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood.* 2002;100:1611-1618.

26. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *N Engl J Med.* 1998;339(22):1565-1577.

27. Yazaki M, Atsuta Y, Kato K, et al. Incidence and risk factors of early bacterial infections after unrelated cord blood transplantation. *Biol Blood Marrow Transplant.* 2009;15:439-446.

28. Kataoka K, Yamamoto G, Nannya Y, et al. Successful engraftment following HLA-mismatched cord blood transplantation for patients with anti-HLA Abs. *Bone Marrow Transplant.* 2008;42(2): 129-130.

29. Uchiyama M, Ikeda T. Successful engraftment following umbilical cord blood transplantation for patients with HLA antibody with or without corresponding HLA in the transplanted cord blood. *Bone Marrow Transplant.* 2010;45(1): 199-200.

30. Fujiwara K, Shimano K, Tanaka H, et al. Application of bead array technology to simultaneous detection of human leucocyte antigen and human platelet antigen antibodies. *Vox Sang.* 2009;96: 244-251.

31. Lubenko A, Rodi KM, Johnson AC. Screening for WBC antibodies by lymphocyte indirect immunofluorescence flow cytometry: superior to cytotoxicity and ELISA? *Transfusion.* 2001;41:1147-1153.

blood

2010 116: 1369-1376
Prepublished online May 17, 2010;
doi:10.1182/blood-2009-10-247510

Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study

Masakatsu Hishizawa, Junya Kanda, Atae Utsunomiya, Shuichi Taniguchi, Tetsuya Eto, Yukiyoshi Moriuchi, Ryuji Tanosaki, Fumio Kawano, Yasushi Miyazaki, Masato Masuda, Koji Nagafuji, Masamichi Hara, Minoko Takanashi, Shunro Kai, Yoshiko Atsuta, Ritsuro Suzuki, Takakazu Kawase, Keitaro Matsuo, Tokiko Nagamura-Inoue, Shunichi Kato, Hisashi Sakamaki, Yasuo Morishima, Jun Okamura, Tatsuo Ichinohe and Takashi Uchiyama

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study

*Masakatsu Hishizawa,[1] *Junya Kanda,[1] Atae Utsunomiya,[2] Shuichi Taniguchi,[3] Tetsuya Eto,[4] Yukiyoshi Moriuchi,[5] Ryuji Tanosaki,[6] Fumio Kawano,[7] Yasushi Miyazaki,[8] Masato Masuda,[9] Koji Nagafuji,[10] Masamichi Hara,[11] Minoko Takanashi,[12] Shunro Kai,[13] Yoshiko Atsuta,[14] Ritsuro Suzuki,[14] Takakazu Kawase,[15] Keitaro Matsuo,[15] Tokiko Nagamura-Inoue,[16] Shunichi Kato,[17] Hisashi Sakamaki,[18] Yasuo Morishima,[19] Jun Okamura,[20] Tatsuo Ichinohe,[1] and Takashi Uchiyama[1]

[1]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto; [2]Department of Hematology, Imamura Bun-in Hospital, Kagoshima; [3]Department of Hematology, Toranomon Hospital, Tokyo; [4]Department of Hematology, Hamanomachi Hospital, Fukuoka; [5]Department of Hematology, Sasebo City General Hospital, Sasebo; [6]Stem Cell Transplantation Unit, National Cancer Center Hospital, Tokyo; [7]Division of Internal Medicine, National Hospital Organization, Kumamoto Medical Center, Kumamoto; [8]Department of Hematology and Molecular Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Science, Nagasaki; [9]Cancer Center, University Hospital, Faculty of Medicine, University of the Ryukyus, Nishihara; [10]Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka; [11]Division of Hematology, Ehime Prefectural Central Hospital, Matsuyama; [12]Japanese Red Cross Tokyo Metropolitan Blood Center, Tokyo; [13]Department of Transfusion Medicine, Hyogo College of Medicine, Nishinomiya; [14]Department of Hematopoietic Stem Cell Transplantation Data Management, Nagoya University, School of Medicine, Nagoya; [15]Division of Epidemiology and Prevention, Aichi Cancer Center, Nagoya; [16]Department of Cell Processing and Transfusion, Research Hospital, Institute of Medical Science, University of Tokyo, Tokyo; [17]Department of Cell Transplantation and Regenerative Medicine, Tokai University, School of Medicine, Isehara; [18]Hematology Division, Tokyo Metropolitan Komagome Hospital, Tokyo; [19]Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya; and [20]Institute for Clinical Research, National Kyushu Cancer Center, Fukuoka, Japan

Allogeneic hematopoietic stem cell transplantation (HSCT) is increasingly used as a curative option for adult T-cell leukemia (ATL), an intractable mature T-cell neoplasm causally linked with human T-cell leukemia virus type I (HTLV-I). We compared outcomes of 386 patients with ATL who underwent allogeneic HSCT using different graft sources: 154 received human leukocyte antigen (HLA)–matched related marrow or peripheral blood; 43 received HLA-mismatched related marrow or peripheral blood; 99 received unrelated marrow; 90 received single unit unrelated cord blood. After a median follow-up of 41 months (range, 1.5-102), 3-year overall survival for entire cohort was 33% (95% confidence interval, 28%-38%). Multivariable analysis revealed 4 recipient factors significantly associated with lower survival rates: older age (> 50 years), male sex, status other than complete remission, and use of unrelated cord blood compared with use of HLA-matched related grafts. Treatment-related mortality rate was higher among patients given cord blood transplants; disease-associated mortality was higher among male recipients or those given transplants not in remission. Among patients who received related transplants, donor HTLV-I seropositivity adversely affected disease-associated mortality. In conclusion, allogeneic HSCT using currently available graft source is an effective treatment in selected patients with ATL, although greater effort is warranted to reduce treatment-related mortality. (*Blood.* 2010;116(8):1369-1376)

Introduction

Adult T-cell leukemia (ATL) is a mature T-cell neoplasm developing in a minority of persons infected with human T-cell leukemia virus type I (HTLV-I), the first retrovirus isolated from a human malignant disease.[1-4] HTLV-I is estimated to infect 10 to 20 million people worldwide and is endemic in some areas of Japan, sub-Saharan Africa, the Caribbean Basin, and South America.[5,6] The area with the highest HTLV-I prevalence is the Kyushu district in southwestern Japan, where more than 10% of the general population is infected and the cumulative incidence of developing ATL among adult virus carriers is estimated at approximately 6.6% for males and 2.1% for females.[7] The onset of ATL after HTLV-I infection appears to require a long latency period because the median age at diagnosis ranges from 40 to 60 years in most endemic regions where mother-to-child viral transmission had been previously common.[4-6]

Clinical manifestation of ATL is heterogeneous and characterized by various degrees of lymphadenopathy, abnormal lymphocytosis, hepatosplenomegaly, skin lesions, and hypercalcemia, dividing the disease into 4 subtypes: acute, lymphomatous, chronic, and smoldering.[8] Patients with acute or lymphomatous type had extremely poor prognosis, mainly because of resistance to a variety of cytotoxic agents and susceptibility to opportunistic infections. Chronic and smoldering forms have relatively indolent clinical courses but can transform into more aggressive subtypes. During the past 3 decades since the clinical discovery of ATL,[1] the results of conventional cytotoxic chemotherapy remain dismal because of low response rates and lack of long-term efficacy. The

Submitted October 5, 2009; accepted May 8, 2010. Prepublished online as *Blood* First Edition paper, May 17, 2010; DOI 10.1182/blood-2009-10-247510.

*M. Hishizawa and J.K. contributed equally to this work.

A part of this work was presented as an abstract at the 49th Annual Meeting of the American Society of Hematology, Atlanta, GA, December 10, 2007.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

median survival time that followed the best clinical results to date is approximately 13 months[9,10]; complete response can only be achieved in 25%-40% of treated cases and most of them eventually relapsed with the median progression-free survival time of 5 to 7 months, whereas available treatment options are extremely limited in those who failed initial chemotherapy.[11-14]

Although the early experience of ablative chemoradiotherapy with autologous hematopoietic stem cell rescue for ATL resulted in a high incidence of relapse and fatal toxicities,[15] allogeneic hematopoietic stem cell transplantation (HSCT) has been explored as a promising alternative that can provide long-term remission in a proportion of patients with ATL.[16-19] Although the mechanisms by which allografting can eradicate HTLV-I–infected neoplastic T cells are not fully elucidated, several reports have suggested the role of graft-versus-HTLV-I or graft-versus-ATL effects.[20-23] Over the past decade, improved access to alternative stem cell sources and the development of less toxic conditioning regimens have led to a rapid increase in the number of cases of ATL treated with allogeneic HSCT, albeit without consistent efficacy.[24-30] Therefore, we conducted a nationwide retrospective cohort study to identify pretransplantation factors that affect survival after allografting for ATL, with special emphasis on the effect of graft source: we compared the outcomes of human leukocyte antigen (HLA)–mismatched related bone marrow or peripheral blood transplantation, unrelated bone marrow transplantation, and unrelated cord blood transplantation with those of HLA-matched related bone marrow or peripheral blood transplantation as treatment for ATL. We also evaluated the effect of donor HTLV-I serostatus on outcomes among patients who received transplants from related donors.

Methods

Collection of data

Data on 417 patients with acute or lymphomatous type ATL who had received T-cell–replete allogeneic bone marrow, peripheral blood, or cord blood transplantation between January 1, 1996, and December 31, 2005, were collected through the 3 largest hematopoietic cell transplant registries in our country: the Japan Society for Hematopoietic Cell Transplantation (JSHCT), the Japan Marrow Donor Program (JMDP), and the Japan Cord Blood Bank Network (JCBBN). The patients were included from 102 transplant centers; the data were updated as of December 2008. To evaluate the effect of HTLV-I infection in donors on transplantation outcomes, additional questionnaires were sent to 77 centers in January 2010 to retrieve data on donor HTLV-I serostatus in 217 related transplants registered with the JSHCT. Our analysis included patients for whom there was data on age at transplantation, sex, donor type, stem cell source, and agents used in the conditioning regimen and graft-versus-host disease (GVHD) prophylaxis. Twenty-two patients who missed any of these data, and 8 patients who had a history of prior autologous or allogeneic stem cell transplantation were excluded from the analysis. One patient who had received an ex vivo T-cell–depleted graft was also excluded. Two independent physicians reviewed the quality of collected data, and a total of 386 patients (209 males and 177 females), with a median age of 51 years (range, 18-79 years), were found to fulfill the inclusion criteria: 197 patients from JSHCT, 99 from JMDP, and 90 from JCBBN. No overlapping cases were identified. Data on engraftment or graft failure were missing in 23 patients. Data on acute GVHD were not available in 53 patients because of early death or missing data.

The JSHCT registry currently includes more than 390 transplant centers variously located in Japan and collects data on transplantation by use of autologous or related stem cell grafts. The JMDP includes more than 190 centers and collects data on unrelated bone marrow transplantation. The JCBBN, a national network of 11 cord blood banks, collects data on unrelated cord blood transplantations reported individually from more than 220 transplant centers to each bank. Participating centers to these registries are requested to report each type of transplantation consecutively and longitudinally. Until 2005, the 3 registries were operated separately from one another: however, a project attempting to unify them has been launched via development of the Transplant Registry Unified Management Program, which enables participating centers to use a shared format for data submission to each registry.[31] All unrelated donor transplants in Japan were facilitated through the JMDP and JCBBN, although peripheral blood donation from unrelated volunteers has not yet been instituted as of March 2010. The study was approved by the data management committees of the JSHCT, JMDP, and JCBBN, as well as by the institutional review boards of Kyoto University, Graduate School of Medicine, where this study was organized.

End points

The primary end point of the study was overall survival, defined as the time from the date of transplantation until date of death from any cause. Patients who remained alive at the time of last follow-up were censored. Reported causes of death were reviewed and categorized into disease-associated or treatment-associated deaths. Disease-associated deaths were defined as deaths from relapse or progression of ATL among patients who survived for at least 30 days after transplantation. Treatment-related deaths were defined as any death other than disease-associated deaths. Neutrophil recovery was considered to have occurred when an absolute neutrophil count exceeded 0.5×10^9/L for 3 consecutive days after transplantation. Primary graft failure was evaluated in patients who survived at least 30 days and was defined as no evidence of neutrophil recovery after transplantation. Acute and chronic GVHD were diagnosed and graded using traditional criteria by the physicians who performed transplantations at each center.[32,33] The incidence of acute GVHD was evaluated in patients who survived for at least 7 days, and that of chronic GVHD was evaluated in patients who survived for at least 100 days.

Statistical analysis

Descriptive statistics were used for summarizing variables related to patient demographics and transplant characteristics. Comparisons among the groups were performed by use of the χ^2 statistic or extended Fisher exact test as appropriate for categorical variables, and the Kruskal-Wallis test for continuous variables. The probability of overall survival was estimated according to the Kaplan-Meier method, and univariable comparisons among the groups were made using the log-rank test. Probabilities of acute and chronic GVHD, treatment-related mortality, and disease-associated mortality were estimated with the use of cumulative incidence curves to accommodate the following competing events[34]: death without GVHD for acute and chronic GVHD, disease-associated death for treatment-related mortality, and treatment-related death for disease-associated mortality. Data on patients who were alive at the time of last follow-up were censored. Cox proportional-hazards regression was used to evaluate variables potentially affecting overall survival, whereas Fine and Gray proportional-hazard model was used to evaluate variables affecting other outcomes.[35] The variables considered were recipient age group ($\leq$ 50 years or $>$ 50 years at transplantation); recipient sex; disease status before transplantation; type of conditioning regimen; type of GVHD prophylaxis; type of graft source; time from diagnosis to transplantation (within 6 months or longer than 6 months); and year of transplantation. Only factors differing in distribution among the graft source groups and factors associated with outcomes by univariable comparison were included in the final models. The effect of donor HTLV-I seropositivity on outcomes after related donor transplantation was also evaluated by univariable and multivariable analysis with the use of data on 156 patients given transplants from siblings or other related family members for whom data on the HTLV-I serostatus were available. Results were expressed as hazard ratios and their 95% confidence interval (CI). All tests were 2-sided, and a P value of less than .05 was considered to indicate statistical significance. All statistical analyses were performed with STATA software (Version 11; Stata Corporation).

Results

Patients

Table 1 shows characteristics of the patients and transplantation procedures. Compared with HLA-matched related bone marrow or