CA, USA), 20 pmol of each primer and 1.25 U of *Taq* polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA). Four microliters of DNA (80 ng) was denatured at 98°C for 5 min, and then 35 cycles of denaturation (96°C, 1 min), annealing (58°C, 1 min) and extension at 72°C for 5 min were applied using an automated PCR thermal cycler (PERKIN ELMER CETUS).

PCR products (10 μl) were digested with 5 U of *Mva*I for CD31 codon 125, *Bfa*I for CD31 codon 563, *Mnl*I for CD49b, *Hph*I for CD62L at 37°C for 4 h and with *Tsp*45I for HA-1 overnight. Fragments were resolved by electrophoresis on 10% polyacrylamide gels for 1 h at 150 V. RFLP profiles in the gel were visualized by silver staining.

Diagnosis of incompatibility of each polymorphic molecule

The combination of HA-1-positive recipients ($HA\text{-}1^{H/R}$ or $HA\text{-}1^{H/H}$) and an HA-1-negative donor ($HA\text{-}1^{R/R}$) was defined as incompatible. HA-1 was restricted to HLA-A2, but patients with the other class I superfamilies were also evaluated. Incompatibility of the other four polymorphic adhesion molecules was defined as a combination of HLA-restricted patients transplanted with material from CD31, CD49b, CD62L-incompatible donors as defined by Maruya *et al*.[6] The CD31 molecules are restricted to the HLA-B44-like superfamily (B37, B41, B44, B45, B47, B49, B50, B60 and B61), CD49b molecules to the HLA-A3-like superfamily (A3, A11, A31, A33 and A*6801) and CD62L molecules to the HLA-A3-like or B44-like superfamilies or both.[13]

Statistical analysis

Acute GVHD was classified according to the described criteria.[14] Relapse was diagnosed as emerging original leukemic cells after allogeneic SCT.

Relapse rate between incompatible and compatible patients was compared by a χ^2 test. Multivariate analysis was performed with logistic regression analysis. Variables included mHag, standard risk at SCT, TBI regimen over 10 Gy, stem cell source (PBSC), UR-BMT, sex incompatibility, a-GVHD and chronic GVHD. Survival rates and curves were estimated using the Kaplan–Meier method, and the log-rank statistical test analyzed differences.

Table 2 Primer sequences

CD31 codon 125 (PECAM-1)
5′-AGTGTTGACATGAAGAGCCTGC-3′
5′-TCAGTTCCAAGGACTCACCTTC-3′
CD31 codon 563 (PECAM-1)
5′-GATGAGGTCCAGATTTCTATC-3′
5′-CTCTGACTGTCAGTATTTTGC-3′
CD49b (VLA-2, GPIα)
5′-GTGACCTAAAGAAAGAGGAAGGA-3′
5′-GATGAAATGTAAACCATACTATCTG-3′
CD62L (LECAM-1)
5′-TGATTCAGTGTGAGCCTTTG-3′
5′-CTTGAGAGGTTGGTTCTG-3′
HA-1
5′-AGGACATCTCCCATCTGCTG-3′
5′-GCCTTTCCCTCCTAATTGCC-3′

Abbreviations: LECAM-1 = L-selectin; PECAM-1 = platelet endothelial cell adhesion molecule-1; VLA-2, GPIα = glycoprotein IA/IIA.

Results

Characteristics of mHag-incompatible and -compatible patients

Table 1 shows comparisons between the two groups. Out of 106 patients, 36 were incompatible with at least one of these molecules, and the other 70 patients were compatible with the donor. Comparison revealed that the relapse rate was lower in the incompatible ($n = 36$) than in the compatible ($n = 70$) patients ($P < 0.013$), although the other characteristics were compatible. The distribution of HA-1 allele type compatibility was identical to published data in Japan.[15]

Multivariate analysis

Table 3 shows the multivariate analysis. Incompatibility of at least one mHag was the most powerful and significant factor to induce a GVL effect among factors evaluated by logistic regression analysis.

Difference of GVL effect among each mHag

Table 4 shows the different intensity of GVL effect among each mHag. Compared with compatible patients, the relapse rate was significantly lower only in those with CD62L incompatibilities. The relapse rate tended to be low

Table 3 Multivariate analysis of anti-leukemia effect[a]

Variables	*Odds ratio (95% CI)*	P-*value*
mHag[b]		
Compatible	1.000**	
Incompatible	0.311 (0.104–0.925)	0.035*
Donor		
Related	1.000**	
Unrelated	0.342 (0.120–0.980)	0.045*
Source of stem cell		
BM	1.000**	
PBSC	0.214 (0.408–3.718)	0.079
a-GVHD (≥II)		
−	1.000**	
+	1.231 (0.408–3.718)	0.712
c-GVHD		
−	1.000**	
+	0.499 (0.184–1.354)	0.712

Abbreviations: a-GVHD = acute GVHD; c-GVHD = chronic GVHD; CI = confidence interval; mHag = minor histocompatibility antigen.
*$P < 0.05$.
**Reference group.
[a]Multivariate analysis performed with logistic regression analysis using computer software.
[b]MHags include HA-1, CD62L, CD31 codon 125, CD31 codon 563 and CD49b where at least one of these mHag mismatches is defined as incompatible.

Table 4 Comparison of relapse rates in each mHag: incompatible versus compatible pairs[a]

mHag	*Number of HLA-restricted patients* N	*Relapse rate (%)*		P-*value*
		Incompatible	*Compatible*	
CD62L	71	5.9% (1/17)	37.0% (20/54)	0.01*
CD31 codon 563	51	11.8% (2/17)	32.4% (11/34)	0.10
CD31 codon 125	50	15.4% (2/13)	29.7% (11/37)	0.26
HA-1	23	16.6% (1/6)	17.6% (3/17)	0.72
CD49b	44	33.3% (2/6)	34.2% (13/38)	0.67

Abbreviation: mHag = minor histocompatibility antigen.
*$P<0.05$.
Numbers in parentheses indicate numbers of relapsed patients divided by numbers of HLA-matched patients.
[a]HLA restrictions of each mHag were analyzed in 106 patients.

Table 5 mHags induce different effects on relapse and a-GVHD[a]

mHag	N	*Ratio*[b]	
		Relapse rate	*a-GVHD*
CD62L	70	0.16	0.6
CD31 codon 563	51	0.37	0.81
CD31 codon 125	50	0.51	1.0
HA-1	23	0.94	1.4
CD49b	44	0.97	0.69

Abbreviations: a-GVHD = acute GVHD; mHag = minor histocompatibility antigen.
[a]HLA restrictions of each mHag were analyzed in 106 patients.
[b]Each value shows the ratio of relapse rate and a-GVHD in patients with incompatible divided by those with compatible mHag, which is calculated as follows:

$$\text{ratio of relapse rate} = \frac{\text{relapse rate (\%) of incompatible pairs}}{\text{relapse rate (\%) of compatible pairs}}$$

$$\text{ratio of a-GVHD} = \frac{\text{rate of a-GVHD of incompatible pairs}}{\text{rate of a-GVHD of compatible pairs}}$$

in those with CD31 and HA-1 incompatibility but this was not statistically significant. The relapse rate of HA-1-incompatible patients with HLA-A2 and with other HLA class 1 superfamilies tended to be low (16.6 and 13.3%) but the value was not statistically significant due to the small numbers. Interestingly, the incidence of a-GVHD ($\geqslant$II) between compatible and incompatible patients did not differ. The incidences of a-GVHD in patients with CD62L, CD31 codon 563, CD31 codon 125, HA-1 and CD49b incompatibilities were 35.3, 35.3, 30.8, 32 and 33.3% respectively, which were comparable to those of compatibilities. Table 5 shows the different effects on GVL and a-GVHD among each mHag. Mismatches of CD62L, CD31 codon 563 and CD31 codon 125 induce a GVL effect rather than a-GVHD.

Long-term effect

Figure 1 shows the accumulated relapse and survival rates in the standard-risk group. The estimated 12-year accumulated relapse rates of incompatible and compatible patients were 22 and 68%, respectively ($P=0.02$). After treatment, the 15-year probability of survival among patients in the standard-risk group who were incompatible and compatible had improved to 88%, and that of compatible patients was 66% ($P=0.03$). In the high-risk group, there was no significant difference between the compatible and incompatible group in relapse rate.

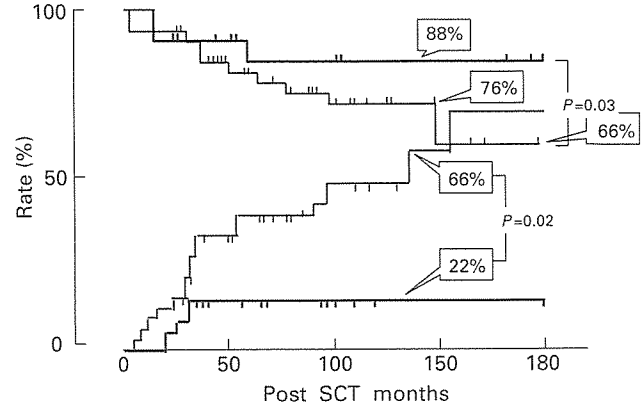

Figure 1 Probability of survival and accumulated rate of relapse among mHag-incompatible and -compatible patients in the standard-risk group. A total of 63 cases were studied where 88% of 10-year survival and 22% of accumulated rate of relapse in mHag-incompatible patients comparing to 64% of 10-year survival ($P<0.03$) and 66% of accumulated of relapse ($P<0.02$) in mHag-compatible patients after HLA-identical SCT.

Discussion

We have demonstrated that mHag incompatibility in HLA-identical stem cell recipients can induce a GVL effect rather than a-GVHD after myeloablative SCT. As neither an increase in a-GVHD nor fatal complications developed during long-term follow-up in the standard-risk group, these mHags, except for CD49b, may be ideal targets for donor-derived T cells after SCT. Differences in CD49b do not influence GVL.

In the standard-risk group, the relapse rate in patients with mHag incompatibility was lower than that of compatible patients (14 versus 44%, $P=0.02$). In the latter group, 18 out of 41 patients relapsed after allogeneic SCT. Nine of the relapsed patients had CML. Four were cytogenetic and three in hematological relapse at 2, 14, 21, 39, 16, 68 and 132 months after SCT. The remaining two were in blastic and extramedullary relapse at 22 and 36 months after SCT. Six of the seven CML in chronic-phase relapse were treated with DLI and the other one patient discontinued CyA to enhance the GVL effect. All of the seven CML patients achieved complete remission 2–6 months after DLI. The other nine of the 18 patients with relapse died owing to relapse-related complications at 8, 32, 34, 43, 44, 48, 55, 59 and 122 months after SCT. Interestingly, none of the seven CML patients with mHag incompatibilities in the standard-risk group has relapsed. This suggests that mHag incompatibility induces a prolonged anti-leukemia effect and induces long-term survival after SCT.

In contrast to the study by Goulmy *et al.*,[2] our data did not show any increase in a-GVHD. These findings are compatible with those of Murata *et al.*,[15] which show that HA-1 mismatch is not significantly associated with a-GVHD in Japanese patients.[6] The difference is probably due to intensified immunosuppression as GVHD prophylaxis and smaller numbers of mHag contributing to GVHD in Japan than in Western counties. Goulmy *et al.* reported that recipient incompatibility with HA-1 is associated with a-GVHD development, as a-GVHD ($\geqslant 2$) developed in all HA-1-positive adult patients ($n = 10$) who received marrow from an HA-1-negative donor. However, they received either MTX or CyA as GVHD prophylaxis. Among our patients, 103 of 106 received short-term MTX + CyA (98) or short-term MTX + FK 506 (five) as prophylaxis for a-GVHD. As the combination of MTX + CyA significantly decreases the incidence of a-GVHD compared with either MTX or CyA alone, the strength of the association might be lower in patients who received either MTX + CyA or FK506 than in those who received either MTX or CyA.

Recently, a large-scale study has been performed to assess the association of ethnicity with the incidence of GVHD. This revealed a lower risk of a-GVHD and early post transplantation toxicity in Japanese and Scandinavian populations,[16] which suggests a less diverse genetic background among HLA-identical pairs in Japan. A large-scale survey of Japanese patients transplanted from an HLA-identical sibling[17] or HLA-identical unrelated donor[12] revealed a similar GVL effect with a lower incidence of a-GVHD than seen in Western countries. Furthermore, the results of donor leukocytes infusion for relapsed patients showed a similar GVL effect, with a relatively low incidence of a-GVHD in Japan compared to Western countries.[10] These results, together with our data, suggest that the number of mismatched mHag is even smaller, and that these molecules have enough power to induce a GVL effect rather than a-GVHD in Japanese patients transplanted from an HLA-identical donor.

Among the four polymorphic adhesion molecules, relapse rates were significantly lower in patients with CD62L incompatibility. Mismatches of CD31 codon 563 and CD31 codon 125 also induced a GVL effect rather than a-GVHD. The anti-leukemia effect of CD62L was associated with only the HLA-A3-like and/or B44-like superfamilies, which are grouped as HLA class I alleles based on the similarity of their peptide binding motifs.[13] As this phenomenon has been described with respect to CTL lines that are specific for melanoma-associated antigens within the A2-like superfamily[18] and to HIV-specific peptides within the A3-like superfamily,[19] molecules from incompatible CD62L and CD31 combinations could be an immunodominant mHag in HLA-identical stem cell recipients.

From these data, we suggest that polymorphic adhesion molecules such as CD62L, CD31 codon 563 and codon 125 could function as immunodominant mHag to induce a GVL effect rather than a-GVHD in Japanese patients transplanted from HLA-identical stem cell grafts contributing to a long-term survival effect. To confirm this hypothesis, a prospective randomized study is needed. Detection of mHag-specific cytotoxic T cells in patients transplanted from an mHag-negative graft is now under investigation.

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References

1 Den Haan JM, Sherman NE, Blokland E, Huczko E, Koning F, Drijfhout JW *et al.* Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science* 1995; **268**: 1476–1480.
2 Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg JH, Vossen J *et al.* Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med* 1996; **334**: 281–285.
3 Tseng LH, Lin MT, Hansen JA, Gooley T, Pei J, Smith AG *et al.* Correlation between disparity for the minor histocompatibility antigen HA-1 and the development of acute graft-versus host disease after allogeneic bone marrow transplantation. *Blood* 1999; **94**: 2911–2914.
4 Dickinson AM, Wang XN, Sviland L, Vyth-Dreese FA, Jedtson GH, Schmacher TN *et al. In situ* dissection of the graft-versus-host activities of cytotoxic T cells specific for minor histocompatibility antigens. *Nat Med* 2002; **8**: 410–414.
5 Mutis T, Verdijk R, Schrama E, Esendam B, Brand A, Goulmy E *et al.* Feasibility of immunotherapy of relapsed leukemia with *ex vivo*-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood* 1999; **93**: 2336–2341.
6 Maruya E, Saji H, Seki S, Fujii Y, Kato K, Kai S *et al.* Evidence that CD31, CD49b and CD62L are immunodominant minor histocompatibility antigens in HLA identical sibling bone marrow transplants. *Blood* 1998; **92**: 2169–2176.
7 Behar E, Chao NJ, Hiraki DD, Krishaswany S, Brown BW, Zehnder JL *et al.* Polymorphism of adhesion molecule CD31 and its role in acute graft-versus-host disease. *N Engl J Med* 1996; **334**: 286–291.
8 Grumet FC, Hiraki DD, Brown BWM, Zehnder JL, Zacks ES, Draksharapu A *et al.* CD31 mismatching affects marrow transplantation outcome. *Biol Blood Marrow Transplant* 2001; **7**: 503–5122.
9 Balduiri CL, Frassoni F, Noris P, Klersy C, Iannore AM, Bacigulupo A *et al.* Donor–recipient incompatibility at CD31-codon 563 is a major factor for acute graft-versus-host-disease after allogeneic bone marrow transplantation from a human leukocytes antigen-matched donor. *Br J Haematol* 2001; **114**: 951–953.
10 Shiobara S, Nakao S, Ueda M, Yamazaki H, Takahashi S, Asano S *et al.* Donor leukocyte infusion for Japanese patients with relapsed leukemia after allogeneic bone marrow transplantation: lower incidence of acute graft versus host disease and improved outcome. *Bone Marrow Transplant* 2000; **26**: 769–774.
11 Morishima Y, Kodera Y, Hirabayashi N, Tanimoto M, Matsuyama T, Horibe K *et al.* Low incidence of acute GVHD in patients transplanted with marrow from HLA-A, B, DR-compatible unrelated donors among Japanese. *Bone Marrow Transplant* 1995; **15**: 235–239.

12 Kodera Y, Morishima Y, Kato S, Akiyama Y, Sao H, Matsuyama T *et al.* Analysis of 500 bone marrow transplants from unrelated donors (UR-BMT) facilitated by the Japan Marrow Donor Program: confirmation of UR-BMT as a standard therapy for patients with leukemia and aplastic anemia. *Bone Marrow Transplant* 1999; **24**: 995–1003.
13 Sidney J, Grey HM, Kubo RT, Sette A. Practical, biochemical and evolutionary implications of the discovery of HLA Class I supermotifs. *Immunol Today* 1996; **17**: 261–265.
14 Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J *et al.* 1994 consensus conference on acute GVHD grade. *Bone Marrow Transplant* 1995; **15**: 825–828.
15 Murata M, Emi N, Hirabayashi N, Hamaguchi M, Goto S, Wakita A *et al.* No significant between HA-1 incompatibility and incidence of acute graft-versus-host-disease after HLA-identical sibling bone marrow transplantation in Japanese patients. *Int J Hematol* 2000; **72**: 371–375.
16 Oh H, Loberiza Jr FR, Zhang MJ, Ringden O, Akiyama H, Asai T *et al.* Comparison of graft-versus-host-disease and survival after HLA-identical sibling bone marrow transplantation in ethnic populations. *Blood* 2005; **105**: 1408–1416.
17 Morishima Y, Morishita Y, Tanimoto M, Ohno R, Saito H, Horibe K *et al.* Low incidence of acute graft-versus-host disease by administration of methotrexate and cyclosporin in Japanese leukemia patients after bone marrow transplantation from human leukocyte antigen compatible siblings: possible role of genetic homogeneity. The Nagoya Bone Marrow Transplantation Group. *Blood* 1995; **74**: 2252–2256.
18 Fleischhauer K, Tanzarella S, Wallny HJ, Bordignon C, Traversari C. Multiple HLA-A alleles can present an immunodominant peptide of the human melanoma antigen Melan-A/MART-1 to a peptide-specific HLA-A*0201 + cytotoxic T cell line. *J Immunol* 1996; **157**: 787–797.
19 Threlkeld SC, Wentworth PA, Kalams SA, Wilkes BM, Ruhl DJ, Keogh E *et al.* Degenerate and promiscuous recognition by CTL of peptides presented by the MHC class I A3-like superfamily: implications for vaccine development. *J Immunol* 1997; **159**: 1648–1657.