flanking region of epitopes has been shown to impair the processing by proteasomes (2, 37). Thus, structural features play an important role in epitope liberation and could influence the working of the five immunoproteasome-associated subunits.

We have previously shown the generation of an HLA-A*2402-restriced CTL epitope in the Epstein-Barr virus (EBV) latent membrane protein 2A (EBV-LMP2A), amino acids 222 to 230 (referred to as $LMP2A_{222-230}$), to be dependent on IFN-γ exposure (18). Differential expression of ip-LMP2, MECL-1, ip-LMP7, PA28α, and PA28β in various combinations has allowed us to selectively address the role of each subunit in the processing of the epitope independently of other IFN-γ-inducible proteins, and we have established that the generation of $LMP2A_{222-230}$ is cooperatively controlled by interplay among ip-LMP2, ip-LMP7, and PA28α. Moreover, these observations were supported by the results of RNA interference experiments. We have now extended our studies to demonstrate that LMP2A structural factors influence epitope liberation in various target cells.

MATERIALS AND METHODS

CTL clones and epitopes. EBV-specific CTL lines and clones were established as described earlier (18). Briefly, EBV-specific T-cell lines were generated from peripheral blood mononuclear cells after stimulation with HLA-A*2402-transfected, TAP-negative T2-A24 cells (18) pulsed with each epitope peptide or autologous EBV-carrying lymphoblastoid cell lines (LCLs). After several rounds of stimulation, CTL clones were established by a limiting dilution method. A polyclonal CTL line that was specific to the epitope $LMP2A_{419-427}$ (TYGPVFMCL) (21) was designated $LMP2A_{419-427}$-CTL, and CTL clones that were specific to the epitope $LMP2A_{222-230}$ (IYVLVMLVL) (18) were designated $LMP2A_{222-230}$-CTL.

Cell lines. T2-A24 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 800 μg/ml of G418 (Invitrogen Corp., Carlsbad, CA). HLA-A*2402-positive LCLs and PT67 cells (BD Bioscience Clontech, Palo Alto, CA), retroviral packaging cell lines, were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml of kanamycin (referred to as LCL medium). HLA-A*2402-positive LCLs expressing short hairpin RNA (shRNA) were maintained in LCL medium in the presence of 0.8 μg/ml of puromycin. HEK293 T cells (referred to as 293T; American Type Culture Collection, Manassas, VA) and HLA-A*2402-positive dermal fibroblast cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Expression vectors. Plasmids expressing various lengths of EBV-LMP2A and EBNA3A, from full-length proteins to minimal epitopes, were constructed as described previously (16, 18). Full-length HLA-A*2402, ip-LMP2, ip-LMP7, MECL-1, PA28α, and PA28β were amplified by reverse transcriptase (RT)-PCR from HLA-A*2402-positive LCLs, cloned into the pcDNA3.1(+) vector (Invitrogen Corp.), and sequenced. A plasmid containing a mutant EBV-LMP2A gene with alanine substituted for leucine at position 231 was constructed by PCR-based mutagenesis as described previously (16). This single amino acid substitution was intended to increase the proteasome cleavage strength, as predicted with the Prediction Algorithm for Proteasomal Cleavages I program (PAProC version 1.0; http://www.paproc.de/) (17, 26).

Transduction of 293T cells. The plasmids encoding HLA-A*2402 and EBV-LMP2A and at least one of those expressing ip-LMP2, ip-LMP7, MECL-1, PA28α, or PA28β were transfected into 293T cells using TransIT-293 transfection reagents (Mirus, Madison, WI). Briefly, 3×10^4 cells were transfected with 100 ng of each plasmid and 0.2 μl TransIT reagent per 100 ng DNA in various combinations in 96-well plates. After 24 h, these cells were used as stimulators in the enzyme-linked immunospot (ELISPOT) assay.

shRNA interference retrovirus vectors. The following small interfering RNA targets were selected in this study: ip-LMP2, AAGUGAAGGAGGUCAGGU AUA; ip-LMP7, AGAUUAACCCUUACCUGCUTT; and PA28α, AAGCCA ACUUGAGCAAUCUGA. shRNA constructs included a TTCAAGAGA-loop separating the sense and antisense sequences followed by a 5T termination signal. These constructs were synthesized as two cDNA oligonucleotides, annealed, and ligated between the BamHI and EcoRI sites of the RNAi-Ready pSIREN-RetroQ vector (BD Biosciences Clontech). In addition, oligonucleotides with sequences selected by the company (BD Biosciences Clontech) as a negative control for gene silencing were annealed and inserted into the same vector.

Retrovirus production and infection. PT67 cells were plated on six-well culture plates, and a 4-μg aliquot of each retrovirus vector plasmid was transfected with Lipofectamine 2000 (Invitrogen Corp) according to the manufacturer's instructions. After culture in the presence of 2.5 μg/ml of puromycin for 14 days, the cells were incubated in medium without puromycin for another 48 h. The culture supernatant was collected, and debris was removed by centrifugation at $1,000 \times g$ for 10 min. A total of 1×10^6 LCLs were suspended in 1 ml of the virus-containing culture supernatant in each well of a 12-well plate, and polybrene was added to a final concentration of 10 μg/ml. Plates were centrifuged at $1,000 \times g$ at 32°C for 1 h and incubated at 37°C in a humidified incubator. The LCLs were then cultured in medium containing puromycin for 14 days. Expression of ip-LMP2, ip-LMP7, or PA28α in these LCLs was analyzed by Western blotting and RT-PCR for gene silencing.

Western blotting. Western blotting was performed as described previously with slight modifications (42). Briefly, aliquots of 130 μg protein were applied to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA), blocked with phosphate-buffered saline containing 10% low-fat dry milk and 0.1% Tween 20 overnight at 4°C, and probed with rabbit polyclonal antibodies specific to ip-LMP2, ip-LMP7, and PA28α (Affinity, Mamhead, United Kingdom), followed by peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed, San Francisco, CA). Proteins were visualized using an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, United Kingdom).

RT-PCR. Total RNA was extracted from LCLs and reverse transcription was performed in 20-μl reactions containing random hexamers and 1-μg aliquots. The specific primer sets used to detect ip-LMP2, ip-LMP7, and PA28α were as follows: ip-LMP2 forward, 5′-GGTGGTGAACCGAGTGTTTGA-3′; ip-LMP2 reverse, 5′-GCCAAAACAAGTGGAGGTTCC-3′; ip-LMP7 forward, 5′-GAT TGCAGCAGTGGATTCTCG-3′; ip-LMP7 reverse, 5′-GACATGGTGCCA AGCAGGTAA-3′; PA28α forward, 5′-ACCAAGACAGAGAACCTGCTCG-3′; and PA28α reverse, 5′-GGCCTTCAGATTGCTCAAGTTG-3′.

ELISPOT assays. ELISPOT assays were performed as previously described (18). In brief, a MultiScreen-HA plate (Millipore) was coated with anti-human IFN-γ monoclonal antibody (Endogen, Rockford, IL) and used as the assay plate. The following stimulator cells in 100 μl of LCL medium were seeded into the wells: (i) 293T cells cotransfected with plasmids expressing HLA-A*2402 and those expressing various lengths of EBV-LMP2A (in some experiments, cells were treated with puromycin at 1 μg/ml for 30 min); (ii) 293T cells cotransfected with plasmids encoding HLA-A*2402 and EBV-LMP2A and at least one of those two expressing ip-LMP2, ip-LMP7, MECL-1, PA28α, or PA28b; and (iii) LCLs transduced by retrovirus vectors expressing shRNA for either ip-LMP2, ip-LMP7, or PA28a.

$LMP2A_{222-230}$-CTLs or $LMP2A_{419-427}$-CTLs in 100 μl medium were introduced into each well and incubated for 20 h. To visualize spots, anti-human IFN-γ polyclonal antibody (Endogen), horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Zymed) and substrate were used. All assays were performed in duplicate.

RESULTS

$LMP2A_{222-230}$ is not presented on target cells expressing full-length EBV-LMP2A. The $LMP2A_{419-427}$-CTL responded to 293T cells pulsed with the epitope peptide and to those expressing full-length EBV-LMP2A cotransfected with HLA-A*2402. However, the $LMP2A_{222-230}$-CTL responded to 293T cells expressing the minimal epitope, but not full-length EBV-LMP2A, cotransfected with HLA-A*2402 (Fig. 1).

As we reported previously, IFN-γ-treated fibroblasts transduced with full-length EBV-LMP2A were recognized by $LMP2A_{222-230}$-CTL, showing $LMP2A_{222-230}$ to be an IFN-γ-dependent epitope. This suggested that IFN-γ-induced immunoproteasome and PA28 subunits generate $LMP2A_{222-230}$ in the target cells (18).

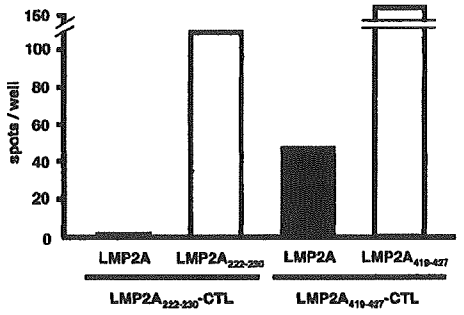

FIG. 1. EBV-specific CTL recognition of target cells measured by the ELISPOT assay. The epitope $LMP2A_{222-230}$ is not presented on 293T cells expressing full-length EBV-LMP2A while $LMP2A_{419-427}$ is presented on these cells. $LMP2A_{222-230}$-CTL is the clone specific for $LMP2A_{222-230}$, and $LMP2A_{419-427}$-CTL is a polyclonal $CD8^+$ T-cell line specific to $LMP2A_{419-427}$. 293T cells were cotransfected with plasmids expressing HLA-A*2402 and full-length EBV-LMP2A or pulsed with the epitope peptide. $CD8^+$ T cells (200/well) were cultured with the indicated stimulators for 20 h. Data from one representative experiment out of three are shown. Each bar demonstrates the average number of spots in duplicate wells.

Generation of $LMP2A_{222-230}$ requires the immunoproteasome subunit ip-LMP7 and PA28α and is enhanced by ip-LMP2. To investigate whether immunoproteasome-associated molecules are involved in generating the $LMP2A_{222-230}$ epitope, we examined the effect of each proteasome immunosubunit (ip-LMP2, ip-LMP7, and MECL-1) and PA28 subunit (PA28α and PA28β) in 293T cells that dominantly have a standard proteasome. First, 293T cells were cotransfected with plasmids encoding HLA-A*2402, the full-length EBV-LMP2A, and immunoproteasome-associated molecules in various combinations, as shown in Fig. 2A. We then evaluated epitope liberation using the ELISPOT assay. Surprisingly, three molecules were found to be involved in the generation of $LMP2A_{222-230}$: ip-LMP7 and PA28α subunits were required, and the ip-LMP2 subunit enhanced its recognition (Fig. 2A). We confirmed the expression of ip-LMP2, ip-LMP7, and PA28α by Western blotting (Fig. 2B).

Inhibition of ip-LMP2, ip-LMP7, and PA28α expression in LCLs by RNA interference decreases the generation of $LMP2A_{222-230}$ in target cells. LCLs predominantly have immunoproteasomes (24), and the $LMP2A_{222-230}$-CTL have recognized HLA-A*2402-positive LCLs, as we reported previously (18). To examine whether ip-LMP2, ip-LMP7, or PA28α is most directly involved in the generation of $LMP2A_{222-230}$, we evaluated the epitope liberation in LCLs in which the expression of each subunit was separately inhibited using a gene-silencing technique. HLA-A*2402-positive LCLs were infected with retrovirus vectors expressing shRNAs for ip-LMP2, ip-LMP7, or PA28α and assessed for the expression of each subunit by Western blotting (Fig. 3A, B, and C) and RT-PCR (data not shown). Then, generation of the $LMP2A_{222-230}$ epitope was probed with epitope-specific CTL using the ELISPOT assay. As expected, epitope liberation was clearly decreased with the inhibition of ip-LMP2, ip-LMP7, or PA28α expression (Fig. 3A, B, and C), demonstrating definitive involvement of all three molecules in the generation of $LMP2A_{222-230}$. To test whether the generation of IFN-γ-independent EBV-LMP2 epitope was influenced in these

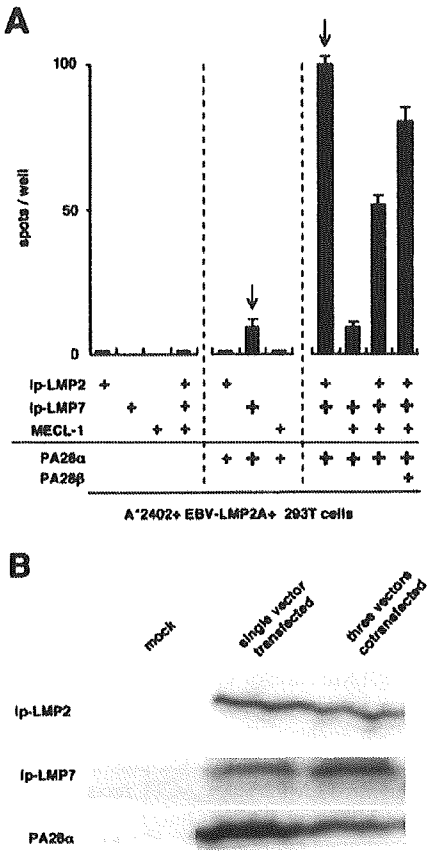

FIG. 2. Involvement of immunoproteasome and PA28 subunits in the generation of $LMP2A_{222-230}$ as analyzed by the ELISPOT assay. (A) Generation of $LMP2A_{222-230}$ requires three immunoproteasome-associated molecules. 293T cells were cotransfected with plasmids encoding HLA-A*2402, full-length EBV-LMP2A, and at least one immunoproteasome-associated molecule (ip-LMP2, ip-LMP7, MECL-1, PA28α, and PA28β). $LMP2A_{222-230}$-CTLs were cultured with stimulators for 20 h as described in Materials and Methods. Data from one representative experiment out of three are shown. Data are means plus or minus standard deviation (SD) of spots in duplicate wells. The arrows indicate noteworthy results. +, presence of immunoproteasome or subunit. (B) Expression of ip-LMP2, ip-LMP7, and PA28α in 293T cells transfected with corrresponding expression vectors. 293T cells were transfected with each of three plasmids or with all these vectors. Expression of each subunit was analyzed by Western blotting. "Single vector-transfected" represents the 293T cells transfected with each plasmid encoding ip-LMP2, ip-LMP7, or PA28α and "three vectors cotransfected" represents the 293T cells cotransfected with the three plasmids. Results of one representative experiment out of two are shown.

LCLs transfected with shRNA expression vectors for ip-LMP2, ip-LMP7, or PA28α, we investigated the generation of $LMP2A_{419-427}$ using the ELISPOT assay. We found that there were no significant differences in the processing of this epitope. (data not shown).

Incomplete or shortened EBV-LMP2A results in efficient generation of $LMP2A_{222-230}$ in target cells. Recently, DRiPs have been reported to be major sources of CTL epitopes (43, 46), suggesting that incomplete antigen proteins allow efficient processing. To test this possibility with regard to $LMP2A_{222-230}$, 293T

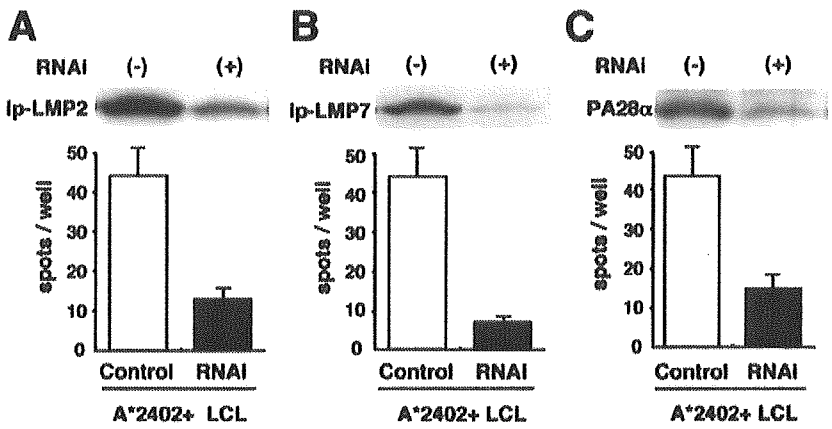

FIG. 3. Generation of $LMP2A_{222-230}$ is inhibited by RNA interference (RNAi) products targeting ip-LMP2 (A), ip-LMP7 (B), or PA28α (C) expression. Immunoproteasome-expressing HLA-A*2402-positive LCLs were infected with retrovirus vectors expressing shRNA for ip-LMP2, ip-LMP7, or PA28α. As a control stimulator, an LCL infected with a retrovirus vector expressing a nonsilencing shRNA was used. Inhibition of each subunit expression in shRNA-expressing LCLs was analyzed by Western blotting. $LMP2A_{222-230}$-CTL (5×10^3 cells/well) was cultured with each shRNA-transduced LCL for 20 h. Results of one representative experiment out of two are shown. Data are means plus or minus SD of spots in duplicate wells. −, absence of immunoproteasome or subunit; +, presence of immunoproteasome or subunit.

cells transduced with HLA-A*2402 and full-length EBV-LMP2A together with ip-LMP7 and/or PA28α were treated with puromycin for 30 min to generate short-lived premature proteins (6, 13, 47). We then analyzed the generation of $LMP2A_{222-230}$ by ELISPOT assay. As shown in Fig. 4A, puromycin treatment remarkably augmented $LMP2A_{222-230}$-CTL recognition on the cells expressing ip-LMP7 and PA28α. Interestingly, puromycin was capable of substituting either effect of ip-LMP7 and PA28α.

Next, we introduced truncated EBV-LMP2A of different lengths starting from isoleucine at position 222, the first amino acid of $LMP2A_{222-230}$, into expression vectors (Fig. 4B). The generation of $LMP2A_{222-230}$ was studied in 293T cells cotransfected with vectors encoding HLA-A*2402 and each truncated EBV-LMP2A without immunoproteasomes and PA28 subunits. Interestingly, the shortest EBV-LMP2A antigen was processed most efficiently and all truncated EBV-LMP2A antigens could be processed to generate $LMP2A_{222-230}$ without the aid of immunoproteasomes and PA28 (Fig. 4C). These data clearly demonstrated that the efficiency of $LMP2A_{222-230}$ generation is, at least in part, dependent on the length of the source protein.

Substitution of amino acid immediately flanking the C terminus of $LMP2A_{222-230}$, increasing the proteasome cleavage strength, results in efficient generation of $LMP2A_{222-230}$ in target cells. Finally, we investigated whether the amino acid cleavage strength at a specific position affects the processing of the $LMP2A_{222-230}$ epitope. To determine the cleavage strength of each amino acid in EBV-LMP2A, the program PAProC was used (http://www.paproc.de/). We focused on the cleavage strength, which is critical for epitope generation, of amino acids in the position immediately flanking the C termini of CTL epitopes (14, 34). First, we constructed a plasmid containing a mutant full-length LMP2A gene in which alanine replaced leucine at position 231; this was predicted to increase the cleavage strength after leucine at position 230, i.e., the C terminus of $LMP2A_{222-230}$ (Fig. 5A). It was thought that this change would facilitate $LMP2A_{222-230}$ generation by proteasomes. Target 293T cells were cotransfected with vectors encoding HLA-A*2402, ip-LMP7, PA28α, and the mutant EBV-LMP2A, and $LMP2A_{222-230}$-CTL recognition was evaluated using the ELISPOT assay. A remarkable increase was evident for cells expressing ip-LMP7 and PA28α (Fig. 5B), suggesting the processing of $LMP2A_{222-230}$ to be accelerated by the amino acid substitution at the specific position in the EBV-LMP2A antigen.

DISCUSSION

IFN-γ induces cells to express the proteasome subunits ip-LMP2, MECL-1, and ip-LMP7, leading to the formation of immunoproteasomes and the proteasome activator subunits PA28α and PA28β, comprising the activator complex. Early experiments with IFN-γ-treated cells demonstrated the generation of a number of epitopes to be affected by immunoproteasomes and PA28 (15, 32, 44). Immunoproteasomes have various cleavage site preferences as well as cleavage rates for the generation of some epitopes, while PA28 up-regulates epitope liberation via conformational changes within the proteasome 20S complex. Following the discovery that the influenza virus matrix-derived epitope required ip-LMP7 expression for its generation (3), the involvement of immunoproteasomes and PA28 subunits with different CTL epitopes received much attention. The results of the studies that investigated the effect of at least two immunoproteasome-associated molecules in the generation of CTL epitopes are summarized in Table 1 (1, 8, 10, 19, 23, 36, 38, 40, 41). The combination patterns of the five immunoproteasome-associated subunits fall into three categories. (i) PA28 alone, (ii) ip-LMP7 alone, and (iii) both ip-LMP2 and ip-LMP7 exerted the epitope generation. It has been hypothesized that immunoproteasomes and PA28 cooperate in antigen processing, but direct experimental evidence has hitherto been lacking. In this study, we found that the $LMP2A_{222-230}$ epitope has two unique features. First, coexpression of ip-LMP7 and one PA28 subunit is necessary for its

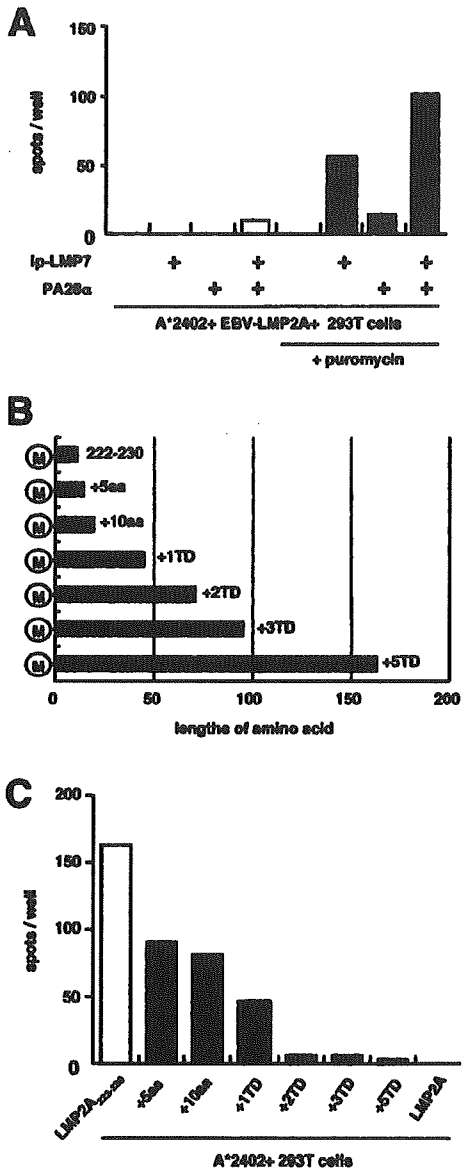

FIG. 4. Generation of $LMP2A_{222-230}$ from incomplete and shortened EBV-LMP2A as analyzed by ELISPOT assay. (A) $LMP2A_{222-230}$-CTL recognition of puromycin-treated (1 μg/ml for 30 min) or untreated target cells expressing EBV-LMP2A. 293T cells, cotransfected with plasmids encoding HLA-A*2402, full-length EBV-LMP2A, ip-LMP7, and/or PA28, were cultured with $LMP2A_{222-230}$-CTL (1×10^4 cells/well) for 20 h. For Ip-LMP7 and PA28 lanes, "+" indicates the presence of the immunoproteasome or subunit. (B) The length and structure of the truncated EBV-LMP2A fragments. Numbers of transmembrane domains (TDs) following $LMP2A_{222-230}$ in the fragments are shown; e.g., +3TD indicates fragment $LMP2A_{222-230}$ plus three transmembrane domains. Each TD located from the C terminus of $LMP2A_{222-230}$ is serially numbered. M, methionine. (C) $LMP2A_{222-230}$-CTL recognition of target cells expressing truncated EBV-LMP2A fragments in the absence of immunoproteasomes. The 293T cells were cotransfected with expression vectors encoding HLA-A*2402 and one truncated EBV-LMP2A and cultured with $LMP2A_{222-230}$-CTL (1×10^3 cells/well) for 20 h. Numbers of amino acids and transmembrane domains (TDs) following $LMP2A_{222-230}$ in the fragments are shown; e.g., +5aa indicates fragment $LMP2A_{222-230}$ plus five C-terminal amino acids. Results of one representative experiment out of two are shown. Each bar represents the average number of spots in duplicate.

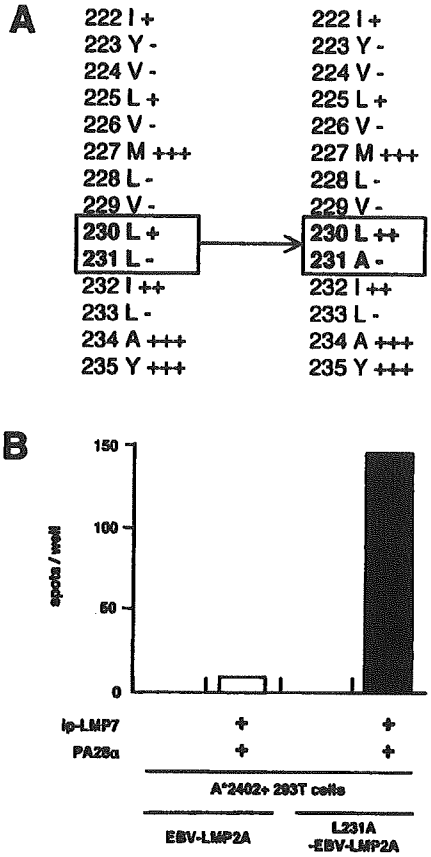

FIG. 5. Comparison of generation of $LMP2A_{222-230}$ from native and mutant EBV-LMP2A with alanine substituted for leucine at position 231 (referred as to L231A-EBV-LMP2A). (A) Partial amino acid sequences of EBV-LMP2A and L231A-EBV-LMP2A. The position numbers, single code letters for amino acids and predicted cleavage strengths are shown. Predictions by the program PAProC are scored as follows: −, no cleavage behind this position; +, ++, +++, cleavage behind this position, with a hint of the strength indicated by the number of +'s. (B) Generation of $LMP2A_{222-230}$ from EBV-LMP2A and L231A-EBV-LMP2A, analyzed by ELISPOT assay. 293T cells were cotransfected with plasmids encoding HLA A*2402, ip-LMP7, PA28α, and full-length EBV-LMP2A or L231A-EBV-LMP2A and cultured with $LMP2A_{222-230}$-CTL for 20 h. Results of one representative experiment out of two are shown. Each bar represents the average number of spots in duplicate. For Ip-LMP7 and PA28 lanes, "+" indicates the presence of the immunoproteasome or subunit.

generation. Second, ip-LMP2 has additional effects on epitope liberation. These data suggest that the processing of an IFN-γ-inducible epitope is controlled differentially by multiple immunoproteasome-associated subunits. To our best knowledge, this is the first documentation of molecular evidence of such cooperation.

Incorporation of the immunoproteasome is reported to be cooperative. The ip-LMP7 is required for immunoproteasome formation and maturation (9, 14). MECL-1 is incorporated if ip-LMP2 is present, while MECL-1 dependency for the incorporation of ip-LMP2 is under dispute (5, 11). Moreover, this cooperativity in forming proteasome complexes results in altered cleavage properties. In the present study, the generation

TABLE 1. Effects of immunoproteasomes and PA28 subunits by species on epitope generation[a]

Species	Source	Antigen	Epitope location	Epitope sequence	Immunoproteasome or subunit					Reference
					ip-LMP2	MECL1	ip-LMP7	PA28α	PA28β	
Mouse	Murine CMV	pp89	168–176	YPHFMPTNL	–	ND[b]	–	+	ND	11
Mouse	Influenza virus	NP	146–154	TYGRTRALV	–	ND	–	+	ND	11
Human	Influenza virus	Matrix	58–66	GILGFVFTL	–	ND	+	ND	ND	9
Human	HIV	RT	346–354	VIYQYMDDL	–	ND	+	ND	ND	38
Human	HBV	HBcAg	141–151	STLPETTVVRR	–	–	+	ND	ND	39
Human	Melanoma	MAGE-3	114–122	AELVHFLLL	–	–	+	ND	ND	36
Human	Melanoma	TRP2	360–368	TLDSQVMSL	–	–	–	+	+	41
Human	EBV	LMP2A	356–364	FLYALALLL	+	–	+	–	–	20
Human	Melanoma	Melan-A	26–35	ELAGIGILTV	+	–	+	ND	ND	23
Human	LCMV	gp	33–41	KAVYNFATC	+	ND	+	–	–	1
Human	LCMV	gp	276–286	SGVENPGGYCL[c]	+	ND	+	–	–	1
Human	EBV	LMP2A	222–230	IYVLVMLVL	+	–	+	+	–	Present study

[a] CMV, cytomegalovirus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; LCMV, lymphocytic choriomeningitis virus; –, absence; +, presence.
[b] ND, not done.
[c] Generation of this epitope was inhibited by the expression of either ip-LMP7 or ip-LMP2.

of $LMP2A_{222–230}$ is enhanced by ip-LMP2 expression. This effect may be exerted through the functions of ip-LMP7 and PA28α, which induce the cleavages properties on the epitope generation.

In this study, we developed a retrovirus vector producing shRNA to confirm the effects of ip-LMP2, ip-LMP7, and PA28α in the generation of $LMP2A_{222–230}$. Generally, the use of chemically synthesized small interfering RNA or expression plasmids for shRNA is a more feasible way to test the involvement of target molecules but we believe that the retrovirus system has advantages in our case, because the effects of RNA interference in the target cells proved stable. After an epitope binds to MHC molecules and is presented on the cell surface, the complex exists for some time. Since there is a wide range in the life spans of the MHC-epitope complex, it is difficult to infer the sufficient duration to maintain inhibition of immunoproteasome-associated subunits to examine their effects in peptide liberation. In our retrovirus system, LCLs were cultured in medium containing puromycin for 14 days after retrovirus vector infection, and we assessed $LMP2A_{222–230}$ presentation on the surface. This procedure should exclude false-positive results that are observed with the ELISPOT assay.

DRiPs are thought to be important sources of CTL epitopes (29, 35, 49), as in the case of EBNA1, for example, for which epitopes are not readily generated from stable mature EBNA1 because of the glycine-alanine repeat domain within the protein (43, 46). EBV-LMP2A has 12 hydrophobic integral membrane sequences, and this hydrophobic-rich structure may inhibit epitope liberation (20). To address the question of whether the incomplete EBV-LMP2A might be superior to the mature complete EBV-LMP2A for epitope generation, we treated target cells with puromycin, which generates short-lived premature termination products from newly synthesized proteins (6, 13, 47). Interestingly, $LMP2A_{222–230}$ production was accelerated in puromycin-treated 293T cells expressing ip-LMP7 or PA28α, in contrast to the limited yield without puromycin treatment, even when the subunits were coexpressed. The data suggest that puromycin treatment is not sufficient to generate $LMP2A_{222–230}$ epitopes via constitutive proteasomes, but rather affects epitope generation by enhancing the effect of ip-LMP7 and PA28α. Next, we expressed a panel of shorter EBV-LMP2A fragments encompassing $LMP2A_{222–230}$ in target cells and compared their recognition to that of $LMP2A_{222–230}$-CTL. Each fragment started from the N terminus of $LMP2A_{222–230}$, as shown in Fig. 4B. This strategy should focus on the cleavage efficiency of the C-terminal side, which is performed exclusively by proteasomes (14, 34). We found that shorter EBV-LMP2A fragments were processed more efficiently. Therefore, the length of the source antigen may be a critical factor. Addition of two consecutive hydrophobic transmembrane domains substantially abrogated the epitope presentation. The obstacles presented by the intrinsic structure of EBV-LMP2A may be overcome by the effects of ip-LMP7 and PA28α in the generation of the $LMP2A_{222–230}$ epitope.

The cleavage efficiency at each amino acid varies widely in antigen proteins (25, 27, 44), and this may explain why one epitope is generated efficiently by proteasomes while another is not, even when processed from the same protein. Previous work showed that even a single amino acid substitution of asparagine for the aspartic acid immediately flanking the C terminus of the Moloney murine leukemia virus epitope SSWDFITV resulted in its abrogation (2). The program PAProC predicts that the cleavage strength of the C-terminal leucine in EBV-LMP2A is weak (17, 26), and substitution of an amino acid to increase the cleavage strength (from "+" to "++", as shown in Fig. 5A) resulted in remarkable up-regulation of $LMP2A_{222–230}$ liberation in cells expressing ip-LMP7 and PA28α.

EBV-LMP2A is thought to be an important antigen in EBV-related malignancies and is targeted by CTLs that recognize multiple epitopes located throughout the membrane-spanning molecules (20, 30, 31). Interestingly, EBV-LMP2A epitopes can be divided into two groups: (i) hydrophobic examples located in the transmembrane domain and processed in a TAP-independent manner and (ii) intertransmembrane hydrophilic epitopes, which are TAP-dependent (20). In addition, the generation of one hydrophobic epitope, $LMP2A_{356–364}$, requires ip-LMP7 and ip-LMP2 (19). Moreover, we here demonstrated that the processing of $LMP2A_{222–230}$ requires immunoproteasome subunits ip-LMP7 and PA28α and is enhanced by immunoproteasome subunit ip-LMP2. These two epitopes belong to

the former group, although the effects of immunoproteasome and PA28 subunits on other epitopes remain to be investigated. Potentially, EBV-LMP2A is a good model for determining the mechanisms by which immunoproteasomes and PA28 affect CTL epitope generation.

In conclusion, the present investigation provided evidence for differential roles of ip-LMP2, ip-LMP7, and PA28α in the generation of the $LMP2A_{222–230}$ epitope, which was most efficiently generated from incomplete EBV-LMP2A fragments and a mutated LMP2A gene with improved cleavage characteristics in cells expressing ip-LMP7 and PA28α. Although the precise function of each of the three subunits could not be clarified, we showed the generation of $LMP2A_{222–230}$ to be controlled by multiple factors. Further investigations on the differential effects of immunoproteasome-associated subunits could provide important information for understanding the presentation of viral and tumor antigens for CTL recognition.

ACKNOWLEDGMENTS

We thank K. Nishida and F. Ando for technical expertise and H. Tamaki and Y. Matsudaira for secretarial assistance.

This work was supported in part by Grants-in-Aid for Scientific Research (C) (no. 17590428) and the Encouragement of Young Scientists (B) (no. 16790281) from the Japan Society for the Promotion of Science, Scientific Research on Priority Areas (no. 17016090) from the Ministry of Education, Culture, Science, Sports, and Technology of Japan and the Third Team Comprehensive Control Research for Cancer (no. 30) from the Ministry of Health, Labor, and Welfare of Japan.

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Bone Marrow Transplantation (2006) 37, 381–386

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ORIGINAL ARTICLE

Increased risk for treatment-related mortality after bone marrow transplantation in GSTM1-positive recipients

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Treatment-related mortality (TRM) is a major obstacle to successful allogeneic hematopoietic stem cell transplantation (HSCT). A variety of drugs are used in allogeneic HSCT, and a genetic polymorphism in metabolic enzymes could affect the metabolism of drugs and potentially influence TRM. Here, we focused attention on GSTM1 and GSTT1 enzymes, which metabolize chemotherapeutic agents, chemical carcinogens and by-products of oxidative stress and are absent from more than 50% of some populations. To assess the significance of homozygous *GSTM1* and *GSTT1* gene deletion in HSCT, we analyzed DNA from 373 patients with hematological disease and their HLA-identical unrelated bone marrow donors using PCR. Homozygous *GSTM1* and *GSTT1* gene deletions were observed in 56 and 45% of patients, respectively, and 57 and 46% of donors, respectively. There was no significant association between GSTT1 polymorphism and any outcome. However, a GSTM1-positive genotype in recipients was significantly associated with higher TRM and lower survival. These results suggest that a *GSTM1*-null genotype in recipients protects against TRM after allogeneic HSCT. Further studies are needed to elucidate a mechanism of increased risk for TRM in GSTM1-positive recipients.

Bone Marrow Transplantation (2006) **37**, 381–386. doi:10.1038/sj.bmt.1705257; published online 16 January 2006

Keywords: hematopoietic stem cell transplantation; GSTM1; GSTT1; polymorphism; treatment-related mortality; survival

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative therapy for hematological diseases but also carries a risk of treatment-related mortality (TRM) resulting from chemoradiotherapy, graft-versus-host disease (GVHD) or its treatment, and infections.[1] A variety of drugs are used in allogeneic HSCT, such as chemotherapeutic agents, immunosuppressants, antibiotics, antifungal and antiviral drugs; thus, a genetic polymorphism in metabolic enzymes could affect the metabolism of drugs and influence subsequent TRM after HSCT.

We recently reported a metabolic enzyme, UDP glycosyltransferase 2 family, polypeptide B17 (UGT2B17), has a null phenotype at frequencies of 11 and 85% among Caucasians and Japanese populations, respectively,[2] and identified the use of a UGT2B17-positive donor as an independent risk factor for higher TRM and lower survival after HSCT.[3] Here, we focused attention on glutathione *S*-transferase (GST) M1 and GSTT1 enzymes. These enzymes mainly metabolize chemotherapeutic agents, chemical carcinogens and by-products of oxidative stress,[4] and are absent from more than 50% of some populations.[5–7] We analyzed the association between homozygous *GSTM1* and *GSTT1* gene deletions in the donor or recipient with various outcomes of transplantation and found GSTM1-positive recipients were significantly associated with higher TRM and lower survival. These results suggest that some reactive intermediates or toxic metabolites generated by GSTM1 may initiate or promote the development of TRM.

Patients and methods

Patients

The study population was selected from the patients who received bone marrow transplantation from an unrelated donor through the Japanese Marrow Donor Program (JMDP) between January 1993 and March 2000. The selection criteria for the patients and donors in the study population were (1) donor/recipient pairs matched for all genotypes of HLA-A, -B, -C and DRB1, (2) an intensive myeloablative pretransplant conditioning regimen, (3) an unmanipulated marrow graft, (4) the use of cyclosporin A (CsA) or tacrolimus as GVHD prophylaxis, (5) DNA samples were stored and available for genotyping, and (6) clinical outcome data were available. The genotypes of

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Received 12 September 2005; revised 15 November 2005; accepted 16 November 2005; published online 16 January 2006

each allele at the HLA-A, -B, -C and DRB1 loci were determined by high-resolution DNA typing as described previously.[8,9]

The characteristics of the 373 patients are summarized in Table 1. Standard-risk disease was defined as acute myeloid leukemia or acute lymphoblastic leukemia in first remission, chronic myeloid leukemia in first chronic phase or myelodysplastic syndrome classified as refractory anemia. All other hematological malignancies including Hodgkin's lymphoma and non-Hodgkin's lymphoma were considered advanced disease. The pretransplant conditioning regimen varied according to disease or stage of disease at transplantation. GVHD prophylaxis consisted of either CsA plus short-term methotrexate (sMTX) ± anti-thymocyte globulin, or tacrolimus plus sMTX.

TRM was defined as any death that occurred while the patient was in remission. The assessment and grading of acute and chronic GVHD were performed as described previously.[10,11] A final clinical survey of these patients was carried out on July 1, 2001, and the median follow-up period was 45 months (range, 0–111 months). Stored DNA was available from all 373 recipients and from 313 donors for analysis of *GSTM1* and *GSTT1* genotype. Informed consent was obtained from all patients and donors, and approval was obtained from the ethics committee at Nagoya University School of Medicine.

Table 1 Patient characteristics

Number (male/female)	373 (211/162)
Median age (years (range))	24 (1–51)
Disease	
Acute myeloid leukemia	101
Acute lymphoblastic leukemia	92
Chronic myeloid leukemia	98
Myelodysplastic syndrome	28
Hodgkin's lymphoma	11
Non-Hodgkin's lymphoma	18
Severe aplastic anemia	25
Status of malignant disease	
Standard	164
Advanced	177
Unknown	7
Pretransplant conditioning regimen	
TBI containing	303
TBI + CY	102
TBI + CY + BU	33
TBI + CY + other(s)	130
TBI + BU	1
TBI + BU + other(s)	13
TBI + other(s)	24
Non-TBI containing	70
BU + CY	33
BU + CY + other(s)	23
BU + other(s)	2
CY + other(s)	12
GVHD prophylaxis	
CsA + sMTX (+ anti-thymocyte globulin)	299 (43)
Tacrolimus + sMTX	31

BU = busulfan; CsA = cyclosporin A; CY = cyclophosphamide; GVHD = graft-versus-host disease; other(s) = chemotherapy drug(s) other than cyclophosphamide and busulfan; sMTX = short-term methotrexate; TBI = total body irradiation.

Determination of homozygous deletion of the GSTM1 *and* GSTT1 *genes*

The homozygous deletion of the *GSTM1* and *GSTT1* genes was determined by PCR on genomic DNA from donor and recipient cells. The sense and antisense primers used for PCR to detect the *GSTM1* gene were 5′-GAACTC CCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTC AAATATACGGTGG-3′, respectively. The sense and antisense primers used for PCR to detect the *GSTT1* gene were 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′, respectively. The sense and antisense primers for PCR to detect the *β-globin* gene as an internal control in each assay were 5′-ACACAACTGTGTTCACTAGC-3′ and 5′-CAACTT CATCCACGTTCACC-3′, respectively. Thirty cycles of amplification were performed using thermalcycler (Model 9600; Perkin-Elmer, Boston, MA, USA) on 0.5 μl genomic DNA extracted from peripheral blood before transplantation or Epstein–Barr virus-transformed lymphoblastoid cells, which were established from pretransplant cryopreserved peripheral blood mononuclear cells. Each reaction contained 0.4 μl of Advantage 2 Polymerase Mix (Clontech Laboratories Inc., Palo Alto, CA, USA), 0.2 mmol/l of each of the four deoxyribonucleotides, 4 pmol of each primer for *GSTT1*, *GSTM1* and *β-globin*, and PCR buffer in a volume of 20 μl. Each cycle consisted of denaturation (95°C; 30 s), annealing (65°C; 15 s) and elongation (72°C; 30 s). A 10 μl volume of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel.

Statistical analysis

A χ^2 test with 2×2 contingency tables was used to evaluate differences of the frequencies of homozygous *GSTM1* and *GSTT1* genes deletion between patients and donors. The Cox proportional-hazard model was applied to multivariate analysis for TRM, acute and chronic GVHD, relapse, disease-free survival (DFS) and overall survival (OS).[12] The following variables were evaluated in a univariate analysis: patient age (continuous variable); disease status of hematological malignancy at the time of transplantation (advanced disease versus standard disease); GVHD prophylaxis (CsA containing regimen versus tacrolimus containing regimen); pretransplant conditioning regimen (TBI containing versus non-TBI containing); incidence of acute GVHD (only for analysis about chronic GVHD) (grade II–IV versus grade 0–I and grade III–IV versus grade 0–II); patient *GSTM1* and *GSTT1* genotype (deleted versus positive); and donor *GSTM1* or *GSTT1* genotype (deleted versus positive). All variables with $P < 0.10$ were entered into the multivariate logistic regression using a backward, stepwise method. $P < 0.05$ was regarded as statistically significant, and those between 0.05 and 0.1 as suggestive of a trend. The TRM, DFS and OS were estimated by using the Kaplan–Meier method, and log-rank test was used to analyze differences.[13]

Results

Frequencies of homozygous deletion of the GSTM1 *and* GSTT1 *genes*

A homozygous deletion of the *GSTM1* gene was found in 207 (55.5%) of 373 patients and in 177 (56.5%) of 313 healthy unrelated donors. A homozygous deletion of the *GSTT1* gene was found in 168 (45.0%) of 373 patients and in 145 (46.3%) of 313 healthy unrelated donors. There were no statistically significant differences in the frequencies of homozygous *GSTM1* and *GSTT1* deletion between each disease group and donor group (data not shown).

TRM

Of 367 evaluable patients for TRM, 115 (31.3%) were dead without relapse at the time of survey. In a univariate analysis, higher patient age, advanced disease and GSTM1-positive patient were associated with higher TRM (Table 2). There was no significant association between *GSTT1* genotype in either patient or donor and TRM. In a multivariate analysis, GSTM1-positive patient as well as higher patient age and advanced disease were significantly associated with higher TRM. TRM was analyzed in relation to *GSTM1* genotype in the patient by the Kaplan–Meier method (Figure 1a). TRM in the GSTM1-positive patients was significantly higher than that in the GSTM1-negative patients (42.9 versus 29.3%; $P=0.04$). Because death after day 200 may include treatment-'unrelated' death, we analyzed TRM within 200 days after transplant in relation to *GSTM1* genotype in the patient (Figure 1b). TRM within 200 days in the GSTM1-positive patients was also significantly higher than that in the GSTM1-negative patients (28.8 versus 18.7%; $P=0.04$).

Forty-four of GSTM1-positive patients died within 200 days after transplant while they were in remission. Causes of death in the patients were rejection/graft failure ($n=3$; 6.8%), GVHD ($n=11$; 25.0%), interstitial pneumonia ($n=8$; 18.2%), sepsis ($n=7$; 15.9%), bleeding ($n=3$; 6.8%), veno-occlusive disease (VOD) ($n=2$; 4.5%), adenovirus infection ($n=1$; 2.3%), myocardiopathy ($n=1$; 2.3%), cerebral infarction ($n=1$; 2.3%), hemolytic uremic syndrome ($n=1$; 2.3%), leukoencephalitis ($n=1$; 2.3%), pneumonia ($n=1$; 2.3%) and unknown ($n=4$; 9.6%). Thirty-seven of GSTM1-negative patients died within 200 days after transplant while they were in remission. Causes of death in the patients were rejection/graft failure ($n=1$; 2.7%), GVHD ($n=7$; 18.9%), interstitial pneumonia ($n=13$; 35.1%), sepsis ($n=6$; 16.2%), bleeding ($n=3$; 8.1%), VOD ($n=1$; 2.7%), heart failure ($n=2$; 5.4%), renal failure ($n=1$; 2.7%), hemosiderosis ($n=1$; 2.7%), thrombotic microangiopathy ($n=1$; 2.7%) and unknown ($n=1$; 2.7%). There was no significant difference in the frequencies of each cause of death between two patient groups ($P=0.52$).

Table 2 Univariate and multivariate analyses of risk factors for transplant outcome

Outcome and significant factor	*Univariate analysis* P-*value*	*Multivariate analysis*	
		Odds ratio (95% CI)	P-*value*
TRM			
Higher patient age	0.001	1.03 (1.02–1.04)	<0.0001
Advanced disease	0.015	1.80 (1.23–2.63)	0.002
GSTM1-positive patient	0.043	1.49 (1.03–2.16)	0.036
Acute GVHD (II–IV)			
Advanced disease	0.025	1.46 (1.04–2.07)	0.031
CsA as GVHD prophylaxis	0.008	6.23 (1.54–25.2)	0.010
Chronic GVHD			
Acute GVHD (II–IV)	0.002	1.81 (1.25–2.61)	0.002
Relapse rate			
Advanced disease	<0.0001	3.64 (2.21–5.99)	<0.0001
DFS			
Higher patient age	0.02	1.02 (1.01–1.03)	0.0002
Advanced disease	<0.0001	2.54 (1.85–3.50)	<0.0001
GSTM1-positive patient	0.025	1.50 (1.08–1.97)	0.013
OS			
Higher patient age	0.0013	1.03 (1.02–1.04)	<0.0001
Advanced disease	<0.001	2.54 (1.83–3.53)	<0.0001
GSTM1-positive patient	0.070	1.41 (1.04–1.92)	0.030

CI = confidence interval.

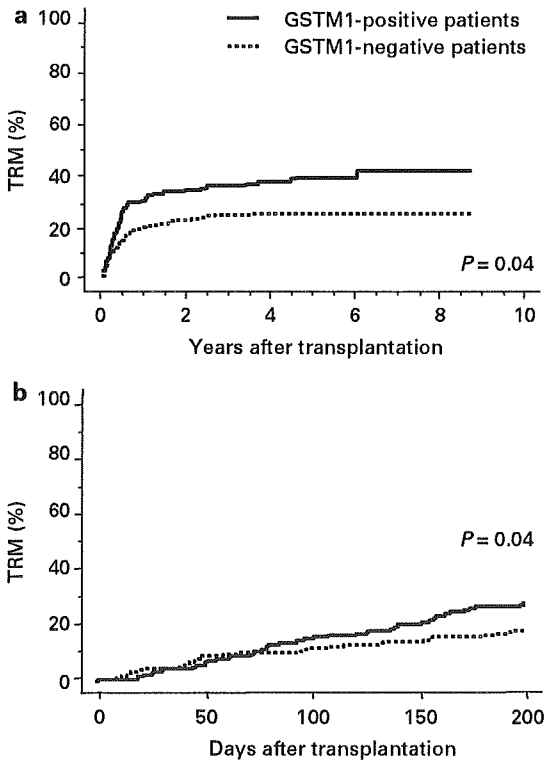

Figure 1 Impact of homozygous deletion of *GSTM1* on TRM after unrelated donor bone marrow transplantation. (**a**) TRM in the GSTM1-positive patients with a hematological disease was 42.9% ($n=166$) and that in GSTM1-negative patients was 29.3% ($n=201$). (**b**) TRM within 200 days after transplantation in the GSTM1-positive patients with a hematological disease was 28.8% ($n=166$) and that in GSTM1-negative patients was 18.7% ($n=201$).

It is suggested that a polymorphism of GSTM1 may be associated with risk of VOD development, especially if the patients received busulfan (BU) and/or cyclophosphamide (CY) as a pretransplant conditioning regimen.[14,15] We therefore analyzed the association between GSTM1 polymorphism and death from VOD in the subgroups of patients. In the patients who received BU (+others) ($n=105$), none of the GSTM1-positive patients ($n=47$) died of VOD, whereas one of the GSTM1-negative patients ($n=58$) died of VOD (not significant). In the patients who received CY (+others) ($n=333$), two of the GSTM1-positive patients ($n=145$) died of VOD, whereas none of the GSTM1-negative patients ($n=188$) died of VOD (not significant).

GVHD

Of 370 evaluable patients, 132 (35.7%) developed grade II–IV acute GVHD. No significant association was detected between the *GSTM1* or *GSTT1* deletion in the patient or donor with the incidence of grade II–IV acute GVHD (Table 2). Forty-one (11.1%) patients developed grade III–IV acute GVHD, but no factor was detected as a risk factor for a higher incidence of grade III–IV acute GVHD.

Of 304 evaluable patients, 124 (40.8%) developed chronic GVHD, including 48 (15.8%) with a limited type and 76 (25.0%) with an extensive type. No significant association was detected between the *GSTM1* or *GSTT1* deletion in the patient or donor with the incidence of chronic GVHD (Table 2).

Relapse

Of 348 evaluable patients with a malignant disease, 74 (21.3%) relapsed after transplantation. No significant association was detected between the *GSTM1* or *GSTT1* deletion in the patient or donor with a relapse rate (Table 2).

DFS and OS

Of 348 evaluable patients with a malignant disease, 177 (50.9%) were alive at the time of survey, including 165 (47.4%) patients who were alive without relapse. In a univariate analysis, higher patient age, advanced disease and GSTM1-positive patient were significantly associated with lower DFS and OS (Table 2). *GSTT1* genotype in either patient or donor was not associated with DFS and OS. In a multivariate analysis, GSTM1-positive patient as well as higher patient age and advanced disease were significantly associated with lower DFS and OS. DFS and OS were analyzed in relation to *GSTM1* genotype in the patient by the Kaplan–Meier method (Figure 2). Both DFS and OS in the GSTM1-positive patients were lower than those in the GSTM1-negative patients (DFS, 38.3 versus 54.0%, $P=0.02$; OS, 41.1 versus 55.1%, $P=0.07$).

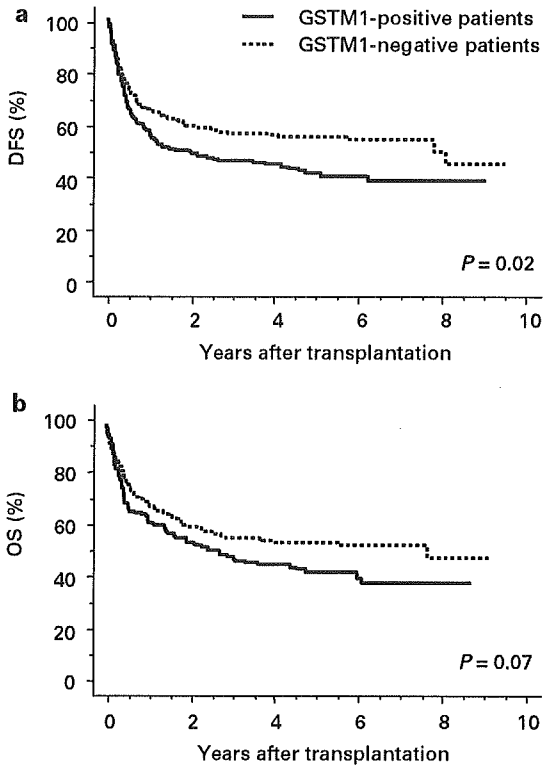

Figure 2 Impact of homozygous deletion of *GSTM1* on DFS and OS after unrelated donor bone marrow transplantation. (**a**) DFS in the GSTM1-positive patients with a malignant disease was 38.3% ($n=152$) and that in GSTM1-negative patients was 54.0% ($n=192$). (**b**) OS in those two groups were 41.1% ($n=152$) and 55.1% ($n=191$), respectively.

Discussion

To evaluate a potential contribution of *GSTM1* and *GSTT1* genotype to allogeneic HSCT outcome, we determined the frequencies of homozygous deletion of *GSTM1* and *GSTT1* genes in the patients with a hematological disease and healthy marrow donors. A homozygous deletion of the *GSTM1* and *GSTT1* genes was found in ~57 and ~46% of healthy donors, respectively, which was comparable to the previous Japanese studies.[7,16] Of patients with a hematological malignancy, homozygous deletion of the *GSTM1* and *GSTT1* genes was found in ~56 and ~45%, respectively, and there was no difference in the frequencies between each disease group and donor group. The findings, of no association between both *GSTM1*- and *GSTT1*-null genotypes and risk of each hematological malignancy, were consistent with our previous study in a different Japanese population;[7] however, many studies have emphasized the importance of these genetic polymorphisms in susceptibility to hematological diseases.[17,18] The analysis in a larger study size may reveal the role of *GSTM1*- and *GSTT1*-null genotypes in the development of hematological malignancies in Japanese people.

The present study demonstrated that a GSTM1-positive patient was significantly associated with higher TRM. GSTs are classified into phase II-metabolizing enzyme group. After phase I, enzymes, in which the cytochrome *P*450 has a central role, convert xenobiotics into a variety of reactive hydrophobic and electrophilic intermediates, phase II enzymes generally detoxify them by neutralizing

reactive electrophiles.[4] GSTs catalyze the nucleophilic addition of thiol of glutathione (GSH) to electrophilic acceptors of various substrates. However, in a minority of the reactions, GSTM1 can activate certain molecules instead of detoxification. GSH conjugates originated from such substrates are relatively unstable and the reaction product requires further detoxification, or is reversely converted into the original compound. Thus, it is possible some reactive intermediates or toxic metabolites can be generated by GSTM1 and initiate or promote the development of TRM.

Although the majority of GST substrates are either xenobiotics or by-products of oxidative stress, GSTs also contribute to the metabolism of endogenous compounds including leukotriene (LT) A4, prostaglandin (PG) D2, PGH2 and PGJ2, as part of their normal biosynthetic pathways.[4] LTB4 and PGI2 were described as markers for the inflammatory processes during GVHD development,[19] suggesting that the absence of GSTM1 may protect the recipient against the production of some inflammatory mediators.

Recently, GSTM1 deficiency in the patients with β-thalassemia major was found to be a risk factor for VOD after bone marrow transplantation using BU plus CY as a pretransplant conditioning regimen.[14] BU is metabolized by GSTs including GSTA1, GSTM1 and GSTP1 to form a positively charged sulfonium ion that is toxic to sinusoidal endothelial cells and hepatocytes. CY is metabolized to 4-hydroxycyclophosphamide (HCY) by cytochrome *P*450, and subsequently to iminocyclophosphamide. Iminocyclophosphamide is conjugated with GSTs and its metabolite is excreted into the bile. As a competing pathway for this detoxification pathway for HCY, the ring-opened form of HCY, aldophosphamide, enters cells and decomposes to phosphoramide mustard, the final cytotoxic metabolite, and acrolein through β-elimination.[15] Thus, BU metabolites and/or CY metabolites may increase risk of VOD. We analyzed the association between GSTM1 polymorphism and death from VOD in the subgroup of patients who received BU (+others) or CY (+others); however, we could not find the effect of GSTM1 polymorphism on fatal VOD. Further studies are clearly needed to determine whether GSTM1 polymorphism is associated with VOD development.

In summary, we assessed the significance of homozygous *GSTM1* and *GSTT1* gene deletions in the donor or recipient with the outcome of unrelated donor bone marrow transplantation. GSTT1 deficiency was not significantly associated with any outcome. However, the GSTM1-positive recipient was an independent risk factor for higher TRM and lower DFS and OS. It is possible that the presence of the *GSTM1*-null genotype in recipient may provide better protection against TRM after allogeneic HSCT. Further studies are needed to elucidate a mechanism of increased risk for TRM in GSTM1-positive recipients.

Acknowledgements

We thank the staff of transplant and donor centers, and JMDP. We also thank Dr Stanley R Riddell for critical reading of the manuscript. This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan (MM and YK).

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Bone Marrow Transplantation (2006) 37, 289–296

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ORIGINAL ARTICLE

Risk and prognostic factors for Japanese patients with chronic graft-versus-host disease after bone marrow transplantation

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The incidence and prognostic factors for chronic graft-versus-host disease (cGVHD) were evaluated for 255 Japanese patients who survived more than 100 days after bone marrow transplantation, and of whom 119 (47%) developed cGVHD. Prior acute GVHD (grade 2–4) and use of an unrelated donor were significantly associated with the onset of cGVHD. Presence of cGVHD did not have an impact on mortality (hazard ratio (HR) = 0.89; 95% confidence interval (CI), 0.59–1.3). Three factors at diagnosis were associated with cGVHD-specific survival: presence of infection (HR = 4.1; 95% CI, 1.6–10.3), continuing use of corticosteroids at the onset of cGVHD (HR = 3.9; 95% CI, 1.7–9.1), and a Karnofsky performance score <80 (HR = 4.7; 95% CI, 2.0–11.3). The probability of cGVHD-specific survival at 4 years was 79% (95% CI, 70–86%). The severity and death rate of Japanese patients with cGVHD was lower than those for populations in Western countries, which might be the result of greater genetic homogeneity of Japanese ethnics. Our patients could not be accurately classified when the proposed prognostic models from Western countries were used, thus indicating the need for a different model to identify high-risk patients.

Bone Marrow Transplantation (2006) **37**, 289–296. doi:10.1038/sj.bmt.1705247; published online 9 January 2006

Keywords: chronic GVHD; Japanese; prognostic model; Karnofsky performance score; corticosteroids; infection

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Received 9 May 2005; revised 1 November 2005; accepted 8 November 2005; published online 9 January 2006

Introduction

Chronic graft-versus-host disease (cGVHD) is an important complication of allogeneic hematopoietic stem cell transplantation (HSCT) with a reported incidence from 30 to 70%.[1,2] To date, a variety of factors have been found to be associated with the development of cGVHD (reviewed by Higman and Vogelsang[3]). The most important factor is the presence of prior aGVHD, which has been repeatedly identified in several studies.[2–7] Other factors include older age, diagnosis of chronic myelogenous leukemia, female donor to male recipient, and the use of an unrelated donor.[2,4,6,7] A recent meta-analysis confirmed that use of peripheral blood stem cells significantly increases the risk of cGVHD.[8]

Chronic GVHD can be classified according to the extent of disease with the most commonly used grading system dividing cGVHD into limited or extensive disease. This system was proposed in 1980 based on the clinicopathologic findings of 20 patients.[9] However, the majority of patients come under the extensive cGVHD category, which is characterized by highly heterogeneous manifestations. For this reason, this grading system, although highly reproducible and useful for clinical decision making as to whether to initiate treatment, provides only limited information on the prognosis of patients.[10,11]

Several clinical and biologic features have been identified as prognostically significant in previous studies of cGVHD, including categorization as extensive cGVHD,[9,12] Karnofsky performance status (KPS),[12] thrombocytopenia ($<100\,000\,mm^3$),[12,13] progressive-type onset of cGVHD,[14] lichenoid histology,[14] and elevated bilirubin.[14] According to recently introduced new clinical grading systems for cGVHD,[10,11] thrombocytopenia, progressive-type onset, and extensive skin GVHD, involving more than 50% of body surface area at the diagnosis of cGVHD, are independent risk factors affecting relapse-free survival.[10] One of these systems uses a novel scoring method based on the results of 151 patients[10] and the validity and significance of this system were confirmed in 1105 patients from

multiple institutions.[15] Lee *et al.*[11] identified high KPS (≥80%) and oral involvement as favorable prognostic signs, and cutaneous involvement, diarrhea, and weight loss as unfavorable prognostic factors.

Because of the historically determined genetic homogeneity of the Japanese population[16] compared to those in Western countries, Japanese patients who receive allogeneic HSCT show a lower incidence of acute GVHD.[17–19] This finding prompted us to analyze a series of Japanese patients in an attempt to identify the incidence and risk factors of cGVHD as well as the prognostic factors for patients who developed cGVHD in the Japanese context.

Patients and methods

Patients

In the 5-year period from January 1995 to December 1999, a total of 400 patients with allogeneic HSCT were treated consecutively at eight centers belonging to the Nagoya Blood and Marrow Transplantation Group (NBMTG). This is a collaborative group consisting of 22 adult and three child HSCT centers. Eight centers of NBMTG which perform allogeneic transplants participated in this study. Of the 400 patients, 301 received bone marrow transplantation (BMT) from either an HLA genotypically matched relative or from HLA A, B, DR genotypically matched unrelated donors. Only patients who underwent BMT were included in this study, and those who received a peripheral blood stem cell transplant or who underwent two or more HSCT procedures were not. The study population comprised 255 patients who survived longer than 100 days post transplant, while patients who developed GVHD after donor leukocyte infusion ($n=2$) or who decided themselves to abandon immunosuppressive agents ($n=1$) were not included.

Diagnosis and staging of cGVHD

Diagnosis of cGVHD was based on clinical criteria of skin, oral and other affected sites as previously described.[9,20] The diagnosis of cGVHD was confirmed by histologic studies of skin, oral, or other affected sites in 60 of 110 patients (54.6%) who were clinically diagnosed. Established criteria were used for the histologic diagnosis.[9,21] Staging of cGVHD was originally performed by institutional physicians, and was reviewed on the basis of these physicians' data sheets by three independent hematologists (YA, RS and KY) using the most commonly used criteria.[9] The stage in agreement was used for the analyses.

Analysis of demographic variables for risk factors of extensive cGVHD

Patient age, sex, diagnosis of primary disease, disease status at transplant, donor type, preparative regimen, GVHD prophylaxis, and prior aGVHD grade were examined for risk factors of cGVHD.

Demographics, clinical, and laboratory variables analyzed for prognostic factors for those who developed cGVHD

The following variables documented at the time of diagnosis of cGVHD were used for our study: age, sex, donor type, donor/recipient sex matching, pretransplant disease status, preparative regimen, GVHD prophylaxis, prior occurrence and severity of aGVHD, positive cytomegalovirus status (donor and/or recipient), mode of presentation of cGVHD, serum alkaline phosphatase and total bilirubin, peripheral blood platelet count, absolute serum immunoglobulin level, blood eosinophil percentage, and use of corticosteroids before initiation of treatment of cGVHD. The extent and severity of cGVHD in the affected organs were also assessed. Other surrogate parameters indicating disease severity, such as weight loss after BMT, presence of chronic diarrhea, performance status measured by KPS, and the presence of infectious complications, were also included for analysis.

Coding system of the clinical variables analyzed for prognostic factors for patients who developed cGVHD

The most common sites of involvement were coded at the time of diagnosis of cGVHD into two or three grades by using a coding system based on the one by Akpek *et al.*[10] with several modifications. These codes were then entered into the database for analyses. The coding system or cutoff points of other variables are listed in Table 1.

Data collection instrument and methods

Subject-, disease-, and transplant-related variables were collected on standardized forms provided by the NBMTG. Survival, relapse, and complication data in the database, including those for cGVHD, are revised annually. Informed consent is obtained from all patients when they are registered with the NBMTG. This study was approved by the board of directors of NBMTG.

Patients diagnosed with cGVHD were selected from the NBMTG database. Data collection forms were sent out for the collection of clinical and laboratory data at the time of diagnosis of cGVHD, at the initiation of primary therapy, and, if used, at the initiation of secondary therapy. Immunosuppressive therapy regimens and responses to primary and secondary therapy were reviewed. Data on survival and relapse were collected as of March 2003.

Statistical analyses

Cox regression model was used for both univariate and multivariate analyses of risk factors for extensive cGVHD. Factors found to be significant ($P<0.05$) in the univariate analysis were included in the multivariate analysis.

To identify the prognostic factors for patients who developed cGVHD, the major statistical end point was 'cGVHD-specific survival' from the time of diagnosis of cGVHD. Although the term 'cGVHD-specific survival' does not completely exclude all deaths without recurrent malignancy, we used this term in its traditional meaning of recurrence censored survival.[10,15,22] Death because of relapse of underlying hematological disorders was censored at the time of relapse. Cox regression model was used to analyze data for the identification of prognostic factors. All clinical factors and laboratory data were initially categorized as several numeric variables (see above), and subsequently binarized during univariate analyses. The cutoff points were chosen to make optimal use of information,

Table 1 Prognostic significance of cGVHD parameters for cGVHD-specific survival

	N	*HR (95% CI)*	P-*value*
Mode of presentation of cGVHD			
De novo	33	1.0	
Quiescent	73	1.1 (0.44–2.6)	0.89
Progressive	4	1.8 (0.83–4.0)	0.14
Grade of cGVHD			
Limited	30	1.0	
Extensive	80	1.6 (0.60–4.3)	0.34
Cutaneous extent			
Code 1 (no skin involvement)	45	1.0	
Code 2 (50% or less of all skin surface area)	54	0.61 (0.26–1.5)	0.27
Code 3 (more than 50% of all skin surface area)	9	1.3 (0.71–2.2)	0.42
Oral involvement			
Code 1 (no clinical evidence of cGVHD)	25	1.0	
Code 2 (clinically evident oral symptoms)	78	0.62 (0.25–1.5)	0.31
Code 3 (severe oral cGVHD causing functional impairment)	4	1.1 (0.37–3.1)	0.91
Thrombocytopenia			
Code 1 ($\geqslant 100\,000/mm^3$)	56	1.0	
Code 2 ($< 100\,000/mm^3$ and $\geqslant 50\,000/mm^3$)	40	1.2 (0.48–2.8)	0.73
Code 3 ($< 50\,000/mm^3$)	14	2.1 (0.74–6.2)	0.16
Eosinophilia (>4%)	45	3.5 (1.3–9.5)	0.01
Hyperbilirubinemia (>1.2 mg/dl)	22	1.7 (0.71–4.2)	0.23
Elevated alkaline phosphatase (>2.5 × ULN IU/l)	26	1.8 (0.79–4.2)	0.16
Weight loss			
None	61	1.0	
<10% from baseline; body weight before BMT	22	0.65 (0.22–2.0)	0.45
⩾10% from baseline; body weight before BMT	9	1.4 (0.75–2.6)	0.29
Chronic diarrhea	7	2.1 (0.62–6.9)	0.24
Presence of infection	20	5.7 (2.5–13.2)	<0.0001
Reduced serum IgG (<500 mg/dl)	33	2.0 (0.84–4.7)	0.12
KPS<80%	30	5.8 (2.5–13.3)	<0.0001
Use of corticosteroids	23	3.9 (1.8–8.7)	0.001

GVHD = graft-versus-host disease; HR = hazard ratio; ULN = upper limit of normal; BMT = bone marrow transplantation; KPS = Karnofsky performance score.

with the proviso that smaller groups contain at least 20% of the patients. A forward stepwise method was applied for detecting sets of independent significant factors. In the previously reported studies of prognostic factor analyses of cGVHD, the statistical end point was either cGVHD-specific survival,[10,15,22] or overall survival.[11] Our analyses were therefore also performed with overall survival as the statistical end point.

Survival distributions were estimated with the method of Kaplan and Meier, and compared by means of the long-rank test. Cumulative incidence of cGVHD was calculated by treating death without cGVHD or relapse as a competing risk for cGVHD. The proportional hazards regression model with cGVHD entered as a time-dependent covariate was used to determine the effect of cGVHD on relapse, non-relapse mortality, and overall survival. All statistical analyses were performed with STATA software version 8.0 (College Station, TX, USA).

Results

Patient characteristics and incidence of cGVHD

Patient characteristics and BMT information are summarized in Table 2.

Of the 255 BMT recipients, 119 (47%) developed cGVHD. The cumulative incidence of cGVHD 2 years after transplant was 42% (95% confidence interval (CI), 35–48%) (Figure 1a). Cumulative incidence of clinical extensive cGVHD 2 years after transplant was 31% (95% CI, 25–37%).

Risk factors for developing cGVHD

Among the factors evaluated as risks for the development of clinical cGVHD, only the presence of prior grades 2–4 aGVHD was identified as significant by univariate analysis (Table 3). When the same clinical characteristics were analyzed for clinical extensive cGVHD, univariate analysis showed two factors to be significant, unrelated donor type (hazard ratio (HR) = 1.8; 95% CI, 1.2–2.7; $P = 0.008$), and prior grades 2–4 aGVHD (HR = 1.9; 95% CI, 1.2–3.0; $P = 0.005$). Multivariate analysis demonstrated that the factors constituting independent risk factors for clinical extensive cGVHD were unrelated donor type (HR = 1.6; 95% CI, 1.1–2.5; $P = 0.02$), and prior grades 2–4 aGVHD (HR = 1.8; 95% CI, 1.1–2.7; $P = 0.02$).

Effect of cGVHD on relapse and mortality

The median follow-up of survivors was 4.1 years. The probability of overall survival 4 years after the transplant

Table 2 Patient characteristics

	N
Total number of patients	255
Median age, years (range)	36 (16–54)
Male/female	174/81
Diagnosis	
Acute leukemia/myelodysplastic syndrome	143
Chronic leukemia	69
Lymphoma/multiple myeloma	27
Aplastic anemia	14
Disease status (malignant disease, only)	
Standard risk (CR1 and chronic phase)	120
Advanced risk (all others)	130
Donor type	
HLA-identical, related	155
HLA-identical, unrelated	100
Preparative regimen	
Chemotherapy alone	21
Total body irradiation based	202
GVHD prophylaxis	
Cyclosporine + short-term methotrexate	189
FK506 + short-term methotrexate	54
Others	12
Acute GVHD grade	
0	96
1	97
2–4	62

CR1 = first complete remission; HLA = human leukocyte antigen; GVHD = graft-versus-host disease.

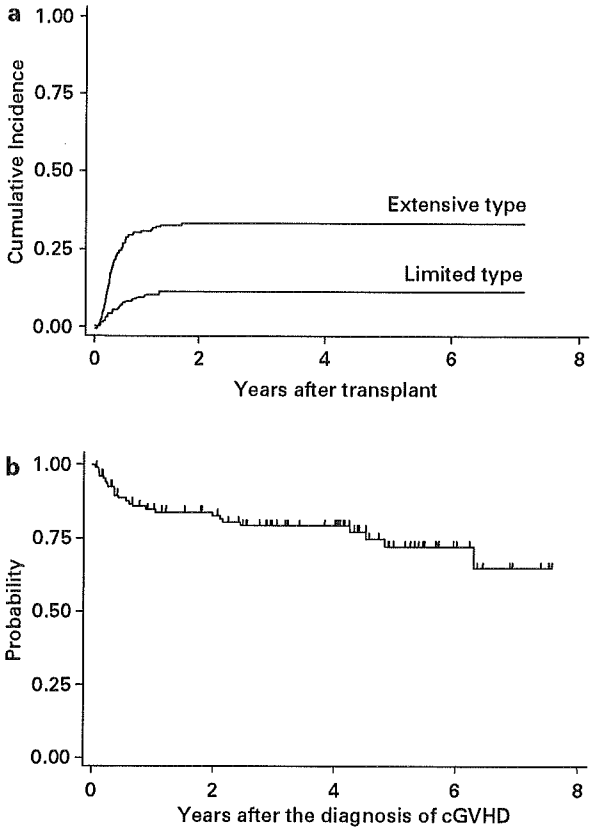

Figure 1 Cumulative incidence of limited and extensive cGVHD after allogeneic hematopoietic stem cell transplantation was 10% (95% CI, 7–14%) and 31% (95% CI, 25–37%) at 2 years, respectively. (**a**) cGVHD-specific survival from the time of diagnosis of cGVHD for 110 patients who developed cGVHD was 79% (95% CI, 70–86%) at 4 years (**b**).

was 60% (95% CI, 53–66%), and cumulative incidence of relapse after 2 years was 21% (95% CI, 16–27%).

The analysis of the effects of cGVHD as a time-dependent covariate with adjustments for patient age, sex, disease status, and donor type showed no statistical significance for relapse (HR = 0.70; 95% CI, 0.37–1.3), non-relapse mortality (HR = 1.4; 95% CI, 0.72–2.6) or survival (HR = 0.97; 95% CI, 0.64–1.5). The results were similar to the results obtained for the effects of clinical extensive cGVHD on relapse (HR = 0.67; 95% CI, 0.33–1.3), non-relapse mortality (HR = 1.6; 95% CI, 0.92–2.7), and survival (HR = 1.05; 95% CI, 0.69–1.6).

Characteristics and survival of patients who developed clinical cGVHD

Detailed information at the time of diagnosis of cGVHD was available for 110 of the 119 patients who developed cGVHD. Their characteristics are shown in Table 4, and their clinical manifestations and laboratory data at the time of diagnosis of cGVHD are given in Table 1.

Follow-up of survivors after the diagnosis of cGVHD (median length: 4.2 years) showed that 69 (63%) of the 110 patients were alive at the time of data collection. The probability of cGVHD-specific survival and overall survival 4 years after the diagnosis of cGVHD was 79% (95% CI = 70–86%) (Figure 1b) and 65% (95% CI = 55–74%), respectively. Of the 41 deaths, 16 (39%) were the result of relapse and 25 (61%) of other complications. The latter consisted of various infections (seven patients) and organ failures associated with cGVHD (12 patients). Detailed information on their cause of death is summarized in Table 5. The patient who died of gastric cancer was censored from the analysis of cGVHD-specific survival when the disease was diagnosed. For the 80 patients with clinical extensive cGVHD, cGVHD-specific survival and overall survival 4 years after the diagnosis of cGVHD were 76% (95% CI = 65–84%) and 62% (95% CI = 50–71%), respectively.

Prognosis of patients with cGVHD

Univariate analysis showed that age of 36 or older (HR = 2.6; 95% CI, 1.1–6.3), <4% eosinophils at the time of diagnosis of cGVHD (HR = 3.5; 95% CI, 1.3–9.5), Karnofsky score of <80% (HR = 5.8; 95% CI, 2.5–13.3), presence of infectious complication (HR = 5.7; 95% CI, 2.5–13.1), and use of corticosteroids before initiating treatment for cGVHD (HR = 3.9; 95% CI, 1.8–8.7) were significant prognostic factors (Table 4, Table 1). Corticosteroids were being administered to 23 patients (22%) at

Table 3 Univariate analysis of risk factors for development of cGVHD

Factor	*No. of patients*	*HR (95% CI)*	P-*value*
Patient age			
Younger than 36 years	117	1.0	
36 years or older	138	0.93 (0.65–1.3)	0.73
Patient sex			
Female	81	1.0	
Male	174	1.1 (0.76–1.6)	0.56
Diagnosis			
Acute leukemia/myelodysplastic syndrome	143	1.0	
Chronic leukemia	69	1.2 (0.81–1.8)	0.38
Lymphoma/multiple myeloma	27	0.85 (0.51–1.4)	0.54
Aplastic anemia	14	—	—
Disease status (malignant disease, only)			
Standard risk (CR1 and chronic phase)	120	1.0	
Advanced risk (all others)	130	0.76 (0.52–1.1)	0.13
Donor type			
HLA-identical, related	155	1.0	
HLA-identical, unrelated	100	1.4 (0.94–1.9)	0.10
Preparative regimen			
Chemotherapy alone	21	1.0	
Total body irradiation based	202	1.00 (0.54–1.9)	1.00
GVHD prophylaxis			
Cyclosporine + short-term methotrexate	189	1.0	
FK506 + short-term methotrexate	54	1.1 (0.68–1.6)	0.84
Acute GVHD grade			
0–1	193	1.0	
2–4	62	1.8 (1.2–2.6)	0.005

GVHD = graft-versus-host disease; HR = hazard ratio; CR1 = first complete remission; HLA = human leukocyte antigen.

Table 4 Patient characteristics and their prognostic significance for cGVHD-specific survival

	N	*HR (95% CI)*	P-*value*
Total number of patients	110		
Median age, years (range): 36 years (17–62)			
Younger than 36 years	52	1.0	
36 years or older	58	2.6 (1.1–6.3)	0.03
Sex			
Female	34	1.0	
Male	76	1.0 (0.44–2.4)	0.96
Donor and recipient, sex mismatch			
None	56	1.0	
Female to male	34	0.96 (0.62–1.5)	0.83
Male to female	15	1.0 (0.29–3.5)	0.99
Disease status			
Standard risk (CR1 and chronic phase)	61	1.0	
Advanced risk (all others)	49	0.74 (0.33–1.7)	0.48
Donor type			
HLA-identical, related	65	1.0	
HLA-identical, unrelated	45	0.84 (0.37–1.9)	0.67
GVHD prophylaxis			
Cyclosporine + short-term methotrexate	84	1.0	
FK506 + short-term methotrexate	24	1.2 (0.47–3.0)	0.73
Others	2		
Acute GVHD grade			
0	33	1.0	
1 (cutaneous)	43	1.3 (0.43–3.8)	0.67
2–4 (systemic)	34	1.6 (0.94–2.7)	0.08

GVHD = graft-versus-host disease; HR = hazard ratio; CR1 = first complete remission; HLA = human leukocyte antigen.

Table 5 Cause of death of patients who developed cGVHD

Cause of death	N
Relapse	16
Other than relapse	25
Infection	7
Organ failures associated with cGVHD	12
Lung failure	10
Liver failure	2
Other causes	6

GVHD = graft-versus-host disease.

the onset of cGVHD. All patients received steroids for treatment of acute GVHD and were in the process of tapering off when the cGVHD developed. Multivariate analysis identified three factors at the time of diagnosis of cGVHD as independent predictors for non-relapse mortality: presence of infection (HR = 4.1; 95% CI, 1.6–10.4), use of corticosteroids (HR = 3.9; 95% CI, 1.7–9.1), and KPS <80% (HR = 4.7; CI, 2.0–11.3) (Table 6). These results enabled us to establish a prognostic model for cGVHD-specific survival or relapse-censored survival in terms of how many of these three risk factors are present. Figure 2 shows the probability of cGVHD-specific survival for patients divided into three risk groups according to this model. The estimated 4-year cGVHD-specific survival was 91.8% for patients without any prognostic factors (PFs) ($n = 51$), 72.6% for those with one or two factors ($n = 42$), and 14.3% for those with all three factors ($n = 7$) ($P < 0.0001$; log rank test). Six of the seven patients who showed all three prognostic factors died within 8 months after the diagnosis of cGVHD. The same three risk groups were used successfully for overall survival. The estimated 4-year overall survival was 80% for patients without any prognostic factors (PFs) ($n = 51$), 60% for those with one or two factors ($n = 42$), and 0% for those with all three factors ($n = 7$) ($P < 0.0001$; log rank test).

Univariate and multivariate analyses confirmed the same three factors as independent prognostic factors when the subjects were limited to 80 patients with clinical extensive cGVHD. These three risk groups thus resulted in successful categorization of patients with clinical extensive cGVHD for prognosis of cGVHD-specific survival ($P < 0.0001$;

Table 6 Multivariate analysis for non-relapse mortality of patients newly diagnosed with cGVHD

Prognostic factor	N	*HR*	*95% CI*	P-*value*
KPS ⩾ 80%	76	1.0	—	
KPS < 80%	30	4.7	2.0–11.3	<0.001
No use of corticosteroid	82	1.0	—	
Use of corticosteroid	23	3.9	1.7–9.1	0.002
Absence of infection	88	1.0	—	
Presence of infection	20	4.1	1.6–10.4	0.001

GVHD = graft-versus-host disease; HR = hazard ratio; CI = confidence interval; KPS = Karnofsky performance score.

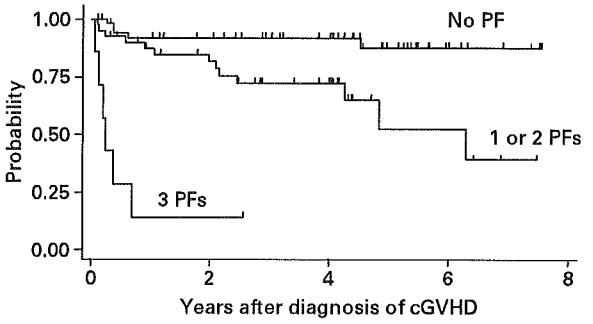

Figure 2 cGVHD-specific survival after the diagnosis of cGVHD for patients grouped by the total number of prognostic factors (PFs) was 92% (95% CI, 80–97%) ($n=51$) for no PF, 73% (95% CI, 55–84%) ($n=42$) for 1 or 2 PFs at 4 years, and 14% (95% CI, 1–46%) ($n=7$) for 3PFs at 2 years.

log rank test) as well as of overall survival ($P<0.0001$; log rank test).

Comparison with other grading systems

The classical grading system of limited/extensive-type cGVHD produced the survival curves in Figure 3a for our patients after the diagnosis of cGVHD. The result shows that the probability of cGVHD-specific survival 4 years after the diagnosis of cGVHD was 76% (95% CI, 65–84%) for the extensive type and 89% (95% CI, 70–96%) for the limited type, without any statistically significant difference (log rank test; $P=0.34$). There was no significant difference either when the statistical end point was overall survival (log rank test; $P=0.25$). In this case, the probability of overall survival 4 years after the diagnosis of cGVHD was 62% (95% CI, 50–72%) for the extensive type and 76% (95% CI, 56–88%) for the limited type.

Another grading system by Akpek *et al.*[15] was also used. None of the factors used in this grading system, for example, progressive-type onset, extensive involvement of more than 50% of total skin surface area, and thrombocytopenia proved to be prognostic for our patients (Table 1). Categorization according to this system for cGVHD-specific survival when matched to the system's statistical end point could not produce a useful stratification of our patients (Figure 3b). Finally, the grading system by Lee *et al.*[11] was used for our patients for overall survival matched to that system's statistical end point. The results in

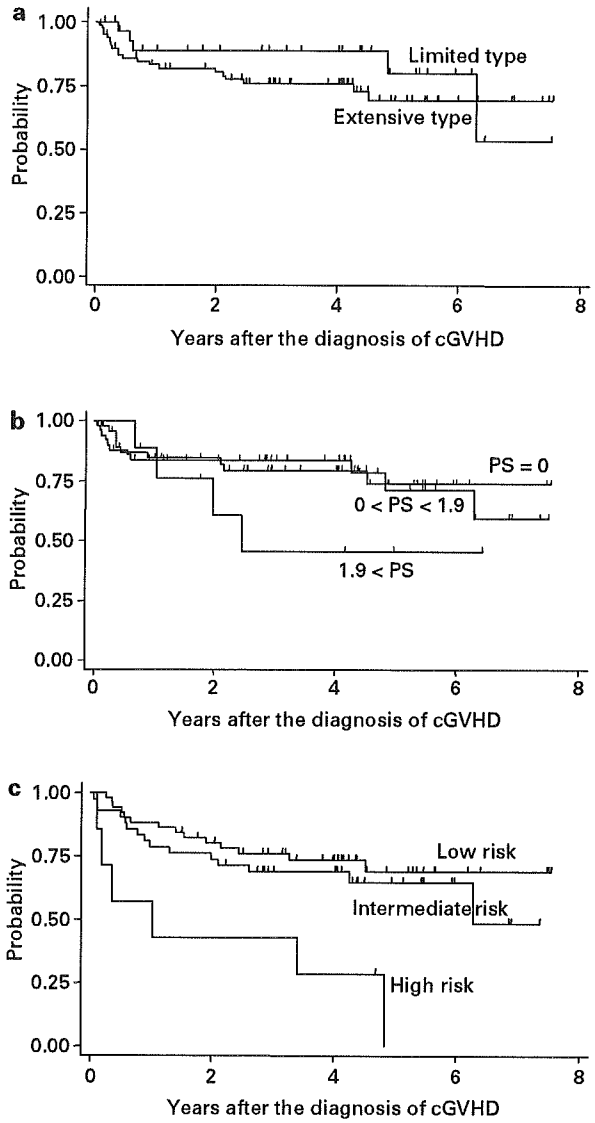

Figure 3 Survival curves after the diagnosis of cGVHD according to the classical limited/extensive grading system. cGVHD-specific survival at 4 years was 89% (95% CI, 70–96%) ($n=30$) and 76% (95% CI, 65–84%) ($n=80$), respectively (**a**). With Akpek's prognostic scoring (PS) system, the cGVHD-specific survival at 4 years was 79% (95% CI, 70–92%) ($n=48$) for PS = 0, 84% (95% CI, 70–92%) ($n=51$) for $0<PS<1.9$, and 46% (95% CI, 11–76%) ($n=9$) for $1.9<PS$ of cGVHD-specific survival at 4 years (**b**). With Lee's grading system, overall survival at 4 years was 73% (95% CI, 59–84%) ($n=51$) for low risk, 69% (95% CI, 53–81%) ($n=42$) for intermediate risk, and 29% (95% CI, 4–61%) ($n=7$) for high risk (**c**).

Figure 3c show that the majority of patients were classified into the low or intermediate risk group, and only seven patients as high risk, with no statistically significant difference between the low- and intermediate-risk groups (log rank test; $P=0.33$). The results were quite similar when Akpek *et al.*'s and Lee *et al.*'s grading systems were applied to the 80 subjects with clinical extensive cGVHD. More than 90% of our patients were classified in the lower

risk groups with no significant difference between the two groups (log rank test; $P = 0.47$ and 0.59, respectively).

Discussion

The aim of this study was to identify the incidence and risk factors of cGVHD, as well as the survival and prognostic factors for patients who developed cGVHD among Japanese BMT patients, who show a lower incidence and severity of acute GVHD than patients in Western countries.[17,19,23] Our analyses concerning cGVHD showed that the incidence and risk factors of our study group were comparable to those of Western patients. The most important risk factor among various factors found to be associated with the development of cGVHD is the existence of prior acute GVHD.[2,7] A recent report comparing GVHD and survival after HLA-identical sibling BMT in ethnic populations showed no differences in the risk for cGVHD.[23] The results of our analysis were consistent with those reported in the literature.

We also used recently reported prognostic scoring systems, which were developed on the basis of clinical findings for Western patients, on our Japanese patients, and unexpectedly found that these scoring systems did not produce an effective categorization. None of the three prognostic factors at the diagnosis of cGVHD in Akpek *et al.*'s scoring system were found to be prognostic in our analyses. When we compared the percentage of patients with Akpek *et al.*'s prognostic factors recorded in published data from Western countries with the corresponding percentages for our patients, we found some major differences. The proportion of progressive type onset of cGVHD is reportedly 20–70% in Western countries,[3,11,15] but was only 4% for our patients. The extent of cutaneous cGVHD was also different, with the incidence of those presenting with cutaneous lesions covering more than 50% of the total skin area was 9.5% for our patients, but 25–72% for those reported by Akpek *et al.*[15] These differences are likely to result in different results for prognostication and point to the need for the establishment of a different model for Japanese patients.

Our analyses identified the ongoing use of corticosteroids at the onset of cGVHD as a significant prognostic factor, which has not been included in previous studies.[10,11,14] Whether the use of a certain agent can be a prognostic factor is associated with how it is used. Approximately 20% of our patients were using corticosteroids at the time of diagnosis of cGVHD, and their steroid use was associated with prior acute GVHD. Wagner *et al.*[4] reported that 59% of patients were still under corticosteroid treatment on day 100, which was higher than for our series. They further showed that steroid use on day 100 was an adverse prognostic factor for patients after HSCT. Przepiorka *et al.* found that 40% of patients were on steroid treatment on day 100 and showed poorer prognosis,[5] but neither of these studies examined steroid use at the time of cGVHD onset. Because the occurrence of cGVHD despite the use of corticosteroids indicates resistance to immunosuppressive therapies and an immunodeficient status, it is reasonable to assume that steroid use at the diagnosis of cGVHD acts as an adverse prognostic factor. We believe therefore that the use of corticosteroids should be more widely examined for its prognostic significance.

The probability of cGVHD-specific survival of our patients was fairly high, 79% at 4 years, while the probability was <60% at 4 years in the study by Akpek *et al.*[10] A recent Korean study reported a cGVHD-specific survival of 74.2% at 5 years, which is similar to our result.[22] The presence of cGVHD did not show prognostic significance for our patients, which might be the result of the neutralization of the positive and negative effects of cGVHD on survival. The result of our analyses limited to those with clinical extensive cGVHD indicate that it is not due to the higher proportion of patients with limited cGVHD in Japanese patients which contribute to the different outcomes from the Westerns.

In conclusion, we identified three prognostic factors for patients who developed cGVHD. Since the manifestations of cGVHD in Japan are different from those in Western countries, the morbidity and mortality of cGVHD were found to be lower for Japanese patients. This indicated the need for a different prognostic model to identify high-risk patients.

Acknowledgements

The authors are grateful for the assistance by and cooperation from all the staff members of the collaborating institutes of the Nagoya Blood and Marrow Transplantation Group.

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