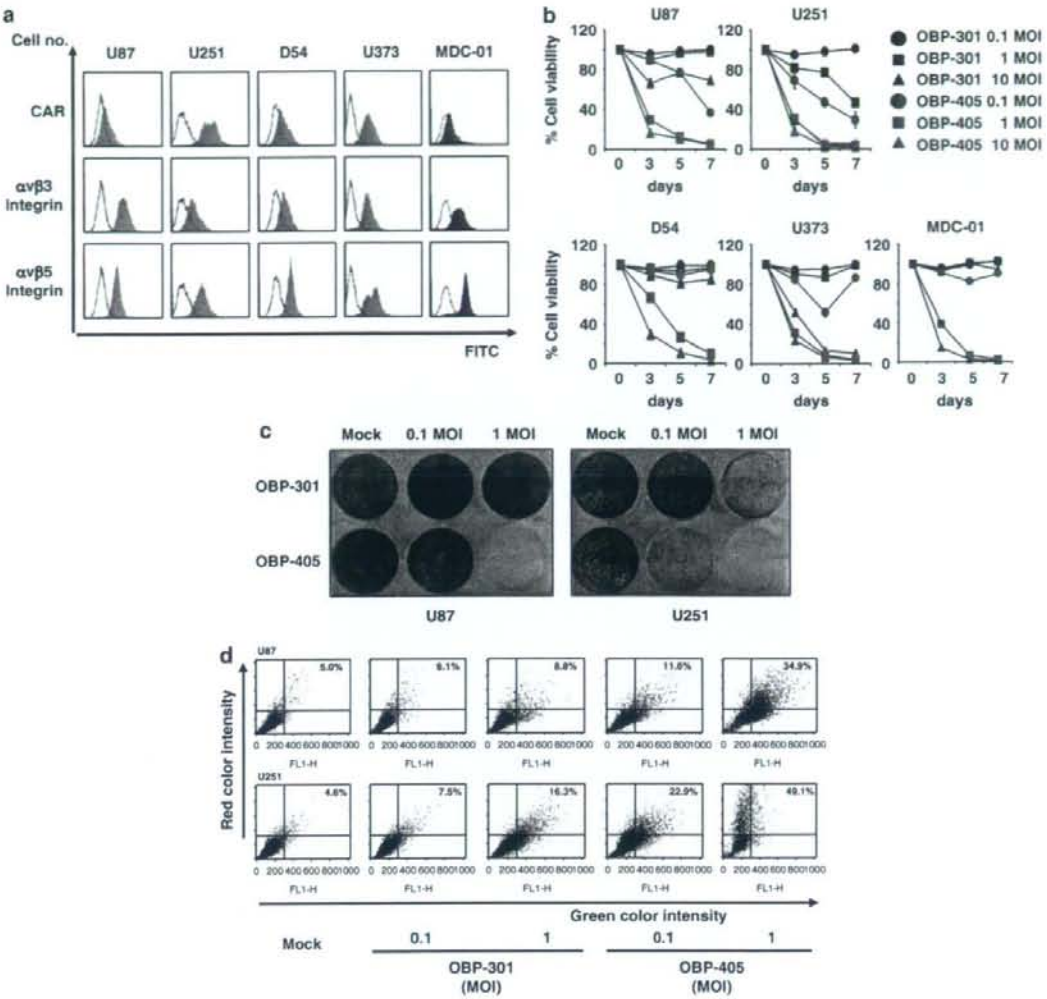

Figure 1 (a) Flow cytometric analysis of CAR and integrin (αvβ3 and αvβ5) expression on glioblastoma cells. Human glioblastoma cell lines U87-MG, U251-MG, D54 and U373-MG were purchased from American Type Culture Collection (Manassas, VA, USA). Primary glioblastoma MDC-01 cells were isolated from surgical specimens of glioblastoma at MD Anderson Cancer Center and were positive for telomerase and glial-fibrillary acidic protein. Cells were incubated with anti-CAR (Upstate Biotechnology, Lake Placid, NY, USA), anti-αvβ3 integrin and anti-á„β5 integrin (Chemicon International, Temecula, CA, USA) monoclonal antibodies and then detected with fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG secondary antibody (Zymed Laboratories, San Francisco, CA, USA). Open areas (control), isotype-matched normal mouse IgG1 conjugated to FITC. (b) Effect of OBP-301 and OBP-405 on the viability of glioblastoma cells. Cells were infected at the indicated MOI values and surviving cells were quantified over 7 days by the use of WST-1 assay (Roche Applied Science, Indianapolis, IN, USA). Results shown are the means ± s.d. of three independent experiments. (c) Oncolytic effect of OBP-301 and OBP-405 on glioblastoma cells. Low-CAR expressing (U87-MG) and high-CAR expressing (U251-MG) cells were stained with 0.5% crystal violet (Sigma-Aldrich, St Louis, MO, USA) 5 days after infection. (d) Development of acidic vesicular organelles (AVOs) in U87-MG and U251-MG cells infected with OBP-301 or OBP-405 at an MOI of 0.1 or 1.0 for 72 h. Mock- or virus-infected cells were stained with 1.0 μg ml^{-1} acridine orange (Polysciences, Warrington, PA, USA) for 15 min at room temperature and analyzed using a flow cytometer (FACScan; Becton Dickinson, San Jose, CA, USA). In acridine orange-stained cells, the cytoplasm and nucleus fluorescence bright green and dim red, whereas acidic compartments fluorescence bright red.[10,11] The intensity of the red fluorescence is proportional to the degree of acidity and volume of AVOs. Top of grid was considered as AVOs. CAR, coxsackievirus and adenovirus receptor; IgG, immunoglobulin G; MOI, multiplicity of infection.

lity of glioblastoma cells (Figure 1b). In addition, OBP-405 killed the cells more efficiently than did OBP-301, and neither OBP-301 nor OBP-405 induced apoptosis (Figure 1c) (Supplementary Figures 1a–c).

Nonapoptotic autophagy is characterized by the development of acidic vesicular organelles (AVOs).[10] Compared with mock infection, both OBP-301 and OBP-405 increased the percentage of AVO-positive cells in a multiplicity of infection (MOI)-dependent manner (Figure 1d). As expected, OBP-405 induced the development of AVOs more efficiently than did OBP-301.

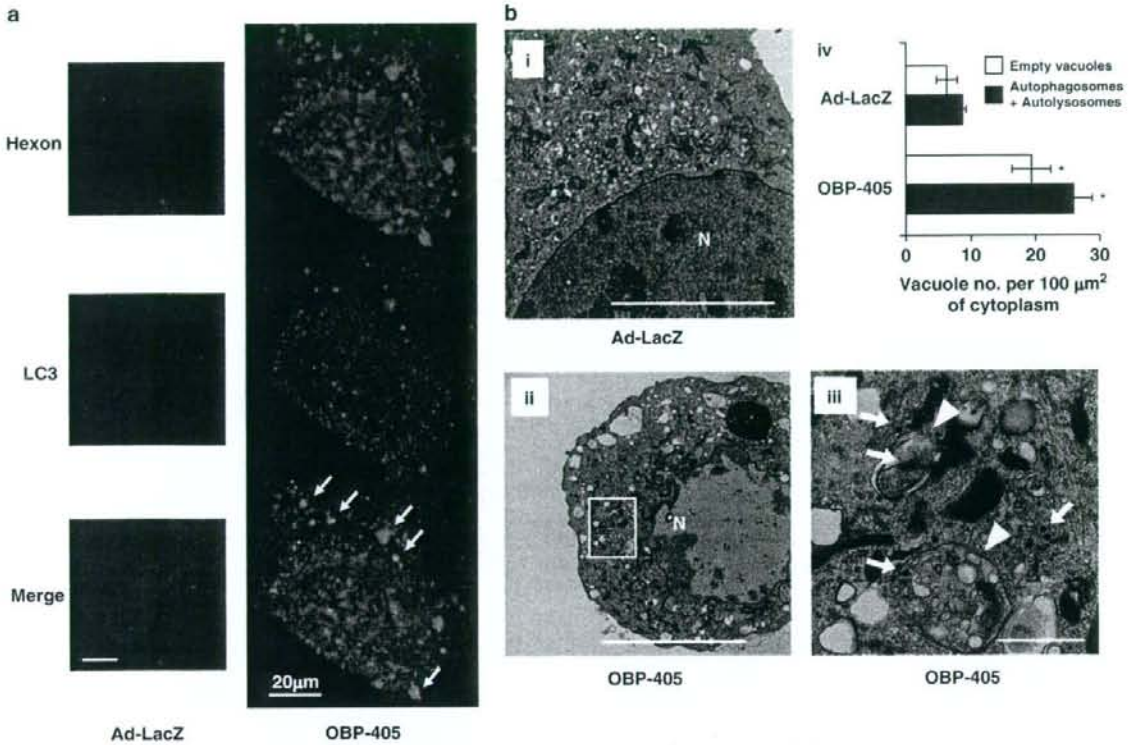

Figure 2 (**a**) Localization of the adenoviral protein hexon and autophagic LC3B protein in glioblastoma cells infected with Ad-LacZ or OBP-405 at an MOI of 0.5. After infection for 72 h, U87-MG cells were processed for fluorescent immunocytostaining with anti-LC3B (1:5000 dilution) and antiadenovirus 1, 2, 5 and 6 hexon (Chemicon International) antibodies. Anti-LC3B antibody was generated as described previously.[14,15] The slides were monitored using inverted microscope (ECRIPSE TE2000-U; Nikon, Melville, NY, USA) and the data were deconvolved and analyzed using AutoQuant's AutoDeBlur software (MediaCybernetics, Bethesda, MD, USA). The arrow shows the colocalization of LC3B and hexon. (**b**) Electron photomicrographs showing the ultrastructure, including the nucleus (N) of glioblastoma cells treated with nonreplicating adenovirus carrying the Ad-LacZ or OBP-405 at an MOI of 0.5 for 72 h. (**i**) Ad-LacZ-infected U87-MG cells; few autophagic vacuoles were observed, scale bar = 10 μm. (**ii**) OBP-405-infected U87-MG cells, scale bar = 10 μm. (**iii**) A magnified view of the area boxed in (ii), scale bar = 1 μm. The arrow indicates viral particles and the arrowhead indicates an autophagosome that includes residual material and virus particles in the cytoplasm. (**iv**) Autophagosomes and autolysosomes were quantified, as described previously.[16,17] $^*P < 0.05$ vs Ad-LacZ. MOI, multiplicity of infection.

The green fluorescent protein (GFP)-tagged expression vector of LC3 is a useful tool with which to detect autophagy.[12] On fluorescence microscopy, GFP-LC3-transfected U87-MG cells showed the diffuse distribution of GFP-LC3 with mock infection, whereas infection with OBP-405 at an MOI of 1.0 resulted in a punctate pattern of GFP–LC3 (Supplementary Figure 2a). This pattern represents autophagic vacuoles and indicated that OBP-405 induced autophagy. With both OBP-301 and OBP-405, the percentage of GFP–LC3 dots increased in an MOI-dependent manner; this increase was considerably higher with OBP-405 than with OBP-301.

The LC3 protein exists in two cellular forms, LC3-I and LC3-II. LC3-I is converted to LC3-II by conjugation to phosphatidylethanolamine, and the amount of LC3-II is closely correlated with the number of autophagosomes.[13] In both U87-MG and U251-MG cells, the amount of LC3-II was increased by infection with OBP-301 or OBP-405 in an MOI-dependent manner and by OBP-405 in a time-dependent manner (Supplementary Figure 2b). These results indicated that OBP-405 caused more autophagy in glioblastoma cells than OBP-301 did.

To analyze the association between adenoviral infection and autophagy, we determined the localization of OBP-405 and autophagic vacuoles. The adenoviral hexon was detected in the cytoplasm 6 h after infection, but at that point, autophagic vacuoles positive for the isoform B of human LC3 (LC3B) were not observed (Supplementary Figure 3), indicating that autophagy was not initiated. Twenty-four hours after infection, hexon was detected in the cytoplasm and nucleus and LC3B-positive autophagic vacuoles were observed. At 48 h, the cell and nucleus had become larger. At 72 h after infection, the majority of the autophagic vacuoles were colocalized with hexon-positive adenoviruses (Figure 2a). In addition, we analyzed the ultrastructure of infected U87-MG cells. U87-MG cells infected with control nonreplicating adenovirus (Ad-LacZ) exhibited few autophagic features, whereas autophagic vacuoles, autolysosomes and empty vacuoles were observed after infection with OBP-405. Most OBP-405-infected cells

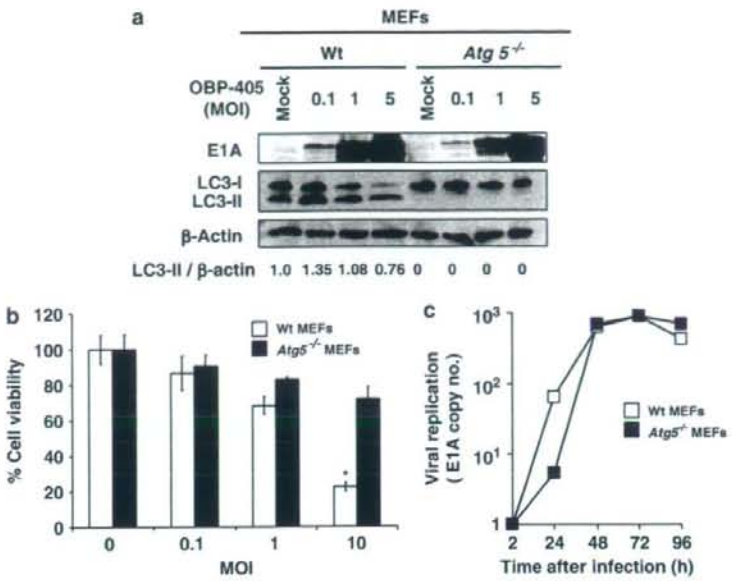

Figure 3 (a) Effect of OBP-405 infection on the wild-type and *Atg5*$^{-/-}$ MEFs (kindly provided by Dr N Mizushima). The expression of E1A (BD Biosciences Pharmingen, San Diego, CA, USA) and an amount of LC3-II in MEFs infected with OBP-405 at an MOI of 0–5.0 for 72 h was assessed by western blotting. The intensities of the amount of LC3-II bands were normalized by the bands' intensities of β-actin (Sigma-Aldrich), using Bio-Rad Fluor-S Multiimager (Bio-Rad Laboratories, Hercules, CA, USA). (b) Effect of OBP-405 on the viability of wild-type and *Atg5*$^{-/-}$ MEFs. MEFs were infected with OBP-405 at an MOI of 0 to 10 for 48 h, and cell viability was assessed by WST-1 assay. *$P<0.01$ vs *Atg5*$^{-/-}$ MEFs. (c) E1A viral replication of wild-type and *Atg5*$^{-/-}$ MEFs infected with OBP-405 at an MOI of 1.0. The cells were incubated at 37 °C for the indicated periods, trypsinized and harvested for intracellular replication analysis at 2, 24, 48, 72 and 96 h. DNA purification was performed with the QIAmp DNA mini kit (Qiagen, Valencia, CA, USA). The E1A DNA copy number was determined by quantitative real-time PCR, using a Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) and 7500 real-time PCR systems (Applied Biosystems), as described previously.[9] The amount of viral E1A copy number is defined as the fold increase for each sample relative to that at 2 h. MEFs, mouse embryonic fibroblasts; MOI, multiplicity of infection

exhibited viral particles in the nucleus and cytoplasm, but they exhibited neither the chromatin condensation nor the DNA fragmentation that is the characteristic of apoptosis (Figure 2b). Interestingly, viral particles were observed inside autophagic vacuoles. These results suggested that OBP-405 infection initiated the autophagic process and that the autophagic vacuoles sequestered replicating OBP-405.

To assess the role of autophagy in OBP-405 infection, we inhibited the OBP-405-induced autophagy pharmacologically by using 3-methyladenine (3-MA);[18] the decreased viability of these cells was significantly reversed ($P<0.01$) (Supplementary Figures 4a and b). However, the inhibition of autophagy did not affect the increase in E1A copy number of OBP-405 (Supplementary Figure 4c). To exclude the possibility that the effects of 3-MA are independent of inhibition of autophagy, we inhibited autophagy specifically by using small interfering RNA (siRNA) directed against *autophagy-related gene 5* (*Atg5*), which is essential for autophagosome formation. Transfection with *Atg5* siRNA effectively inhibited OBP-405-induced autophagy and recovered the OBP-405-inhibited viability of U87-MG and U251-MG cells (Supplementary Figure 5a–c). Together, the results indicated that OBP-405 induced cell death through autophagy.

In addition, *Atg5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were significantly more resistant to OBP-405-induced death than the wild-type MEFs ($P<0.01$) (Figures 3a and b). This observation supported our results with 3-MA and *Atg5* siRNA. Similar to 3-MA, viral replication of OBP-405 did not differ significantly between the wild-type and *Atg5*$^{-/-}$ MEFs (Figure 3c).

The above observations prompted us to hypothesize that the antitumor effect of OBP-405 would be augmented by the combinatorial therapy with other autophagy-inducing agents. To test our hypothesis, we combined OBP-405 with rapamycin, an inhibitor of the mammalian target of rapamycin and the DNA-alkylating agent temozolomide (TMZ), both of which efficiently induce autophagy.[19,20] Rapamycin and TMZ not only enhanced OBP-405-induced autophagy *in vitro* but also synergized with OBP-405 to induce the death of glioblastoma cells (Figures 4a and b; Supplementary Figure 6). In contrast, TMZ or rapamycin did not alter viral replication (Figure 4c). Thus OBP-405 synergizes with autophagy-inducing agents to increase cell death *in vitro*.

To determine whether the *in vitro* effect of OBP-405 with TMZ or rapamycin translates to greater activity *in vivo*, we established intracranial tumors in nude mice. Compared with mice treated with Ad-LacZ, mice treated with OBP-405 lived significantly longer (mean survival = 27.5 vs 34.0 days; difference (95% confidence interval) = 6.0 (3.0–9.1) days; $P=0.0008$) (Figure 5a). Mice treated with TMZ also survived significantly longer (mean survival = 27.5 vs 37.0 days; difference = 10

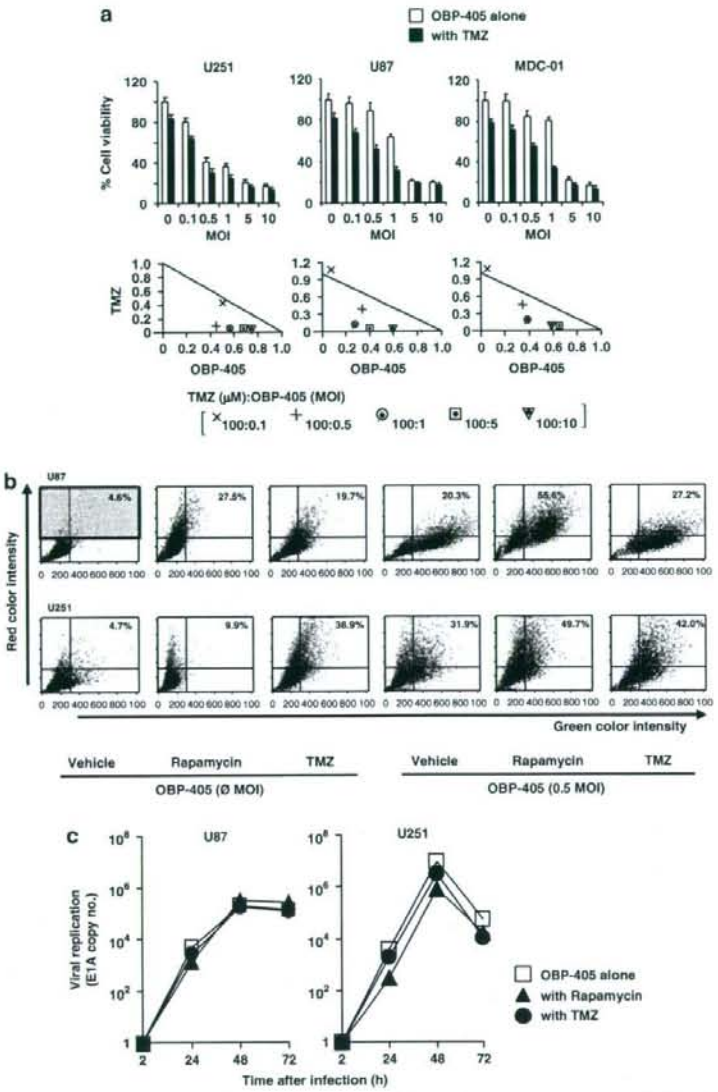

Figure 4 (**a**) Combined effects of OBP-405 with TMZ on glioblastoma cells. U251-MG, U87-MG and MDC-01 cells were infected with OBP-405 at an MOI of 0 to 10 in the presence of 100 μM TMZ (purchased from a pharmacy in The University of Texas MD Anderson Cancer Center) for 72 h for the WST-1 assay. The combined effect of OBP-405 with TMZ was analyzed with the combination index (CI)-isobologram using CalcuSyn software (Biosoft, Ferguson, MO, USA), as described previously.[19] In the isobologram, a plot on the diagonal line indicates that the combination is simply additive. A plot to the left under the line indicates that the combination is synergistic, whereas a plot to the right above the line indicates that it is antagonistic. Each plot represents values generated in at least three independent experiments for the simultaneous treatment of cells. (**b**) Development of acidic vesicular organelles (AVOs) in U87-MG and U251-MG cells infected with OBP-405 at an MOI of 0.5 in the presence of 1 nM rapamycin (Sigma-Aldrich) or 100 μM TMZ were stained with 1.0 μg ml^{-1} acridine orange as described previously.[10,11] Top of grid was considered as AVOs. (**c**) E1A viral replication of U87-MG and U251-MG cells infected with OBP-405 at an MOI of 0.5 alone or with 1 nM rapamycin or 100 μM TMZ over 72 h as described previously.[9] MOI, multiplicity of infection; TMZ, temozolomide.

(7.0–12) days; $P<0.001$), but RAD001-treated mice did not (mean survival = 27.5 vs 30.5 days; difference = 4 (−1 to 9) days; $P=0.14$). Strikingly, mice treated with OBP-405 and TMZ survived significantly longer than those treated with TMZ alone (mean = 53.0 vs 37.0 days; difference = 15.1 (7.2–23.1) days; $P=0.0015$), and mice treated with OBP-405 and RAD001 survived significantly longer than those treated with RAD001 alone (mean = 38.0 vs 30.5 days; difference = 9.3 (1.7–16.8) days, $P=0.021$). Finally, compared with results using OBP-405 alone, the survival time of mice was significantly prolonged by combination with TMZ (difference = 19 (11–26) days, $P=0.0001$) or RAD001 (difference = 7 (1–13) days, $P=0.025$).

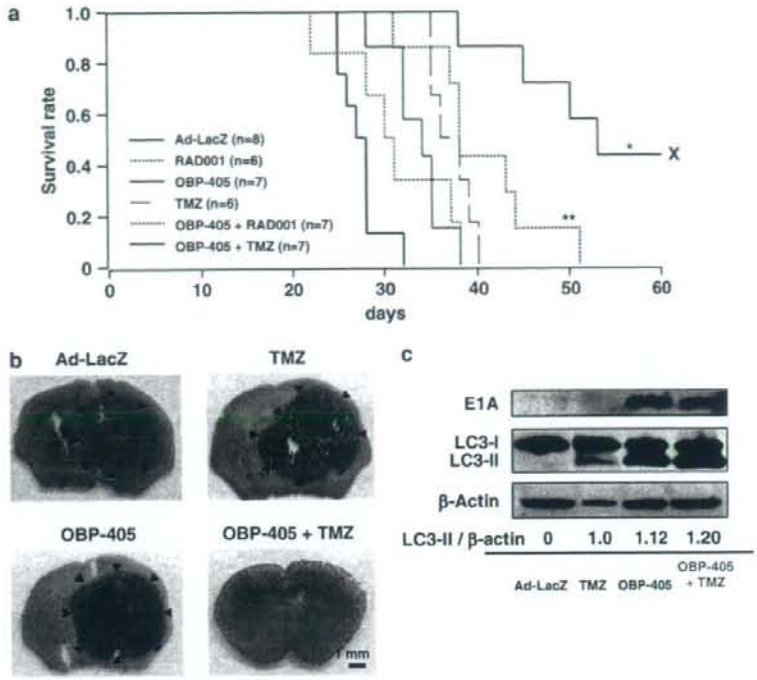

Figure 5 (**a**) Curves showing overall survival of mice bearing U87-MG intracranial tumors treated with Ad-LacZ, RAD001, TMZ, OBP-405, OBP-405 plus RAD001 or OBP-405 plus TMZ. The Kaplan–Meier method and pooled-variance two-tailed t-test were used to assess the statistical significance of differences in survival time; $^{*}P = 0.0015$ vs TMZ; $^{**}P = 0.021$ vs RAD001; 8- to 12-week-old female nude mice (Department of Experimental Radiation Oncology, MD Anderson Cancer Center) were used. The intracranial tumor model using U87-MG cells (5×10^5) was established as described previously.[3] Three days after the inoculation of U87-MG cells (day 0), the treatments were initiated as follows. On days 3, 5 and 7, through a 10 μl Hamilton syringe fitted with a 26-gauge needle connected to a microinfusion pump, Ad-LacZ (2.2×10^9 pfu ml^{-1}) or OBP-405 (2.2×10^9 pfu ml^{-1}) in 10 μl of sterile PBS was infused into the tumors through the screw guide at a depth of 3.5 mm from the skull. Two hundred microliters of TMZ (7.5 mg kg^{-1} in PBS with 5% dimethyl sulfoxide) was injected intraperitoneally five times a week for 2 weeks, and RAD001 (5 mg kg^{-1} in water, kindly supplied by Norvartis, Basel, Switzerland) was administered orally every day until the animals became moribund and were killed. (**b**) Hematoxylin and eosin-stained brain tissues of mice bearing intracranial U87-MG tumors treated with Ad-LacZ (day 32), TMZ (day 40), OBP-405 (day 38) or OBP-405 plus TMZ (day 60). Scale bar = 1 mm. (**c**) Western blots showing induction of autophagy and expression of E1A in intracerebral tumors treated with Ad-LacZ (day 28), TMZ (day 39), OBP-405 (day 35) or OBP-405 plus TMZ (day 53). Anti-LC3B antibody and anti-E1A antibody were used. The intensities of the amount of LC3-II bands were normalized by the bands' intensities of β-actin. PBS, phosphate-buffered saline; TMZ, temozolomide.

The intracranial tumors of mice treated with Ad-LacZ, OBP-405, TMZ or RAD001 alone grew extensively, with midlines shifted laterally. Strikingly, tumors were undetectable in brain tissue harvested from three mice treated with OBP-405 plus TMZ that survived 60 days after inoculation (Figure 5b). Hexon was detected in the intracranial tumor treated with OBP-405 plus TMZ (day 45) but not in the tumor treated with Ad-LacZ (day 28) (Supplementary Figure 7). This finding was supported by western blotting results showing detectable E1A protein expression in intracranial tumors treated with OBP-405 alone or with TMZ (Figure 5c). These results indicated that OBP-405 replicated and spread through the intracranial tumors but not through the normal brain tissues and supported the contention that the effect of OBP-405 is specific to tumors, likely due to the hTERT promoter activity. Then, we determined whether the induction of autophagy is detected under *in vivo* settings using an anti-LC3B-specific antibody. As shown in Figure 5c, the amount of LC3-II was higher in intracranial tumors of mice treated with TMZ, OBP-405 and OBP-405 plus TMZ than in Ad-LacZ-treated mice. These results indicated that autophagy was induced in intracranial tumors of mice as well as *in vitro* and that the extent of autophagy was enhanced by the combination treatment. However, intracranial tumors established from noninvasive glioblastoma cell lines may limit the clinical relevance of studies assessing the efficacy of novel therapies.[21] Therefore, we will further assess whether the treatment with OBP-405 plus TMZ or RAD001 prolong the survival of the mice carrying invasive intracranial tumors.[21]

In conclusion, we found that the fiber-modified hTERT-Ad OBP-405 has a marked antitumor effect on glioblastoma cells regardless of the cellular expression level of CAR. Moreover, autophagy-inducing agents (TMZ and rapamycin) increase the *in vitro* and *in vivo* antitumor activity of OBP-405 through the enhancement of autophagic pathway. A recent clinical study showed that TMZ had antitumor activity both as a single agent

and as adjuvant chemotherapy for patients with malignant gliomas, although its efficacy was modest.[22] Our study results might indicate a new way to treat glioblastomas with a combination of autophagy-inducing agents.

Acknowledgements

We thank Dr Noboru Mizushima for the GFP-LC3 expression vector and the wild-type and *Atg5−/−* MEFs. We also thank E Faith Hollingsworth for technical support and Elizabeth L Hess for editing the manuscript. This work was supported in part by Grant CA-088936 from the National Cancer Institute (to S Kondo), by a generous donation from the Anthony D Bullock III Foundation (to Y Kondo, R Sawaya and S Kondo) and the Institutional Core Grant CA-16672 High Resolution Electron Microscopy Facility, MD Anderson Cancer Center.

Grant support: This study was supported in part by National Cancer Institute Grants CA088936 and CA108558, a start-up fund from The University of Texas MD Anderson Cancer Center (SK), a generous donation from the Anthony D Bullock III Foundation (YK, RS and SK) and the cancer center support grant (CCSG)/shared resources of MD Anderson Cancer Center.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)

Bone Marrow Transplantation (2009), 1–7

www.nature.com/bmt

ORIGINAL ARTICLE

Intensive glucose control after allogeneic hematopoietic stem cell transplantation: a retrospective matched-cohort study

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Some studies have shown that intensive glucose control (IGC) improves outcome in the intensive care unit setting. However, it is the benefit of IGC in hematopoietic SCT (HSCT) that is not well defined. Between June 2006 and May 2007, IGC was maintained prospectively after allogeneic HSCT and clinical outcomes were compared with a cohort matched for conditioning regimen, source of stem cells, age and relation to donor. A stratified Cox regression model was used. There were no significant differences in baseline clinical characteristics. The median age was 43.5 years in both groups. The primary diagnosis was a hematologic malignancy. Patients in the IGC group had a lower glucose level (least-square mean, 116.4 vs 146.8 mg per 100 ml, $P<0.001$) compared to the standard glucose control group. The incidences of documented infections and bacteremia were significantly lower in the IGC group (14 vs 46%, $P=0.004$, 9 vs 39%, $P=0.002$, respectively). IGC tended to reduce the incidence of renal dysfunction (19 vs 37%, $P=0.36$) and the elevation of C-reactive protein (18 vs 38%, $P=0.13$). This study suggests that IGC has may have a beneficial effect after HSCT. IGC should be evaluated further in a large prospective, randomized study.

Bone Marrow Transplantation advance online publication, 19 January 2009; doi:10.1038/bmt.2008.431

Keywords: intensive glucose control; allogeneic transplantation; hyperglycemia; C-reactive protein

Introduction

Previous studies showed that intensive glucose control (IGC), in which the target blood glucose level was set within 80–110 mg per 100 ml, reduced infections, dysfunction of organs including the liver and kidney and mortality compared to patients who received standard glucose control.[1,3] Although these results have been confirmed in several subsequent studies,[4–7] the precise mechanism that underlies this association is unclear. In animal models, it has been shown that insulin itself has a direct inhibitory effect on the inflammation process.[8,9] However in human studies, it has been suggested that these benefits could be directly attributed to IGC rather than to any pharmacological activity of administered insulin *per se*.[3,4]

Recipients of allogeneic hematopoietic SCT (HSCT), which is the most drastic therapeutic modality in patients with hematological malignancies, often suffer from serious complications including infectious diseases, GVHD and multiple organ failure. They are also at higher risk of hyperglycemia because of the use of steroids for the treatment of GVHD, the use of total parenteral nutrition (TPN), immunosuppressive drugs and infectious complications,[10,11] which makes them further susceptible to numerous serious complications including infectious diseases and multiple organ failure.[12–14] Our group previously reported that hyperglycemia during neutropenia was associated with an increased risk of acute GVHD and nonrelapse mortality (NRM) after myeloablative allogeneic HSCT,[15] and that hyperglycemia during neutropenia was associated with a higher incidence of subsequent acute GVHD. It is well known that an increase in the levels of circulating cytokines may aggravate hyperglycemia, and hyperglycemia itself could increase the levels of cytokines. This vicious cycle could lead to elevated cytokine levels, which could lead to subsequent acute GVHD. With this background, it can be hypothesized that IGC would reduce the incidence of infectious diseases, acute GVHD and organ dysfunctions after allogeneic HSCT. Therefore, we prospectively investigated the effect of IGC after allogeneic HSCT, and compared the clinical outcomes to those in a matched cohort to address whether IGC following allogeneic HSCT could improve the clinical course of patients, that is, reduction of infectious diseases and organ dysfunction, as has been shown in the intensive care unit (ICU) setting.

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Received 22 May 2008; revised 28 October 2008; accepted 21 November 2008

Patients and methods

Patients

From June 2006 to May 2007, a total of 73 patients received allogeneic HSCT at the National Cancer Center Hospital (Tokyo, Japan); 60 patients were eligible for participation in this trial. Finally, 22 patients (36.7%) were enrolled in this IGC study to keep the blood glucose level at 80–110 mg per 100 ml, as shown in Figure 1.

Study center and organization

The National Cancer Center Hospital in Tokyo holds 600 beds. The transplant team consists of 4 full-time physicians and 26 nursing staff who oversee 26 beds in the HSCT, and the entire ward is covered by high-efficiency particulate air-filters. We regularly perform 90–120 transplants per year: 80% allogeneic and 20% autologous.

Study design

This was a case–control study to investigate the clinical benefits of comprehensive nutritional support including IGC and parenteral nutrition (PN) management, which was approved by the Institutional Review Board. A matching control group was selected among patients who received HSCT from January 2002 to March 2007 (ratio of 1:2 compared to the study group) according to the following criteria: (1) conditioning regimen (conventional myeloablative or reduced intensity), (2) source of stem cells (BM, peripheral blood or cord blood), (3) age and (4) source of donor (related or unrelated). Criteria (1–4) were essential for inclusion. As a result, 42 matched controls were selected, and a total of 64 patients were subjected to further analysis (Table 1).

Exclusion criteria

Exclusion criteria were as follows: (1) patients who received a reduced-intensity conditioning regimen for an HLA-matched related donor, as we applied GVHD prophylaxis without short-term MTX in this setting, and they had much less need for TPN and less need for intense glucose control,[16] (2) those with a poor performance status (Eastern Cooperative Oncology Group) $\geqslant 2$, (3) those with uncontrolled infectious diseases at the beginning of the conditioning regimen and (4) those with preexisting neutropenia. We previously reported that the incidence of severe stomatitis (Common Terminology Criteria for Adverse Events (CTCAE) grade (3) was 0% after reduced-intensity SCT (RIST) from a related HLA-matched donor.[16] In this situation, the need for TPN and the incidence of hyperglycemia were quite low, compared to RIST from an unrelated donor, which included additional low-dose TBI or antithymocyte globulin (ATG) and short-term MTX or conventional SCT with a myeloablative regimen. Hence, we only included patients who received a RIST regimen from an unrelated donor, who had a higher probability of glucose-control intervention, to evaluate the beneficial effects of IGC.

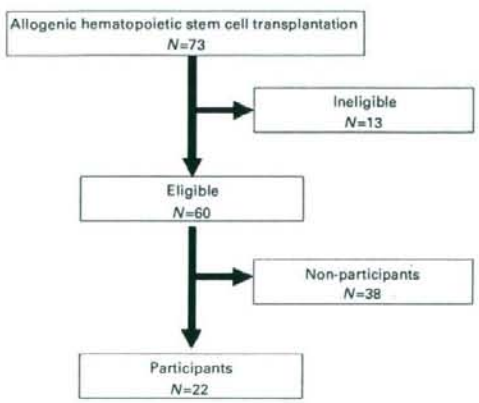

Figure 1 Trial profile.

Table 1 Patients' characteristics

Variable	*N (%)/median (range)*		*P-value*
	Intensive glucose control (n = 22)	*Standard glucose control* (n = 42)	
Age (years)	43.5 (17–64)	43.5 (20–66)	
<40	8 (36)	18 (43)	0.62
≥40	14 (64)	24 (57)	
Sex			
Male	9 (41)	22 (52)	0.38
Female	13 (59)	20 (48)	
Disease risk[a]			
Standard	6 (27)	16 (38)	0.39
High	16 (73)	26 (62)	
Conditioning			
CST	14 (64)	27 (64)	
BU/CY	9 (40)	18 (43)	
CY/TBI (12 Gy)	4 (18)	6 (14)	
Other	1 (5)	3 (7)	
RIST	8 (36)	15 (36)	0.96
2CdA/BU	1 (5)	1 (2)	
Flu/BU	7 (32)	14(33)	
Low-dose TBI (2–4 Gy)	3 (14)	7 (17)	
Low-dose ATG	5 (23)	10 (24)	0.92
GVHD prophylaxis			
Cyclosporin-based	7 (32)	27 (64)	
Tacrolimus-based	15 (68)	15 (36)	0.01
Short-term MTX (+)	22 (100)	40 (95)	0.30
Relation to donor			
Related	6 (27)	12 (29)	
Unrelated	16 (73)	30 (71)	0.91
Stem cell source			
Bone marrow	15 (68)	30 (71)	
PBSC	5 (23)	10 (24)	
Cord blood	2 (9)	2 (5)	0.19
HLA match			
Match	11 (50)	28 (67)	
Mismatch	11 (50)	14 (33)	0.19

Abbreviations: ATG = antithymocyte globulin; 2CdA = cladribine; CST = conventional stem cell transplantation; Flu = fludarabine; RIST = reduced-intensity stem cell transplantation.
[a]Standard-risk patients included those with acute leukemia in first complete remission, chronic leukemia in first chronic phase, MDS in refractory anemia and NHL in complete remission, and the remaining patients were categorized as high risk.

Transplantation procedures

Forty-one patients received a myeloablative conditioning regimen that included BU (orally 4 mg/kg per day × 4 days or i.v. 3.2 mg/kg per day × 4 days) plus CY (60 mg/kg per day × 2 days, $n=27$), CY plus 12 Gy TBI ($n=10$) or other ($n=4$). Twenty-three patients received a reduced-intensity conditioning regimen that included fludarabine (30 mg/m^2 per day × 6 days) or cladribine (0.11 mg/kg per day × 6 days) plus BU (oral 4 mg/kg per day × 2 days or i.v. 3.2 mg/kg per day × 2 days). Low-dose TBI (2 or 4 Gy, $n=10$) and/or low-dose ATG (total dose 5–10 mg/kg ATG-F or 5 mg/kg thymoglobulin, $n=15$) were added. GVHD prophylaxis included CYA- ($n=13$) and tacrolimus-based regimens ($n=51$), with an additional short course of MTX. G-CSF was administered in all patients from day +6 after transplantation until engraftment. Most patients received ciprofloxacin (200 mg orally three times daily) for bacterial prophylaxis after the beginning of the conditioning regimen until neutrophil engraftment. Fluconazole (100 mg once daily) was administered for fungal prophylaxis after the beginning of the conditioning regimen. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and VZV after the beginning of the conditioning regimen until immunosuppressive agents were discontinued. Prophylaxis against *Pneumocystis jiroveci* infection consisted of trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day −3 of transplantation, and from day +28 until day +180 or the cessation of immunosuppressive agents. Patients who developed fever during the neutropenic period were treated with cefepime or other cephalosporin, and additional agents including vancomycin, aminoglycosides and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the ANC exceeded 0.5×10^9 per l.

Glucose management protocol

In the IGC group, the blood glucose level was routinely tested every morning to adjust the dose of insulin so as to keep the level within the range of 80–110 mg per 100 ml. Owing to the presence of fewer nursing staff in the HSCT unit than in the ICU, we replaced the continuous infusion of insulin with the addition of Humulin R to the bottle of PN to control the glucose level within the target range. In TPN, we universally added at least 1 unit of Humulin R per 10 g glucose. In patients who had an elevated blood glucose level, we also added Humulin R to the bottle of PN. We monitored the glucose level at least once a day in the morning as long as the level remained within the target range of 80–110 mg per 100 ml. When the glucose level became elevated, we increased the frequency of monitoring up to 2–4 times daily. In most patients, we adjusted the dose of insulin added to the bottle of PN as described in Table 2. When the blood glucose level was >180 mg per 100 ml or the dose of insulin was high, we manually adjusted the dose of Humulin R and administered insulin subcutaneously according to the attending physician's discretion. S.c. insulin administration usually consisted of 3–5 units at the beginning, and, if this was insufficient, the dose was manually adjusted by 2–4 units. When the patients received high-dose systemic steroid such as methylprednisolone 1–2 mg/kg per day for GVHD, we used the preprandial s.c. injection of insulin Aspart (NovoRapid) three times daily to avoid postprandial hyperglycemia and adjusted the dose according to the amount of food intake and the postprandial glucose level. When patients exhibited nausea, anorexia or vomiting, the amount of food intake became unstable. In such situations, insulin Aspart was injected immediately after the meal. When food intake was <50%, the dose was reduced or discontinued. Routine glucose monitoring was continued until PN was stopped, whereas the blood glucose level was maintained within the target range. Daily caloric intake was calculated by the dietitians. We tried to maintain oral intake as much as possible by using a suitable diet in jelly or liquid form. A dietitian adjusted the dose of supplemental PN to maintain the total caloric intake over 1.0 × basal energy expenditure (BEE), and if the glucose level was stable, the nutritional intake could be increased up to 1.5 × BEE. The glucose concentration in PN was usually started at 7.5% glucose as supplemental PN. The concentration was gradually increased to 12%, and, if necessary, this was further increased up to 18% to meet the target caloric intake. A lipid emulsion was also used to supply 10–30% of total caloric intake. The minimal total nutritional intake was set at 1.0 × BEE because a retrospective analysis at our institute showed that caloric intake of more than 1.0 × BEE was not associated with clinically significant wt loss.[17] To improve the glucose control, this level was set to be slightly lower

Table 2 Protocol for adjustment of Humulin R

Glucose level (mg per 100 ml)	Adjustment of Humulin R
BS ≤ 40	i.v. 50% glucose 20 ml and recheck the glucose level Reduce the dose of Humulin R to 40–60% of the original dose
40 ≤ BS < 60	i.v. 50% glucose 20 ml and recheck the glucose level Reduce the dose of Humulin R to 60–80% of the original dose
60 ≤ BS < 80	i.v. 50% glucose 20 ml and recheck the glucose level Reduce the dose of Humulin R to 70–90% of the original dose
80 ≤ BS ≤ 110	No change
110 < BS < 130	Increase the dose of Humulin R to 110–120% of the original dose
130 ≤ BS < 150	Increase the dose of Humulin R to 120–130% of the original dose
150 ≤ BS < 180	Increase the dose of Humulin R to 130–150% of the original dose
BS ≥ 180	Manually adjust the dose of Humulin R combined with sliding subcutaneous insulin administration

Abbreviation: BS = blood sugars.

than the recommendation in the HSCT setting (1.3–1.5 × BEE,[18]). There are two beneficial aspects of this protocol: we could maintain the minimal caloric intake with supplemental PN and we could immediately start insulin as required after the introduction of PN. The SGC group was managed without a specific protocol for nutrition practice and glucose control, although we routinely monitored blood glucose at least three times weekly to avoid severe hyperglycemia (blood glucose >200 mg per 100 ml).

Outcome measures

Serially monitored glucose values were compared between the IGC group and the SGC group. We also analyzed the association between the mean glucose level during monitoring and the infection rate in both the SGC group and IGC group. Mean glucose levels were estimated for each patient and were categorized as follows: 80–110, 111–140, 141–179 and >180. Glycemic variability, defined as the s.d. of the mean glucose value, was also analyzed. The outcome measures were time to the occurrence of documented infectious complications within 100 days after HSCT, time to each organ dysfunction defined as described below, time to grades II–IV and grades III–IV acute GVHD and time to NRM. These were calculated from the date of the start of the conditioning regimen. Organ dysfunction was defined with reference to van den Berghe[5,7] as follows: (1) hypercreatininemia; serum creatinine level ⩾2.0 mg per 100 ml or more than twice the baseline, (2) hyperbilirubinemia; serum total bilirubin level ⩾2.0 mg per 100 ml and (3) increased inflammatory markers; serum C-reactive protein (CRP) level ⩾15 mg per 100 ml. In our institute, the CRP level was routinely monitored at least three times a week, as we previously reported that the preengraftment CRP level may predict a subsequent occurrence of acute GVHD and NRM after allogeneic HSCT.[19] These results suggested that CRP might be useful not only as a marker of infectious diseases but also as a surrogate marker for produced cytokines. Therefore, the serial changes of CRP level were compared between the two groups. Acute GVHD was graded by the consensus criteria.[20]

Statistical analyses

Baseline characteristics were summarized using descriptive statistics. The Student's t, χ^2 and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. The probability of documented infectious complications and organ dysfunction were calculated using Kaplan–Meier estimates. A stratified Cox regression model, which accounts for the matched-cohort design, was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). On the basis of 64 patients, the study has an approximately 80% power to detect a HR of 0.5 for documented infections. The glucose values, measured repeatedly, were compared between groups using a repeated-measure analysis with a linear mixed-effect model. A level of $P<0.05$ was defined as statistically significant. All P-values are two-sided. All analyses were performed using SAS version 9.1.3 (Cary, NC, USA).

Results

Patient characteristics

Table 1 lists the patients' clinical and transplantation characteristics. Patients and transplantation characteristics were well balanced with the application of matching criteria. Nevertheless, in the IGC group, more patients received tacrolimus for GVHD prophylaxis (68 vs 36%, $P=0.01$) and more had a previous transplantation (32 vs 7%, $P=0.01$). The median duration of follow-up in surviving patients was 299 days (range, 78–607 days) in the IGC group and 1146 days (range, 329–1774 days) in the SGC group.

Glycemic control

Duration of monitoring and number of tests. The median duration of glucose monitoring and intervention in the IGC group was 38 days (range, 24–70 days) after the start of the conditioning regimen. The total number of glycemic monitorings was 867 and 1094 in the SGC group and IGC group, respectively.

Mean values and distribution of values. Patients in the IGC group had a lower glucose level (least-square mean, 116.4 vs 146.8 mg per 100 ml, $P<0.001$) than the SGC group. The trend of the glucose value is shown in Figure 2a. All glycemic results for the SGC and IGC groups were stratified into six levels: <40, 40–79, 80–110, 111–140, 141–179 and ⩾180, as shown in Figure 2b.

Hypoglycemia

In the IGC group, the incidence of mild hypoglycemia (CTCAE grades 1–2, glucose level 40–69 mg per 100 ml) was significantly higher than that in the SGC group (11 vs 3 patients, $P<0.001$). Although one patient (4.5%) in the IGC group who was diagnosed as type 2 diabetes mellitus developed severe hypoglycemia (CTCAE grade 3, glucose level 30–39 mg per 100 ml) with faintness, no patient developed seizure or loss of consciousness.

Glycemic variability

The mean glycemic variability in the SGC group and IGC group was 37.2 mg per 100 ml (range, 10.1–121.7 mg per 100 ml) and 27.5 mg per 100 ml (range, 11.3–46.6 mg per 100 ml), respectively, and glycemic variability in the IGC group tended to be lower than that in the SGC group ($P=0.07$).

TPN and insulin dosing

The percentage of patients who received TPN was 60% (25 patients) and 77% (17 patients) in the SGC group and the IGC group, respectively. The mean duration of TPN was 9 days (range, 0–35) and 13 days (range, 0–38) in the SGC group and IGC group, respectively. There was a tendency for more patients in the IGC group to receive TPN compared to the SGC group, but this difference was not statistically significant. The mean maximal dose of insulin (median (range), 51 (0–100) vs 2 (0–110) IU, $P<0.001$) and the mean maximal dose of insulin per 1 g parenteral glucose

were significantly higher in the IGC group (median (range), 0.22 (0–0.71) vs 0.003 (0–0.4) IU/g glucose, $P<0.001$).

Infections

Table 3 summarizes the results. In the IGC group, dramatically fewer patients developed documented infections within 100 days compared to the SGC group, as shown in Figure 3.

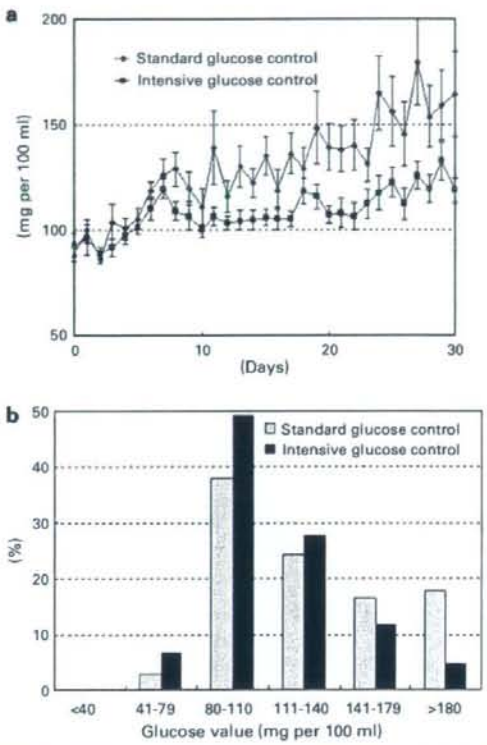

Figure 2 Serial changes in the mean glucose level in the intensive glucose control (IGC) and standard glucose control (SGC) groups. Values are mean + s.e. (**a**). The distribution of the glucose values in IGC and SGC is shown as a histogram (**b**).

Relation to mean glucose level

We also analyzed the association between the mean glucose level during monitoring and the infection rate in both the SGC and IGC groups. The incidence of infection was 34, 17, 67 and 40%, respectively, with mean glucose levels of 80–110, 111–140, 141–179 and ⩾180. When we compared a lower glucose-level group (mean glucose level of 80–140) with a higher glucose-level group (mean glucose level of >140), the incidence of infection was significantly higher in the latter group (28 vs 57%, $P=0.042$). When we assessed only patients with a lower glucose level, the IGC group tended to show a lower incidence of infectious diseases than the SGC group (14 vs 41%, $P=0.061$).

Relation to glycemic variability

We also analyzed the association between glycemic variability and the infection rate. The mean glycemic variability in patients with and without infection was 34.6 mg per 100 ml (range, 10.5–121.7 mg per 100 ml) and 33.3 mg per 100 ml (range, 10.1–110.6 mg per 100 ml), respectively, with no significant difference. As the importance of glycemic variability could vary among patients

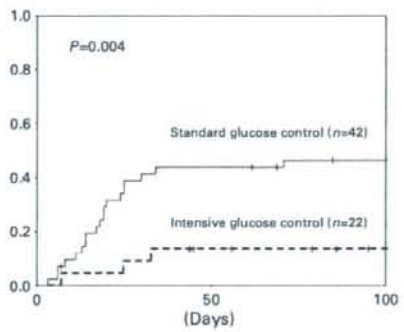

Figure 3 Probability of documented infections in the IGC and SGC groups.

Table 3 Incidence of infectious diseases and organ dysfunction

Variable	N (%)/median (range)			
	Intensive glucose control n = 22 (%)	*Standard glucose control* n = 42 (%)	*HR (95% CI)*	P-value
Documented infection	13	46	0.17 (0.04–0.75)	0.004
Bacteremia	9	39	0.10 (0.01–0.74)	0.002
Organ dysfunction				
Hypercreatininemia[a]	19	37	0.60 (0.19–1.88)	0.36
Hyperbilirubinemia[b]	28	31	1.05 (0.38–2.91)	0.93
Increased inflammatory markers[c]	18	38	0.45 (0.15–1.37)	0.13

Abbreviations: CI = confidence interval.
[a]Serum creatinine level ⩾2.0 mg per 100 ml or more than twice of baseline.
[b]Serum bilirubin level ⩾2.0 mg per 100 ml.
[c]Serum C-reactive protein level ⩾15 mg per 100 ml.

with different mean glucose levels,[21] we divided the patients into two groups based on mean glucose level 80–140 or 140+ and then determined whether glycemic variability was associated with an increased incidence of infections. However, there was no significant association between glycemic variability and the incidence of infections in both groups.

CRP levels

Figure 4 shows serial changes in the CRP level. Even though there was no difference in the CRP level between the two groups at the beginning of the conditioning regimen, the CRP level was significantly elevated in the SGC group compared to that in the IGC group 15 days after the beginning of the conditioning regimen, and this trend continued up to 40 days ($P<0.05$). The maximal CRP level during the neutropenic period in the IGC group was significantly lower than that in the SGC group (median (range), 6.9 (0.9–16.3) vs 11.5 (1.6–37.3), $P=0.007$).

Other clinical outcomes

The probability of grades II–IV acute GVHD within 100 days was 28 and 37% in the IGC and SGC groups (HR 1.05, 95% CI 0.38–2.91, $P=0.93$). The incidences of grades III–IV acute GVHD and NRM within 100 days were low in both groups (one and two patients, and one and one patient, in the IGC and SGC groups, respectively).

Discussion

This is the first study to evaluate the outcomes in allogeneic HSCT patients who were treated with a glucose management protocol. A salient finding of this study is that the incidence of documented infections, especially the incidence of bacteremia, was significantly lower in the IGC group than in the SGC group, as in a previous report in the ICU setting.[1] Moreover, there tended to be fewer organ dysfunctions in the IGC group, albeit this difference was not statistically significant. Furthermore, the CRP level, which might be a surrogate marker for produced cytokines,[19] was significantly lower in the IGC group than in the SGC group, as shown in Figure 4. Even though this study did not have enough power to detect a decrease in acute GVHD and NRM, it could be anticipated that IGC could reduce the CRP level, which would lead to a reduced incidence of acute GVHD and NRM.

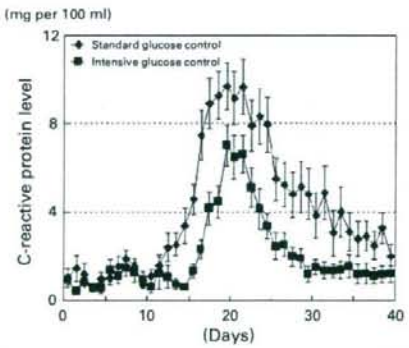

Figure 4 Serial change in the CRP level in the IGC and SGC groups. Values are mean + s.e.

This study has several limitations. One limitation is that only 64 patients were analyzed with no sufficient power to demonstrate any statistically significant changes in the incidences of organ dysfunctions, which was similar to the result in a previous report in the ICU.[1,2] An additional limitation was that the control of the glucose level could be suboptimal. This could be because of the glucose control protocol, which included monitoring of glucose level and the administration of insulin. With regard to the administration of insulin, we replaced the continuous infusion of insulin with the addition of Humulin R to the bottle of PN to control the glucose level within the target range because of the presence of fewer nursing staff in the HSCT unit than in the ICU. This could delay the normalization of hyperglycemia. Even though severe hyperglycemia (>180 mg per 100 ml) was reduced, a glucose value within the normal range (80–110 mg per 100 ml) could be achieved in only 49% of the IGC group as shown in Figure 1b. From a methodological point of view, it might be inappropriate to simply count the number of glucose value measurements, as patients with hyperglycemia were monitored more frequently, as defined in this protocol. Furthermore, as the mode of glucose monitoring was quite different between the IGC group and the SGC group, it could be inappropriate to compare the glucose values. A future protocol should include a more appropriate monitoring of glucose level and administration of insulin system that assures the fine tuning of glucose levels within the target range. Finally, there was a possible selection bias that may have affected the results, as this study was not a randomized-control study and there were many nonparticipants. However, the incidence of documented infections in nonparticipants within 100 days after allogeneic HSCT was 42%. Therefore, the reduction in the incidence of documented infections in the IGC group could not simply be explained by other causes such as the selection of antibiotics or catheter management.

With these limitations in mind, we took several steps to improve the quality of the study. First, we carefully matched patients and transplantation characteristics. Second, the IGC strategy was applied prospectively. Third, the low rate of patients who developed clinically significant hypoglycemia should be emphasized. As previously reported, the IGC procedure becomes very difficult in the medical ICU, especially in patients who have sepsis, a high APACHE score or mechanical ventilation.[1,2,22,23] The low rate of hypoglycemia could be because the medical acuity of our patients were relatively mild compared to those of patients in the medical ICU. Moreover, patients undergoing HSCT are younger and might have better β-cell function. The low rate of hypoglycemia could be important for maximizing the benefit of IGC because severe hypoglycemia could be associated with an increased risk of mortality.[23]

The biological plausibility of the intervention should be discussed. The reduction in infectious diseases by IGC may reflect the deleterious effects of hyperglycemia on macrophage or neutrophil function or insulin-induced protective effects on mucosal and skin barriers.[24 27] The improvement of innate immunity could be quite important, especially during the period of granulocytopenia after allogeneic HSCT. The protection of mucosal tissues could reduce bacterial translocation, which might lead to a reduced incidence of sepsis.

In conclusion, our results suggest that prospective IGC reduced the incidences of infectious diseases and organ dysfunction after allogeneic HSCT. To confirm these findings, a larger, prospective randomized-controlled trial is warranted.

Acknowledgements

We thank the medical, nursing, data processing, laboratory and clinical staffs at the National Cancer Center Hospital for their important contributions to this study through dedicated care of the patients. We are indebted to Y Iisaka for assisting with data collection. We also thank S Saito for helping to prepare the article. This study was supported in part by grants from the Ministry of Health, Labor and Welfare, Japan.

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Biology of Blood and Marrow Transplantation 14:510-517 (2008)

1083-8791/08/1405-0001$32.00/0
doi:10.1016/j.bbmt.2008.02.008

Preengraftment Serum C-Reactive Protein (CRP) Value May Predict Acute Graft-versus-Host Disease and Nonrelapse Mortality after Allogeneic Hematopoietic Stem Cell Transplantation

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Received September 29, 2007; accepted February 10, 2008

ABSTRACT

In a mouse model, inflammatory cytokines play a primary role in the development of acute graft-versus-host disease (aGVHD). Here, we retrospectively evaluated whether the preengraftment C-reactive protein (CRP) value, which is used as a surrogate marker of inflammation, could predict posttransplant complications including GVHD. Two hundred twenty-four adult patients (median age, 47 years; range: 18-68 years) underwent conventional stem cell transplantation (CST, n = 105) or reduced-intensity stem cell transplantation (RIST, n = 119). Patients were categorized according to the maximum CRP value during neutropenia: the "low-CRP" group (CRP < 15 mg/dL, n = 157) and the "high-CRP" group (CRP ≥ 15 mg/dL, n = 67). The incidence of documented infections during neutropenia was higher in the high-CRP group (34% versus 17%, $P = .004$). When patients with proven infections were excluded, the CRP value was significantly lower after RIST than after CST ($P = .017$) or after related than after unrelated transplantation ($P < .001$). A multivariate analysis showed that male sex, unrelated donor, and HLA-mismatched donor were associated with high CRP values. The high-CRP group developed significantly more grade II-IV aGVHD ($P = .01$) and nonrelapse mortality (NRM) ($P < .001$), but less relapse ($P = .02$). The present findings suggest that the CRP value may reflect the net degree of tissue damage because of the conditioning regimen, infection, and allogeneic immune reactions, all of which lead to subsequent aGVHD and NRM.

KEY WORDS

C-reactive protein • Allogeneic transplantation • Acute graft-versus-host disease • Nonrelapse mortality

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is associated with high treatment-related mortality (TRM) because of acute graft-versus-host disease (aGVHD) and infections [1,2]. Inflammatory cytokines, for example, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 [3-11], are produced following conditioning and play a primary role in activating T cells, leading to GVHD and resultant target tissue destruction [12,13]. An acute-phase protein, C-reactive protein (CRP), is produced by hepatocytes downstream of IL-6 [14] and is widely used as a reliable surrogate marker of infectious diseases [15-19]. This process is further stimulated by other cytokines including TNF-α [12,13]. After allogeneic HSCT, the elevation of CRP was observed with infectious complications, but not in uncomplicated aGVHD [8,20]. On the other hand, elevation of CRP has been shown to be associated with TRM [21-24]. Nevertheless, these previous studies adopted the sporadic measurement of CRP and mostly focused on patients undergoing conventional HSCT (CST) with a myeloablative regimen. It has been hypothesized that recently developed reduced-intensity HSCT (RIST) decreases regimen-related toxicities and, hence, may reduce inflammation

that augments the subsequent allogeneic immune reaction to induce GVHD and nonrelapse mortality (NRM).

In this study, the correlation between the preengraftment CRP value and subsequent clinical events was analyzed to test whether high CRP reflected the degree of tissue damage because of the conditioning regimen, infections, and allogeneic immune reactions and/or inflammation, all of which could contribute to subsequent aGVHD and NRM.

MATERIALS AND METHODS

Patient Characteristics

The data from a cohort of 224 consecutive adult patients with hematologic malignancies, who were treated between January 2002 and July 2006 at the National Cancer Center Hospital (NCCH, Tokyo, Japan), were reviewed retrospectively. Patients who developed graft failure or who had previous allogeneic transplantation were excluded. Their characteristics are listed in Table 1. The median age of the patients was 47 years (range: 18-68 years), and their diagnosis included acute myeloid leukemia (AML, n = 94), acute lymphoblastic leukemia (ALL, n = 23), non-Hodgkin lymphoma (NHL, n = 62), myelodysplastic syndrome (MDS, n = 27) and chronic myeloid leukemia (CML, n = 12). Standard risk included acute leukemia in first complete remission, chronic leukemia in the first chronic phase, MDS in refractory anemia, and NHL in complete remission, with the rest of the patients categorized as a high-risk group. Stem cell sources used for transplantation included bone marrow (BM, n = 108), peripheral blood stem cells (PBSC, n = 98) and cord blood cells (CB, n = 18). One-hundred five patients received a CST regimen including total-body irridiation (TBI)-based (n = 50) and non-TBI-based busulfan-containing regimens (n = 55), whereas 119 patients received a RIST regimen including fludarabine or cladribine plus busulfan or melphalan (Table 1). CMV serostatus was positive in 157 patients and negative in 67 patients. The median age of the patients was 49 years in the high-CRP group (range: 19-67) and 47 years in the low-CRP group (range: 18- 68). Written informed consent was obtained according to the Declaration of Helsinki.

Transplantation Procedures

GVHD prophylaxis included cyclosporine- (n = 174) and tacrolimus-based regimens (n = 50), with an additional short course of methotrexate (MTX) in 165 patients. Granulocyte colony-stimulating factor (G-CSF) was administered in all patients from day +6 of transplantation until engraftment was confirmed. Most patients received ciprofloxacin (200 mg orally 3 times daily) for bacterial prophylaxis until neutrophil engraftment. Fluconazole (100 mg once daily) was administered for fungal prophylaxis. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and varicella zoster virus until the cessation of immunosuppressive agents. Prophylaxis against *Pneumocystis jiroveci* infection was provided with trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day −3 of transplantation, and from day +28 until day +180 or the discontinuation of immunosuppressive agents. Patients with fever during the neutropenic period were treated with cefepime, and additional agents including vancomycin and aminoglycosides, and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the absolute neutrophil count exceeded 0.5×10^9/L. In our institute, the CRP level was serially measured as part of our routine checkup at least 3 times a week. Hence, all serially admitted patients were subjected to this analysis. Every patient had started CRP measurement

Table 1. *Patients' Characteristics*

Variable	N (%)/ Median Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	P Value
Age (year)	47 (18-68)	49 (19-67)	.85
<40	53 (34)	26 (39)	
≥40	104 (66)	41 (61)	.47
Patient sex			
Male	84 (54)	48 (72)	
Female	73 (46)	19 (28)	.01
Donor sex			
Male	81 (52)	30 (45)	
Female	76 (48)	37 (55)	.35
CMV serostatus			
Positive	140 (89)	64 (96)	
Negative	17 (11)	3 (4)	.20
Disease risk			
Standard	35 (22)	17 (25)	
High	122 (78)	50 (75)	.62
Conditioning			
CST	72 (47)	33 (50)	
RIST	85 (53)	34 (50)	.64
GVHD prophylaxis			
Cyclosporin-based	122 (78)	52 (78)	
Tacrolimus-based	35 (22)	15 (22)	.99
Short term MTX (+)	107 (68)	58 (87)	.004
Relation to donor			
Related	94 (60)	13 (19)	
Unrelated	63 (40)	54 (81)	<.001
Stem cell source			
Bone marrow	63 (40)	45 (67)	
PBSC	87 (55)	11 (16)	
Cord blood	7 (5)	11 (16)	<.001

CRP indicates C-reactive protein; CMV, cytomegalovirus; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; GVHD, graft-versus-host disease; MTX, methotrexate; PBSC, peripheral blood stem cells; HLA, human leukocyte antigen.

Table 2. *Comparison of Preengraftment CRP Value Stratified According to the Conditioning Regimen (CST versus RIST) and the Relation to Donor (Related versus Unrelated)*

Patients' Characteristics	CRP Value Median (Range)
All patients	8.9 (0.1-42.7)
CST	10.5 (0.3-31.3)*
Related	9.4 (0.6-30.0)†
Unrelated	10.6 (0.3-31.3)†
RIST	6.2 (0.1-42.7)*
Related	1.6 (0.1-9.7)‡
Unrelated	16.2 (0.5-42.7)‡

CST indicates conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation.
*$P = .017$.
†$P = .33$.
‡$P < .001$.

before the initiation of the conditioning regimen, and the median pretransplant CRP level was 0.3 mg/dL (range: 0.0-20.5 mg/dL). The median maximum CRP value during neutropenia was 8.9 mg/dL (0.1-42.7, Table 2).

The "maximum CRP level" was determined by measuring both the CRP level and the neutrophil count, as shown in the example in Figure 1A. The average number of levels assessed for each patient was 8 (range: 1-30). The median day of the maximum CRP level was day 10 of HSCT (range: 0-25), with 79% of patients developing this in later days (≥8 days). The patients were categorized according to the maximum CRP level after the threshold CRP level was determined following a preliminary analysis of the maximum CRP level after CST using an ROC curve analysis (data not shown). The "low-CRP" group (CRP <15 mg/dL) included 157 patients and the "high-CRP" group (CRP ≥15 mg/dL) included 67 patients.

Statistical Analyses

The primary endpoint of this study was the occurrence of grade II-IV and grade III-IV aGVHD, according to the Consensus Criteria [25]. The secondary endpoints were overall survival (OS) and nonrelapse mortality (NRM). Standard descriptive

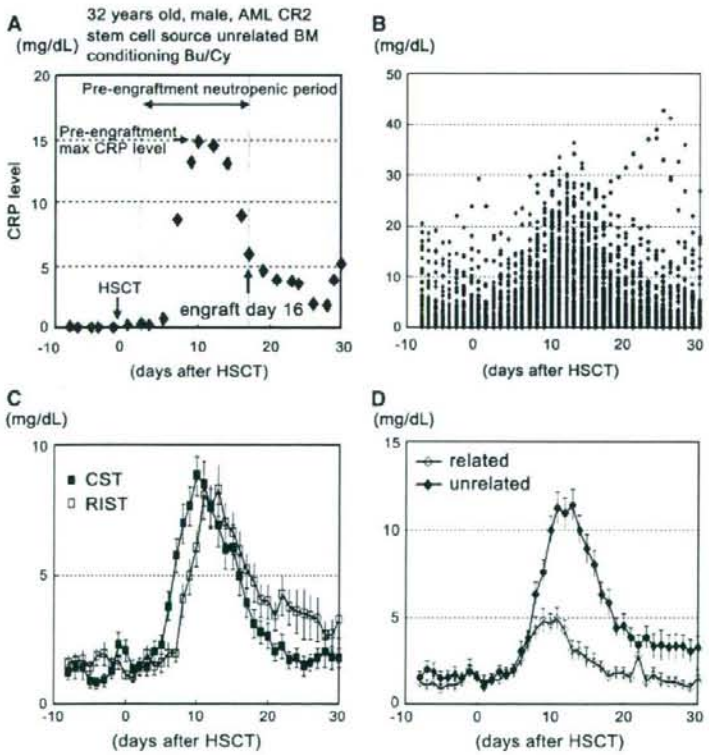

Figure 1. An example of how we measured CRP in a representative patient (A). Dot plot of the CRP level. All patients (B), CST versus RIST (C) and related versus unrelated (D).

statistics were used. Student *t*, chi-square, Fisher's exact test, and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. To analyze the pretransplant risk factors for a high CRP level, logistic analysis was used. OS was estimated using Kaplan-Meier curves. The cumulative incidence of aGVHD and NRM was estimated based on a Cox regression model for cause-specific hazards by treating progressive disease or relapse as a competing event. Cox proportional hazard models were used for the multivariate analysis of variables in aGVHD, NRM, and OS after HSCT. Clinical factors that were assessed for their association with aGVHD included patient age, patient sex, donor sex, CMV serostatus, conditioning regimen (CST versus RIST), donor (human leukocyte antigen [HLA]-matched versus HLA-mismatched, related versus unrelated), GVHD prophylaxis (cyclosporine-based versus tacrolimus-based, short-term MTX versus no MTX) and disease risk (standard versus high risk). NRM and OS were also assessed for their association with these factors. Factors with $P < .10$ in the univariate analyses were subjected to a multivariate analysis using a multiple logistic analysis and Cox proportional hazard modeling. In Japan, only BM and CB are allowed for unrelated transplantation, and most transplantations with a related donor use PBSC as a stem cell source. Therefore, the stem cell source was not included as a factor in the multivariate analysis. A level of $P < .05$ was defined as statistically significant. All P values are 2-sided. All analyses were made with SPSS ver 10.0 statistical software (Chicago, IL). This analysis was approved by the institutional review board.

RESULTS

Infections

The median duration of follow-up in surviving patients was 965 days (61 to 1432 days) in the high-CRP group and 915 days (76 to 1803 days) in the low-CRP group, and the incidence of total documented infections during neutropenia was, respectively, 23 cases in the high-CRP group (34%) and 27 cases in the low-CRP group (17%, $P = .004$). The incidence of bacteremia was, respectively, 20 cases (30%) and 20 cases (13%, $P = .002$), and the incidence of pneumonia was 7 cases (10%) and 4 cases (3%, $P = .01$). The incidence of central venous catheter infection was, respectively, 4 cases (6%) and 7 cases (4%, $P = .63$).

Serial changes in the CRP level are shown in Figure 1B; in most cases, the CRP level was elevated within 2 weeks of HSCT. Stratified data according to conditioning regimen (CST versus RIST) or relation to donor (related versus unrelated) are shown in Figure 1C and D, respectively.

To clarify the pretransplant risk factors for high CRP values during neutropenia, we performed a logistic regression analysis, which showed that male, unrelated donor, stem cell source with BM or CB transplantation (versus PBSCT), HLA-mismatched donor, and immunosuppression with MTX were associated with high CRP values during neutropenia (Table 1). Factors that showed significant associations ($P < .1$) were subjected to a multiple logistic regression analysis, and the results showed that unrelated donor, HLA mismatch and male sex were associated with high CRP ($P < .001$, $P = .005$, $P = .028$, respectively), as shown in Table 3. The median CRP levels after CST and RIST were 10.5 (0.3-31.3) and 6.2 (0.1-42.7), respectively, with a significant difference ($P = .017$) (Table 2). Notably, within the RIST group, the median CRP level was significantly lower in related than in unrelated transplantation (1.6 mg/dL [0.1-9.7] veruss 16.2 mg/dL [0.5-42.7]: $P < .001$). However, the logistic analysis failed to disclose any overall significant difference between CST and RIST.

Primary Outcomes

The cumulative incidences of aGVHD grade II-IV and grade III-IV are shown, respectively, in Figure 2A and B. Grade II-IV and grade III-IV aGVHD were both more frequent in the high-CRP group than in the low-CRP group ($P = .001$ and $P = .04$, respectively). A Cox proportional hazard model showed that a high CRP level and CMV serostatus were associated with an increased risk of grade II-IV aGVHD (Table 4). Similar results were obtained when we included only the patients who received a myeloablative conditioning regimen (grade II-IV aGVHD 25% in the low-CRP group and 58% in the high-CRP group, $P < .001$, grade III-IV aGVHD 7% in the low-CRP group and 21% in the high-CRP group, $P = .047$).

Secondary Outcomes

OS and NRM are shown, respectively, in Figure 3A and B. OS was significantly worse in the

Table 3. *Multiple Logistic Regression Analysis of Risk Factors for High CRP during Neutropenia*
*Factors with $P < .10$ in a Multivariate Analysis Was Shown**

	Multiple Logistic Regression Analysis		
Outcomes and Variables	Odds	95% CI	P Value
Unrelated donor	4.6	2.2-9.6	<.001
HLA mismatch	2.6	1.3-5.0	.005
Patient sex (male)	2.1	1.1-4.2	.0028

CRP indicates C-reactive protein; CI, confidence interval; HLA, human leukocyte antigen; CMV, cytomegalovirus.
*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source.

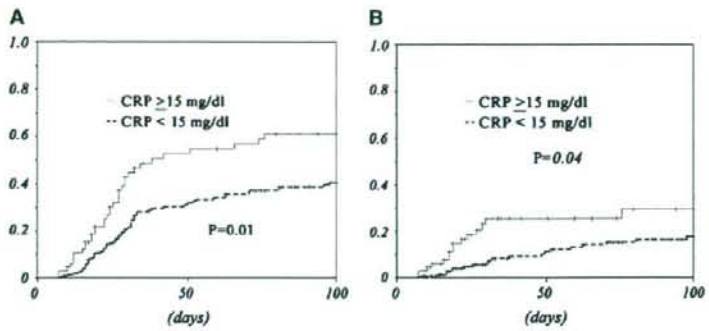

Figure 2. Cumulative incidence of grade II-IV aGVHD (A) and grade III-IV aGVHD (B) stratified according to the maximal CRP level during neutropenia.

high-CRP group than in the low-CRP group (1-year OS 47% versus 75%, $P = .001$). NRM was significantly higher in the high-CRP group than in the low-CRP group (1-year NRM 47% versus 13%, $P < .001$). Similar results were obtained when we included only patients who received a myeloablative conditioning regimen (1-year NRM 8% in the low-CRP group and 38% in the high-CRP group, $P = .007$). A Cox proportional hazard model showed that the risk factors for poor OS were high CRP ($P = .002$, hazard ratio [HR] 2.0, 95% confidence interval [CI] 1.3-3.1) and high-risk disease ($P = .015$, HR 2.2, 95% CI 1.2-4.0), whereas those for high NRM were high CRP ($P < .001$, HR 4.0, 95% CI 2.0-8.0) and high-risk disease ($P = .029$, HR 2.6, 95% CI 1.1-6.2), as shown in Table 4. When the threshold was set at 15 mg/dL, the sensitivity and specificity of the CRP level for prediction of grade II-IV aGVHD, NRM, or OS were 37% and 75%, 59% and 79%, and 40% and 78%, respectively. The relapse rate was significantly lower in the high-CRP group than in the low-CRP group (1-year relapse 21% versus 33%, $P = .02$).

Causes of death are summarized in Table 5. A total of 57 patients (36%) in the low-CRP group and 39 patients (58%) in the high-CRP group died ($P = .002$, OR 2.4 [1.4-4.4]). Six patients (4%) in the low- and 5 (7%) in the high-CRP group died because of aGVHD, for example, death because of infectious diseases associated with aGVHD and its treatment. Seven patients (4%) in the low- and 11 (16%) in the high-CRP group ($P = .003$, OR 4.2 [1.6-11.4]) died because of chronic GVHD (cGVHD), including death because of infectious diseases associated with cGVHD and its treatment. No patient (0%) in the low- and 5 (7%) in the high-CRP group ($P = .002$) died because of infectious diseases excluding infectious disease concomitant with GVHD. No patient in the low-CRP group and 4 (6%) in the high-CRP group ($P = .008$) died because of multiple-organ failure (MOF) excluding MOF because of GVHD and infectious disease.

DISCUSSION

The results of this retrospective study suggested that higher CRP values during the neutropenic period may reflect net inflammation secondary to tissue damage because of the conditioning regimen, infection, and subsequent allogeneic immune reactions, all of which lead to aGVHD/cGVHD and ultimate NRM. In a mouse model, the concept that the production of inflammatory cytokines plays an important role in the development of aGVHD, by affecting the afferent and effector phase [12,13], has been accepted. Cooke et al. [26] showed that LPS antagonism reduced aGVHD in a mouse model, as indicated by Ferrara et al. [4]. However, in human studies, the value of determining individual levels of cytokines to monitor aGVHD has not been fully explored, because this approach is very costly and requires sophisticated techniques, which impedes its universal applicability. On the other hand, CRP is already being widely used

Table 4. *Multiple Variate Analysis for aGVHD, NRM, and OS**

Outcomes and Variables	Hazard Ratio	95% CI	P value
Grade II-IV aGVHD			
High CRP	1.7	1.1-2.6	.02
CMV positivity	3.1	1.0-9.8	.5
Disease risk (high)	1.6	0.9-2.7	.10
NRM			
High CRP	4.0	2.0-8.0	<.001
Age (≥40 years old)	1.9	0.9-3.9	.07
Disease risk (high)	2.6	1.1-6.2	.03
OS			
High CRP	2.0	1.3-3.1	.002
Disease risk (high)	2.2	1.2-4.0	.02

CRP indicates C-reactive protein; CI, confidence interval; CMV, cytomegalovirus; GVHD, graft-versus -host disease; TBI, total body irradiation; NRM, nonrelapse mortality; OS, overall

*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source

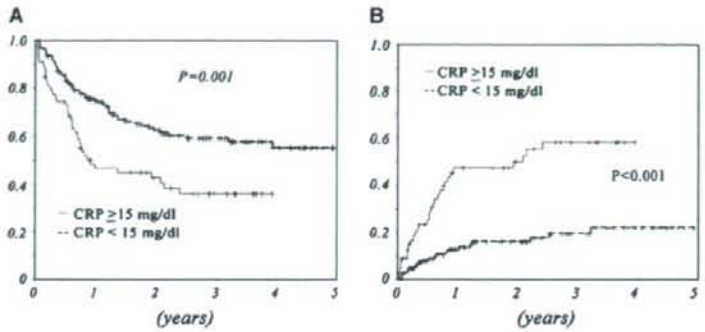

Figure 3. OS stratified according to the maximal CRP level during neutropenia (A). Cumulative incidence of TRM stratified according to the maximal CRP level during neutropenia (B).

worldwide, especially in Japan, to distinguish bacterial infections from other causes of fever [15-19]. Based on this practice, we reviewed the value of the CRP level after HSCT, and our data suggest that it might be useful to monitor the CRP value as a net surrogate marker for produced cytokines, and for predicting the subsequent development of aGVHD and NRM.

Our patients had various interacting backgrounds, and it is still difficult to predict whether a patient with a high CRP level is destined to suffer from GVHD or major infectious complications. Infectious diseases were previously reported to be a primary cause of elevated CRP [8,20], which might, in turn, affect the severity of aGVHD. In this study, we made every effort, including intense culture studies, to exclude infection as a primary cause of increased CRP, and showed that there were significantly more documented infections in the high-CRP group than in the low-CRP group. Current practice for the prevention of infection mostly focuses on the effective control of Gram-negative bacteria, considering the potent immediate pathologic effect of the organisms. However, if the hypothesis that decreasing the net production of cytokines is important for the prevention of subsequent GVHD is correct, more effort should be paid to broadly cover other types of organisms or even clinically less significant infection, that is, stomatitis, at least during the early period of neutropenia, particularly in patients carrying risk factors for high CRP, which included unrelated donor, HLA mismatch, BM, and CB transplantation in this study. The addition of other markers, such as procalcitonin, may be useful for identifying the risk of major infectious complications [24].

Table 5. *Causes of Death Stratified According to CRP Value during Neutropenia*

Causes of death	Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	P Value
Total	57 (36%)	39 (58%)	.002
Relapse/progressive disease	34 (22%)	8 (12%)	.09
acute GVHD (total)	6 (4%)	5 (7%)	.25
acute GVHD	5 (3%)	3 (5%)	.63
acute GVHD + infection	1 (1%)	2 (3%)	.16
chronic GVHD (total)	7 (4%)	11 (16%)	.003
chronic GVHD	3 (2%)	7 (10%)	.005
chronic GVHD + infection	4 (3%)	4 (6%)	.21
Infection*	0 (0%)	5 (7%)	.002
MOF†	0 (0%)	4 (6%)	.008
Respiratory failure‡	3 (2%)	4 (6%)	.11
Others	Stroke 2	VOD 1	
	VOD 2	Myocardial infarction 1	
	Secondary cancer 1		
	Unknown 2		

CRP indicates C-reactive protein; GVHD, graft-versus-host disease; TBI, total-body irradiation; MOF, multiple organ failure; VOD, veno-occlusive disease.

*Excluding infection during GVHD or GVHD treatment.

†Excluding MOF due to GVHD, infection.

‡Excluding respiratory failure because of GVHD, infection, and MOF.

Tissue damage caused by the conditioning regimen, complicated infections, and allogeneic immune reactions are the primary factors that are associated with the initial elevation of CRP early in the course of allogeneic HSCT. Consequently, it can be speculated that a reduced-intensity conditioning regimen results in decreased cytokine release and a resultant lower CRP value, which may lead to less chance of developing GVHD. Although the RIST regimens we used were relatively dose-intense, in this retrospective review we still found that CRP levels tended to be decreased after RIST compared to conventional myeloablative transplantation, particularly in a related compared to an unrelated transplantation setting. Because augmentation of allogeneic immune and inflammation reactions may induce a higher CRP value, we speculate that the benefit of RIST is diminished when a strong allogeneic reaction is induced, as in cases of unrelated transplantation.

To further evaluate the relationship between a higher CRP value during neutropenia and common risk factors associated with transplantation, we performed a multivariate analysis and showed that unrelated donor, HLA mismatch, and male sex were associated with higher CRP values. Additionally, from the finding in the multivariate analysis that unrelated donor and HLA mismatch were independently associated with high CRP, we surmised that the degree of genetic disparity might be associated with higher CRP during neutropenia. Based on a consideration of these findings together, we think that a higher CRP value may reflect the degree of tissue damage because of the transplant regimen and the subsequent magnitude of allogeneic immune reactions. Nevertheless, our analysis was hampered, because in Japan only BM and CB are allowed for unrelated transplantations, and most transplantations with a related donor use PBSC as a stem cell source. In these settings, a theoretically longer neutropenic period after unrelated BM or CB transplantation might be associated with a higher risk of infection, which could lead to higher CRP, as shown in this study.

In this study, the primary causes of death in the low-CRP group were mainly relapse and progression, whereas in the high-CRP group this was NRM. Notably, the observation that the relapse rate was higher in the low-CRP group than in the high-CRP group, as previously suggested by Min et al. [23], may further support our hypothesis that serum CRP values represent overall inflammation and cytokine production, which paves the way to GVHD and related graft-versus-leukemia (GVL) effects. A possible reason for this finding is that a low CRP level resulted in a lower incidence of GVHD and a resultant decrease in the GVL effect, or the high-CRP group developed earlier and more-frequent death from NRM compared to the low-CRP group, which left fewer patients for evaluation of the later occurrence of relapse.

In conclusion, our results suggest that the CRP value in the neutropenic period before engraftment in patients undergoing allogeneic HSCT may be a net surrogate marker of early inflammation that leads to the development of aGVHD/cGVHD and subsequent NRM, as has been proposed in mouse models. The intensity of the conditioning regimen, infectious diseases, and degree of allogeneic immune response attributed to HLA compatibility and the stem cell source may be the major factors that predict higher CRP values. Based on the results of this retrospective study, future clinical studies to evaluate the feasibility of earlier intervention and adjustment of the procedure for preventing GVHD and NRM based on monitoring of the early CRP value are warranted.

ACKNOWLEDGMENTS

This work was presented in part as a poster presentation at the annual Meeting of EBMT, Lyon, March 2007. This study was supported in part by grants from the Ministry of Health, Labor and Welfare, and Advanced Clinical Research Organization, Japan. There is no potential conflict of interest to declare.

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