TABLE III. Patient Characteristics of Intervention Group

| Characteristics | No aggressive Tx (%) | CTx and/or DLI (%) | Second HCT (%) | P |
|---|---|---|---|---|
| Total no. of patients | 25 | 48 | 20 | |
| Diagnosis | | | | 0.053 |
| AML | 10 (40) | 32 (67) | 15 (75) | |
| MDS | 7 (28) | 3 (6) | 3 (15) | |
| CML | 2 (8) | 3 (6) | 0 (0) | |
| ALL | 6 (24) | 10 (21) | 2 (10) | |
| Age | | | | 0.333 |
| <50 | 11 (44) | 28 (58) | 13 (65) | |
| ≥50 | 14 (56) | 20 (42) | 7 (35) | |
| Matched related donor | | | | 0.143 |
| Yes | 8 (32) | 27 (56) | 9 (45) | |
| No | 17 (68) | 21 (44) | 11 (55) | |
| Disease status at first HCT | | | | 0.105 |
| CR | 7 (28) | 26 (54) | 9 (45) | |
| non-CR | 18 (72) | 22 (46) | 11 (55) | |
| Time from first HCT to relapse | | | | 0.938 |
| ≥100 days | 16 (64) | 31 (65) | 12 (60) | |
| <100 days | 9 (36) | 17 (35) | 8 (40) | |

Tx, therapy; CTx, chemotherapy; HCT, hematopoietic cell transplantation; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; ALL, acute lymphoid leukemia; CR, complete remission.

### *Interventions after relapse/progression*

After the diagnosis of relapse or progression, the need for salvage therapy was determined at a multiprofessional conference, at which the clinical circumstances and the opinions of physicians and patients were weighed. The various therapeutic options used after the diagnosis of relapse are summarized in Table II and Fig. 1.

At the diagnosis of relapse or progression, 70 patients had been receiving immunosuppression (median days after initial HCT, 125; range 15–705) and 63 of them had it withdrawn before receiving any other therapies.

After the diagnosis of relapse or progression, 63 patients received reinduction chemotherapy with disease-specific regimens, which included imatinib mesylate (CML, $n = 4$), all trans-retinoic acid and arsenic trioxide (APL, $n = 1$), gemtuzumab ozogamicin (AML, $n = 3$), and intrathecal chemotherapy alone for isolated CNS relapse (AML, $n = 3$; ALL, $n = 1$; CML, $n = 1$). Overall, 27 of the 63 patients who received reinduction chemotherapy achieved CR (43%). Among the 27 patients who achieved CR, 18 proceeded to DLI ($n = 7$) or second HCT ($n = 11$). The remaining nine received no further therapy other than chemotherapy; three patients with CNS relapse were in remission, and the remaining six patients subsequently progressed. Among the 36 patients who did not achieve CR, 14 proceeded to DLI ($n = 7$) or second HCT ($n = 7$), and the remaining 22 did not receive further treatment because of various reasons (disease progression, $n = 15$; infection and/or graft-versus-host disease (GVHD), $n = 4$; refusal, $n = 3$). Two other patients proceeded to second HCT directly after disease relapse with concomitant graft failure.

To compare the outcomes of the interventions after relapse/progression, we divided the 93 patients into three cohorts according to the intervention, that is, no aggressive therapy (Cohort 1, $n = 25$), reinduction chemotherapy and/or DLI without second HCT (Cohort 2, $n = 48$), and second HCT (Cohort 3, $n = 20$). There were no significant differences among the three groups in clinical characteristics such as patient age at the initial HCT, diagnosis, donor in the initial HCT, disease status at the initial HCT, and interval from the initial HCT to relapse (Table III).

### *No aggressive therapy (Cohort 1)*

Among the 93 patients who relapsed, 25 (27%) received no aggressive therapy with curative intent other than WIS or less-intensive chemotherapy, mostly because of comorbidities and/or refractoriness of leukemia/MDS. Among the 10 patients who received WIS alone, only one achieved CR, but this patient subsequently died of bronchiolitis obliterans. All of the remaining eight patients who were given less-intensive chemotherapy alone and seven who received no therapy after relapse/progression died of disease progression without achieving CR. The median OS of the patients in Cohort 1 was 61 days after relapse/progression and the cause of death was primarily disease progression.

### *Reinduction chemotherapy and/or DLI without second HCT (Cohort 2)*

Of the 63 patients who received reinduction chemotherapy after relapse, 45 patients did not receive a second HCT; these 45 patients with or without subsequent DLI and three other patients who received DLI without preceding chemotherapy were placed in Cohort 2.

Overall, 16 (36%) of the 45 patients achieved CR as the best response after reinduction chemotherapy. All three patients with isolated CNS relapse were alive in remission, whereas 11 of 13 patients who had marrow relapse eventually relapsed.

After reinduction chemotherapy, 14 patients (AML, $n = 9$; MDS, $n = 1$; ALL, $n = 3$; CML, $n = 1$) received DLI from the same donor as in the initial HCT. The initial CD3-positive cell dose of DLI ranged from 0.03 to 161 $\times$ $10^8$/kg (median: 2.9 $\times$ $10^8$/kg), and the number of courses of DLI was one to four, which were chosen according to the donor source or the disease status of patients at the discretion of physicians. Although the remission rate of patients who received DLI after chemotherapy was 50%, the incidence of NRM was also rather high (29%, GVHD with or without infection). The median OS of patients who received DLI after relapse/progression was 194 days (range: 52–1,254), which was similar to that of patients without DLI (167 days, range; 19–1,456).

Among the three patients who received DLI without preceding chemotherapy (AML, 1; MDS, 2), two achieved CR but all of them eventually died: one with toxicity and two with disease progression.

### *Second HCT (Cohort 3)*

Table IV summarizes the profiles of 20 patients who underwent a second HCT. The median age at the initial HCT was 38 years (21–66 years) and 65% of the patients were younger than 50 years. The median time from the initial HCT to relapse/progression was 152 days (range: 21–1,211), and the median interval between the initial HCT and the second HCT was 325 days (range: 126–1,310). Six patients received HCT from the same donor as in the initial HCT (HLA-matched related donor, $n = 5$; unrelated bone marrow donor, $n = 1$), and the remaining 14 received the second HCT from a different donor (unrelated bone marrow donor, $n = 7$; cord blood, $n = 6$; haploidentical related do-

**TABLE IV. Characteristics of Second Transplantation**

| Characteristics | No of patients second HCT (%) |
|---|---|
| Total | 20 |
| Age | |
| <50 | 13 (65) |
| ≥50 | 7 (35) |
| Diagnosis | |
| AML | 15 (75) |
| MDS | 3 (15) |
| CML | 0 (0) |
| ALL | 2 (10) |
| Gender | |
| Male | 9 (45) |
| Female | 11 (55) |
| Time from first HCT to relapse | |
| <100 days | 8 (40) |
| ≥100 days | 12 (60) |
| Time from first HCT to second HCT | |
| <1 year | 12 (60) |
| ≥1 year | 8 (40) |
| Donor for first/second HCT | |
| Same | 6 (30) |
| MRD-MRD | 5 |
| UBM-UBM | 1 |
| Different | 14 (70) |
| UBM-UBM | 4 |
| MRD/CB-UBM | 3 |
| MRD/UBM-CB | 6 |
| Other | 1 |
| Conditioning for first/second HCT | |
| Myeloablative | 8 (40) |
| Myeloablative-RIC | 7 (35) |
| RIC-RIC | 5 (25) |
| Stem cell source | |
| BM | 8 (40) |
| PBSC | 6 (30) |
| CB | 7 (35) |
| Remission at second HCT | |
| No | 9 (45) |
| yes | 11 (55) |
| GVHD prophylaxis | |
| CSP-based | 8 (40) |
| TAC-based | 3 (15) |
| Others | 3 (15) |
| GVHD | |
| No | 10 (50) |
| Yes | 10 (50) |

HCT, hematopoietic cell transplantation; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; ALL, acute lymphoid leukemia; MRD, matched-related donor; UBM, unrelated bone marrow; CB, cord blood; RIC, reduced-intensity conditioning; PBSC, peripheral blood stem cell; CSP, cyclosporin; TAC, tacrolimus.

nor, $n = 1$). Among the 15 patients who had received myeloablative conditioning for the initial HCT, eight received myeloablative conditioning and seven received RIC for the second HCT. The remaining five patients received both HCT with RIC. Although the 1-year OS after relapse was better in patients who received myeloablative conditioning for the second HCT than in patients who received RIC (100 vs. 37%, $P = 0.015$), patients who received myeloablative conditioning for the second HCT were younger and had a longer interval between the initial and the second HCT than those who received RIC ($P < 0.001$ and $P = 0.006$, respectively). There was no difference in OS between patients who received a second HCT from the same donor and those who had a different donor (1-year OS: 44 vs. 60%, $P = 0.48$).

Two patients underwent immediate HCT after relapse with concomitant graft failure. Among the other 18 patients who received reinduction chemotherapy before the second HCT, 11 had achieved CR at the second HCT and seven were not in CR. Four of the nine patients with nonremission disease at the second HCT, including two patients who did not receive reinduction chemotherapy, subsequently achieved CR; only one of the nine patients is currently alive in CR.

Of the 20 patients who underwent a second HCT, eight are alive with a median follow-up after relapse of 335 days (range: 181–997); five are in CR and three have recurrent disease.

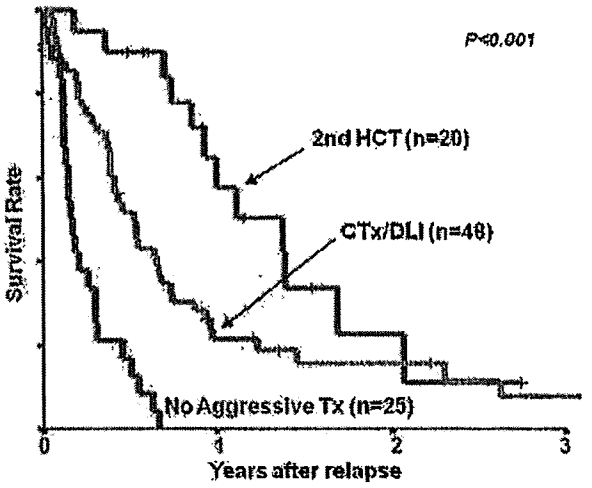


Figure 2. Overall survival. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

GVHD was newly diagnosed or interpreted to progress after the second HCT in 10 of the 20 patients. The median OS after relapse in patients with GVHD after the second HCT was 422 days (range: 181–997), and all of these patients achieved CR as a best response. The median OS after relapse for the remaining 10 patients without GVHD was 314 days (range: 66–757), and five of them failed to achieve CR as a best response.

### *Comparison of CR, NRM, and OS after relapse following the initial HCT*

The median OS after the development of relapse in the 93 patients who had relapse/progression was 184 days (range: 5–1,456). Overall, 15 patients (16%) are currently alive with a median follow-up of 346 days (range; 33–1,456 days), and 10 of these patients are still in CR. Among the 78 patients who died, 69 died of disease progression and nine died of NRM (10%). The causes of NRM were GVHD and/or infection in eight (Cohort 1, one patient; Cohort 2, seven patients), and one early death after the second HCT with hepatic failure, which accounts for the one case of NRM for second HCT (Table II).

We compared the rate of CR, NRM, and OS after relapse among the three different cohorts (Table II). As the maximum response, the probabilities of achieving CR were 4% in Cohort 1, 38% in Cohort 2, and 75% in Cohort 3. The NRM rates were 4, 15, and 5% for each group, respectively. The median duration of remission after achieving CR was 177 days (range, 17–1,167). The median OS after relapse/progression in patients who underwent a second HCT (Cohort 3, 502 days) was significantly longer than those in Cohort 1 (61 days) and Cohort 2 (194 days, $P < .001$, Fig. 2). The 1-year OS after relapse was significantly better in patients with a second HCT (Cohort 3) than in the other patients (Cohorts 1 and 2) (58 vs. 14%). However, there was no significant difference in the 2-year OS, which suggests that it is difficult to maintain CR after a second HCT.

A multivariate analysis showed that CR after intervention (HR 3.83, 95% CI 2.06–7.11, $P < .001$), reinduction chemotherapy (HR 2.83, 95% CI 1.65–4.86, $P < .001$), a second HCT (HR 3.02, 95% CI 1.58–5.79, $P < .001$), and a longer time from the initial HCT to relapse (HR 1.99, 95% CI 1.21–3.28, $P = 0.007$) were associated with an improved OS after relapse/progression (Table V). Diagnosis, patient age at initial HCT, gender, conditioning regimen, or donor in the initial HCT and DLI were not significant factors.

TABLE V. Univariate and Multivariate Analysis of risk Factors for OS after Relapse

| Variables | Univariate analysis HR (95%CI) | P | Multivariate analysis HR (95%CI) | P |
|---|---|---|---|---|
| Diagnosis | | | – | – |
| CML | 1.00 | | | |
| AML | 2.03 (0.62–6.65) | 0.241 | | |
| ALL | 2.54 (0.71–9.00) | 0.150 | | |
| MDS | 3.39 (0.94–12.24) | 0.062 | | |
| Age | | | – | – |
| <50 | 1.00 | | | |
| ≥50 | 1.53 (0.98–2.41) | 0.063 | | |
| Gender | | | – | – |
| Male | 1.00 | | | |
| Female | 0.92 (0.59–1.43) | 0.701 | | |
| Conditioning | | | – | – |
| Myeloablative | 1.00 | | | |
| RIC | 1.34 (0.84–2.12) | 0.216 | | |
| Donor | | | – | – |
| MRD | 1.00 | | | |
| Others | 1.26 (0.80–1.97) | 0.322 | | |
| Disease Status at first HCT | | | | |
| Standard | 1.00 | | | |
| High | 1.23 (0.70–2.12) | 0.465 | | |
| Time from first HCT to relapse | | | | |
| ≥100 days | 1.00 | | 1.00 | |
| <100 days | 1.74 (1.09–2.79) | 0.020 | 1.99 (1.21–3.28) | 0.007 |
| Reinduction CTx | | | | |
| Yes | 1.00 | | 1.00 | |
| No | 3.79 (2.24–6.40) | <.001 | 2.83 (1.65–4.86) | <.001 |
| CTx Intensity | | | – | – |
| Reinduction | 1.00 | | | |
| Less Intensive | 4.44 (2.00–9.88) | <.001 | | |
| DLI | | | – | – |
| Yes | 1.00 | | | |
| No | 1.00 (0.67–1.72) | 0.968 | | |
| Second HCT | | | | |
| Yes | 1.00 | | 1.00 | |
| No | 2.89 (1.55–5.38) | <.001 | 3.02 (1.58–5.79) | <.001 |
| CR after Interventions | | | | |
| Yes | 1.00 | | 1.00 | |
| No | 3.54 (2.06–6.09) | <.001 | 3.83 (2.06–7.11) | <.001 |

OS, overall survival; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; MDS, myelodysplastic syndrome; RIC, reduced-intensity conditioning; MRD, matched-related donor; HCT, hematopoietic cell transplantation; CTx, chemotherapy; DLI, donor lymphocyte infusion; CR, complete remission.

## Discussion

With this retrospective single-center survey in which we compared the outcomes of interventions for relapse/progression after allo-HCT, we showed that a second HCT significantly improved the remission rate and survival. In contrast to previous reports (8–13, 15), NRM after a second HCT was observed in an acceptable percentage of patients (5%), even though 40% of the patients received myeloablative conditioning regimen for the second HCT.

As salvage interventions for leukemia/MDS relapsing after allo-HCT, chemotherapy, DLI either alone or in combination, and second HCT have been considered with different degrees of success. Consistent with reports from other groups [1,4–6], we found that patients who did not undergo intensive chemotherapy had significantly shorter survival. Even though 43% of the patients who were given reinduction chemotherapy achieved CR, all of the relapsed patients who did not receive further intervention eventually relapsed unless relapse is isolated to CNS, and all but one patient died. Prior reports have also suggested that, instead of a certain probability of obtaining remission with reinduction chemotherapy, subsequent relapse is frequently observed and the prognosis is poor when further immunotherapy is suspended [1,4,6,19].

Although DLI has been recognized as an effective treatment for relapsed CML, the efficacy of DLI for relapsed acute leukemia is rather discouraging [3,7,20–22]. Although the remission rate has been reported to be 15–42%, the survival rate has not improved (3-year OS less than 20%), mostly because of a high incidence of uncontrolled GVHD (10–50%). In our cohorts, survival was not improved by adding DLI after chemotherapy, although half of the patients had achieved transient remission. The incidence of NRM after DLI was 29%, which was mostly explained by GVHD. Compared to DLI, a second HCT yielded an even better remission rate and lower NRM in our cohort, which could be respectively explained by the efficacy of the use of conditioning radiochemotherapy and GVHD prophylaxis in the second HCT.

In our data, a second HCT significantly improved the remission rate and survival compared to other interventions, as proven by a multivariate analysis. Although Arellano et al. [1] indicated that immunotherapy including a second HCT was effective compared to chemotherapy or supportive care, other reports that compared interventions after relapse following initial HCT failed to show the advantage of a second HCT [2,6,22]. Prior reports that focused on a second HCT have also expressed concerns about the negative impact of NRM, which has ranged from 24 to 75% (8–13, 15). In contrast, our data revealed a 5% incidence of NRM after a second HCT, which led to improved OS. This unexpectedly low incidence of NRM may reflect the advances in GVHD prophylaxis and supportive care over the past several years. Another possible explanation would be a selection bias of fitter patients that led to less NRM after the second HCT, although there were no significant differences in available characteristics of patients in each intervention group.

Concerning the conditioning regimen for the second HCT, we found that patients who received myeloablative conditioning had a better OS than patients who received

RIC. Eapen et al. [9] indicated the importance of a tumor-killing effect of myeloablative conditioning for the second HCT compared to RIC. Other groups also reported a superior outcome of TBI-based myeloablative conditioning in the second HCT [8,11]. On the other hand, several recent reports have shown that RIC offers a toxicity-reducing benefit in the second HCT [10]. In our cohort, patients who received myeloablative conditioning for the second HCT were younger and had a longer interval from the initial HCT to the second HCT, which could reflect a selection bias in the choice of myeloablative conditioning. Therefore, myeloablative conditioning for the second HCT could be considered beneficial for selected patients.

Consistent with several previous reports, we demonstrated that remission status [4,6,8–12,14,22,23], the use of reinduction chemotherapy [2,6], and a longer interval from the initial HCT to relapse [1,2,4,8–12,14,15,19,22–24] were associated with improved OS after relapse by multivariate analysis. Most prior reports have shown that an interval of 6 months or longer was associated with better OS. We found that patients who relapsed after 100 days following the initial HCT had better OS. However, relapses after intervals of 6 months or 1 year were not significantly associated with improved OS (data not shown).

Prior reports have also suggested that the development of GVHD after a second HCT [2,7–9,13,15,24] and the use of a different donor for the second HCT were associated with a better outcome after the second HCT [10]. Our data showed that both the remission rate and OS tended to be improved in patients who developed newly diagnosed GVHD after the second HCT. However, the use of a different donor for the second HCT did not appear to offer any advantage. Nevertheless, the small number of patients who received a second HCT in our study limits our ability to draw definite answers.

Although the 1-year OS after the second HCT was significantly better than that with other interventions (58 vs. 14%), there was no significant difference in 2-year OS (22 vs. 10%). The substantial decline in the survival curve in the second HCT group after 1 year from relapse was clearly related to recurrence of the underlying diseases. Previous reports also showed a decline in survival in the later period (<30% at 3–5 years from the second HCT) and a substantial relapse rate after the second HCT (>40%) [9–11]. This evidence suggests the need for the effective management of disease recurrence after the second HCT.

Our study is limited by several inherent selection biases. Most importantly, this is a retrospective study that compared the outcomes of interventions that were chosen at the discretion of physicians, although there were no significant differences in patient characteristics among the three cohorts. For example, patients who successfully received intensive intervention such as a second HCT had to survive long enough after relapse to be able to undergo adequate salvage chemotherapy with a rather controlled disease and less comorbidity. Other limitations include the small number of patients, a short follow-up period, and other transplant variables that may have affected the outcomes. Nevertheless, the present data in a consecutive-case series from a single center that reviewed various interventions after relapse allowed us to identify the factors that influenced the prognosis of patients with relapse/progression after allo-HCT.

In summary, these observations may have important implications for the selection of interventions in patients who relapse after allo-HCT. Our data indicated that reinduction chemotherapy with curative intent is required for prolonged survival, if feasible. However, when CR is not available with chemotherapy, long-term survival may be unlikely even with a second HCT. The second HCT may produce improved survival without excessive toxicity. However, the substantial incidence of a later relapse after the second HCT was revealed to be a major concern. Further studies are warranted to identify innovative post-transplant strategies to reduce disease recurrence, including immunotherapy such as a vaccination strategy.

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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.**

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**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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## 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) ⩾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.

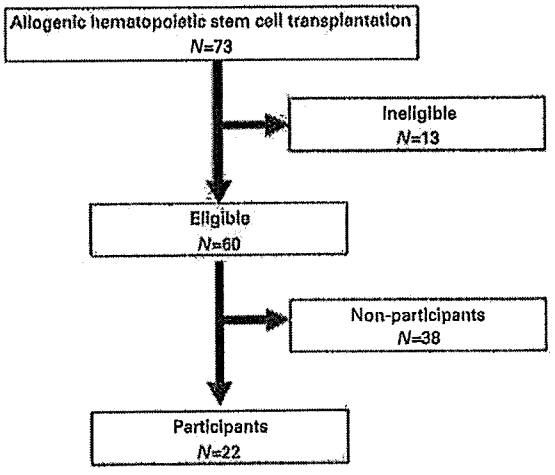


**Figure 1** Trial profile.

**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<br>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<br>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<br>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 $t$, $\chi^2$ 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.

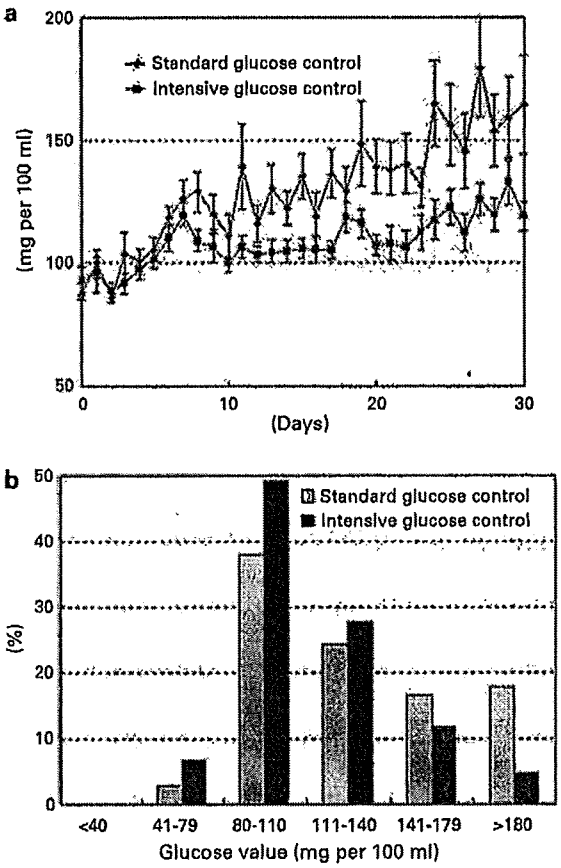


**Figure 2** Serial changes in the mean glucose level in the intensive glucose control (IGC) and standard glucose control (SGC) groups. Values are mean + s.e. (**a**). The distribution of the glucose values in IGC and SGC is shown as a histogram (**b**).

*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

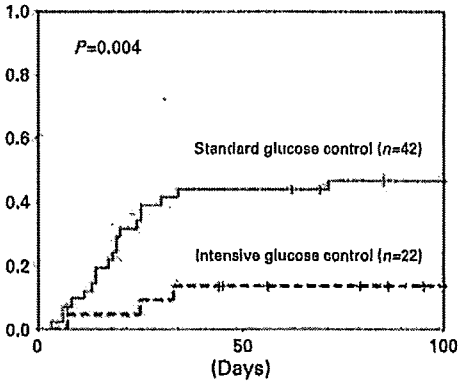


**Figure 3** Probability of documented infections in the IGC and SGC groups.

**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.

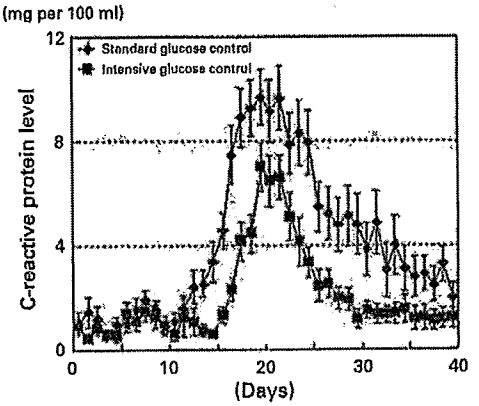


Figure 4 Serial change in the CRP level in the IGC and SGC groups. Values are mean + s.e.

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.

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# A Novel Antiangiogenic Effect for Telomerase-Specific Virotherapy through Host Immune System[1]

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**Soluble factors in the tumor microenvironment may influence the process of angiogenesis; a process essential for the growth and progression of malignant tumors. In this study, we describe a novel antiangiogenic effect of conditional replication-selective adenovirus through the stimulation of host immune reaction. An attenuated adenovirus (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase promoter element drives expression of E1 genes, could replicate in and cause selective lysis of cancer cells. Mixed lymphocyte-tumor cell culture demonstrated that OBP-301-infected cancer cells stimulated PBMC to produce IFN-γ into the supernatants. When the supernatants were subjected to the assay of in vitro angiogenesis, the tube formation of HUVECs was inhibited more efficiently than recombinant IFN-γ. Moreover, in vivo angiogenic assay using a membrane-diffusion chamber system s.c. transplanted in *nu/nu* mice showed that tumor cell-induced neovascularization was markedly reduced when the chambers contained the mixed lymphocyte-tumor cell culture supernatants. The growth of s.c. murine colon tumors in syngenic mice was significantly inhibited due to the reduced vascularity by intratumoral injection of OBP-301. The antitumor as well as antiangiogenic effects, however, were less apparent in SCID mice due to the lack of host immune responses. Our data suggest that OBP-301 seems to have antiangiogenic properties through the stimulation of host immune cells to produce endogenous antiangiogenic factors such as IFN-γ. *The Journal of Immunology,* 2009, 182: 1763–1769.**

Angiogenesis is the development of new capillaries from preexisting capillary blood vessels and is necessary for the growth of solid tumors beyond 1–2 mm in diameter (1). Targeting the angiogenic process is therefore regarded as a promising strategy in cancer therapy. Angiogenesis consists of dissolution of the basement membrane, migration and proliferation of endothelial cells, canalization, branching and formation of vascular loops, and formation of a basement membrane (2). These steps might be regulated by the local balance between the amount of angiogenic stimulators and inhibitors (3–5). As cells undergo malignant transformation, angiogenic mitogens such as vascular endothelial growth factor (VEGF),[3] basic fibroblast growth factor, platelet-derived epithelial cell growth factor, and TGF become dominant, causing the aberrant angiogenesis. In contrast, many endogenous angiogenic inhibitors such as platelet factor 4, thrombospondin 1, angiostatin, endostatin, various antiangiogenic peptides, hormone metabolites, and cytokines constitutively suppress angiogenesis in normal tissues (6). These scenarios suggest the possibility that endogenous angiogenic inhibitors that outweigh the stimulators could turn off the angiogenic switch.

Recent studies have demonstrated that the tumor microenvironment, which orchestrates with the host immune system, is a critical component of both tumor progression and tumor suppression (7). Indeed, the production of cytokines at tumor sites can either stimulate or inhibit tumor growth and progression (8). These findings provide a unique therapeutic opportunity based on selective and locoregional production of endogenous antitumor mediators such as angiogenic inhibitors. We reported previously that telomerase-specific replication-competent adenovirus (Telomelysin, OBP-301), in which the human telomerase reverse transcriptase promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosomal entry sequence, induced selective E1 expression and efficiently killed human cancer cells, but not normal human fibroblasts (9–12). Although the precise molecular mechanism of OBP-301-induced cell death is still unclear, the process of oncolysis is morphologically distinct from apoptosis and necrosis. We found that tumor cells killed by OBP-301 infection could stimulate host immune cells more efficiently compared with chemotherapeutic drug-induced apoptotic cells and necrotic cells by freeze/thaw, thus enhancing the antitumor immune response (13). These results suggest that oncolytic virus is effective not only as a direct cytotoxic drug but also as an immunostimulatory agent that could modify the tumor microenvironment.

In the present article, we explored whether OBP-301-infected oncolytic cells can activate host immune cells and influence tumor

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[3] Abbreviations used in this paper: VEGF, vascular endothelial growth factor; MLTC, mixed lymphocyte-tumor cell culture; MOI, multiplicity of infection.

cell-mediated angiogenesis in vitro and in vivo. Antineoplastic effect of intratumoral administration of OBP-301 on s.c. murine colon tumors transplanted was compared in syngenic immunocompetent mice and SCID mice. Finally, we examined the effect of neutralizing anti-IFN-γ Ab on OBP-301-mediated antiangiogenic potential in vivo.

## Materials and Methods

### *Cell lines and reagents*

The human colorectal carcinoma cell lines SW620 (HLA-A02/A24) and the murine colon adenocarcinoma cell line Colon-26 were maintained in vitro in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Recombinant human IFN-γ was purchased from Peprotech.

### *Mice*

Female BALB/c (BALB/cAnNCrlCrlj), BALB/c *nu/nu* (CAnN.Cg-*Foxn1*$^{nu}$/CrlCrlj), and SCID (CB17/Icr-*Prkdc*$^{scid}$/CrlCrlj) mice, 5–6 wk of age, were purchased from Charles River Japan Breeding Laboratories. Animals were housed under specific pathogen-free conditions in accordance with the guidelines of the Institutional Animal Care and Use Committee.

### *Adenovirus*

The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin), in which the human telomerase reverse transcriptase promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosomal entry sequence, was constructed and previously characterized (9–12). The virus was purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation.

### *Cell viability assay*

XTT assay was performed to measure cell viability. Briefly, cells were plated on 96-well plates at $5 \times 10^3$ per well 24 h before treatment and then infected with OBP-301. Cell viability was determined at the times indicated by using a Cell Proliferation kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

### *Mixed lymphocyte-tumor cell culture (MLTC) and cytokine production assay*

For MLTC, SW620 tumor cells were infected with OBP-301 at a multiplicity of infection (MOI) of 10, washed three times in PBS 72 h after infection, and then cocultured with PBMC at a ratio of 1:40. The supernatant was collected at the indicated times and stored at −80°C until assay. The concentration of IFN-γ was measured with ELISA kits (BioSource International).

### *In vitro angiogenesis assay*

In vitro angiogenesis was assessed based on the formation of capillary-like structures by HUVECs cocultured with human diploid fibroblasts according to the instructions provided with the angiogenesis kit (Kurabo). In brief, the HUVECs were incubated in a medium containing the diluted supernatants of MLTC or recombinant IFN-γ in the presence or absence of VEGF (10 ng/ml). The medium was replaced at days 4, 7, and 9. At day 11, the HUVECs were fixed and stained by using an anti-human CD31 Ab (Kurabo) according to the instructions provided. The formation of the capillary network was observed with a microscope at a magnification of ×40.

### *In vivo assay for tumor angiogenesis*

In vivo angiogenesis was determined using the dorsal air-sac method (14). Briefly, $2 \times 10^6$ SW620 cells were suspended in PBS containing the diluted supernatants of MLTC or control medium, and placed into round-shaped chambers that consisted of a ring covered with cellulose ester filters (pore size, 0.45 μm; Millipore) on both sides. These chambers were implanted into a dorsal air sac produced in female BALB/c *nu/nu* mice by the injection of 10 ml of air. Five mice in each group were sacrificed on day 5, and the formation of a dense capillary network in s.c. regions was examined under a dissecting microscope. The neovascularization was assessed semiquantitatively by counting the number of cork screw vessels. For each slide, a total of three fields at a magnification of ×4 were selected at random, and the scores were averaged.

### *In vivo tumor growth and determination of microvessels*

Female BALB/c and SCID mice were s.c. implanted with $2 \times 10^6$ Colon-26 cells. When tumors grew to ~5–6 mm in diameter, the mice were randomly assigned into three groups and a 100 μl of solution containing $1 \times 10^8$ PFU of dl312 or OBP-301, or PBS was injected into the tumor on days 1, 3, and 5. Tumors were measured for perpendicular diameters every 3 or 4 days, and tumor volume (in cubic millimeters) was calculated using the following formula: $a \times b^2 \times 0.5$, where $a$ is the longest diameter, $b$ is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. For histological analysis, 2 wk after treatment, the tumors were harvested, embedded in Tissue Tek (Sakura), cut into 5 μm-thick sections, and assessed by a standard H&E and immunohistochemical staining using a rat anti-mouse mAb against CD31 (BD Pharmingen). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.

### *In vivo inhibition of IFN-γ with neutralizing Abs*

For neutralizing IFN-γ, mice were i.p. administered 200 μg of rat anti-mouse IFN-γ mAb (XMG1.2; BD Pharmingen) 1 day before the first injection of OBP-301 and on days 1 and 3 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG1 (BD Pharmingen).

### *Statistical analysis*

Determination of significant differences among groups was assessed by calculating the value of Student's t test using the original data analysis. Statistical significance was defined at $p < 0.01$.

## Results

### *Effect of OBP-301-infected human colorectal cancer cells on PBMC in vitro*

First, we examined whether OBP-301 infection affects the viability of human colorectal cancer cells using the XTT assay. SW620 cells were either mock-infected with culture medium or infected with OBP-301 at an MOI of 1 or 10. As shown in Fig. 1*A*, OBP-301 infection induced death of SW620 cells in a dose-dependent manner. Next, we examined the ability of OBP-301-infected oncolytic cells to stimulate PBMC in MLTC. For this purpose, SW620 cells (HLA-A02/A24) treated with 10 MOI of OBP-301 for 72 h were cocultured with HLA-matched PBMC obtained from HLA-A24$^+$ healthy volunteers at a ratio of 1:40. The production of IFN-γ in the supernatants was then explored by ELISA analysis at the indicated time points. PBMC incubated with OBP-301-infected oncolytic SW620 cells secreted large amounts of IFN-γ as early as 24 h after MLTC, whereas PBMC alone induced little IFN-γ secretion (Fig. 1*B*). The maximum level of IFN-γ was ~250 pg/ml. We previously confirmed that addition of OBP-301 alone without target tumor cells did not affect the cytokine secretion from PBMC into the supernatant, indicating that infection of OBP-301 itself had no apparent effect on PBMC (13). These results suggest that PBMC stimulated with oncolytic tumor cells preferentially secrete high-level IFN-γ.

### *Inhibition of in vitro and in vivo angiogenesis by MLTC supernatants with OBP-301-infected human tumor cells*

In the next step, we investigated the effects of MLTC supernatants with oncolytic SW620 tumor cells and HLA-matched PBMC on VEGF-induced angiogenesis in vitro. The addition of VEGF enhanced the formation of vascular-like structures of HUVECs, although tubule formation was almost absent without VEGF. This VEGF-induced angiogenesis was completely impaired by the addition of MLTC supernatants even at 1/4 dilution (Fig. 2). In contrast, although MLTC supernatants were confirmed to contain ~250 pg/ml IFN-γ, 10-fold more concentration of recombinant IFN-γ was needed to attenuate the tubule formation close to basal levels. The supernatants of PBMC

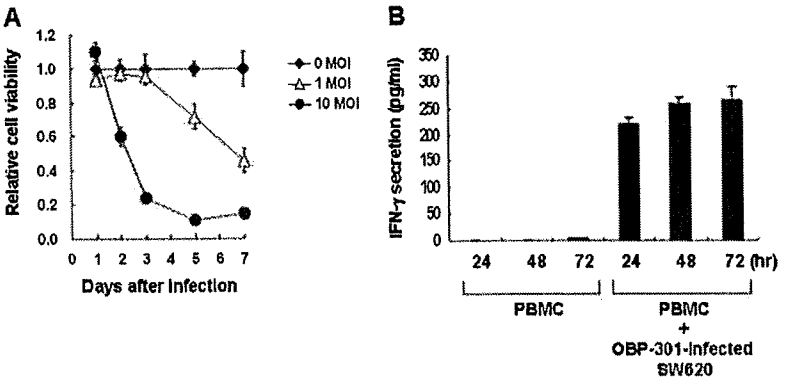


**FIGURE 1.** In vitro cytopathic effects of OBP-301 and IFN-γ secretion by oncolytic cell-stimulated PBMC. *A*, SW620 human colorectal cancer cells were infected with OBP-301 at indicated MOI values, and surviving cells were quantitated over 7 days by XTT assay. The cell viability of mock-treated cells on day 1 was considered 1.0, and the relative cell viability was calculated. Data are mean ± SD of triplicate experiments. *B*, IFN-γ concentrations in the supernatants of MLTC analyzed by ELISA. SW620 cells were treated with 10 MOI of OBP-301 for 72 h, and then cocultured with PBMCs obtained from HLA-A24$^+$ healthy volunteers for the indicated time periods in MLTC. The culture supernatants were harvested and tested by ELISA for IFN-γ concentrations. As a control, the supernatants of PBMC alone were also examined. Data are mean ± SD of triplicate experiments.

alone had no effect on in vitro angiogenesis. These results suggest that MLTC supernatants may contain more antiangiogenic factors in addition to IFN-γ.

We also assessed whether MLTC supernatants inhibited in vivo angiogenesis induced by human cancer cells. SW620 cells in PBS containing supernatants of OBP-301-infected SW620 cells, PBMC, or both, which were packed into membrane chambers, were implanted into a dorsal air sac produced in *nu/nu* mice. The chambers consisted of membranes that allowed the passage of macromolecules such as IFN-γ, but not cells. Five days after implantation, neovascularization, as demonstrated by the development of capillary networks and curled microvessels in addition to the preexisting vessels, occurred in the dorsal subcutis touched by the chamber, which contained SW620 cells alone. The addition of MLTC supernatants, however, reduced the size and tortuosity of the preexisting vessels, and significantly reduced the development of curled microvessels (Fig. 3). Although the preexisting vessels became thinner by supernatants of OBP-301-infected SW620 cells or PBMC, the number of curled microvessels, which is characteristic of tumor neovasculature, was consistent in these two groups with that in the group compared with SW620 cells alone. Thus, MLTC supernatants exhibited a profound antiangiogenic activity in vivo.

*Involvement of host immune activity on antiangiogenic effect of OBP-301*

The finding that OBP-301-infected tumor cells stimulated PBMC to produce antiangiogenic factors prompted us to study whether immunodeficiency of host animals could affect the antitumor effect of OBP-301 in vivo. When $2 \times 10^6$ Colon-26 murine colon adenocarcinoma cells were inoculated s.c. into BALB/c and SCID mice, palpable tumors appeared in 100% of the mice within 2 wk after tumor injection. Fourteen days after tumor inoculation, animals bearing Colon-26 tumors with a diameter of 5–6 mm were treated with the direct intratumoral injection of $10^8$ PFU OBP-301 every 2 days for three cycles. As shown in Fig. 4, treatment with OBP-301 resulted in a significant growth suppression compared with tumors injected with PBS at least for 12 days starting on day 4 after last virus injection ($p < 0.01$) in BALB/c mice; however, OBP-301-mediated antitumor effect was partially impaired in SCID mice, as significant inhibition was observed only for 6 days starting on day 10. Intratumoral injection of replication-deficient dl312 adenovirus had no effect on the tumor growth in BALB/c or SCID mice (data not shown). These results indicate the partial involvement of the host immune system in the OBP-301-mediated antitumor effect.

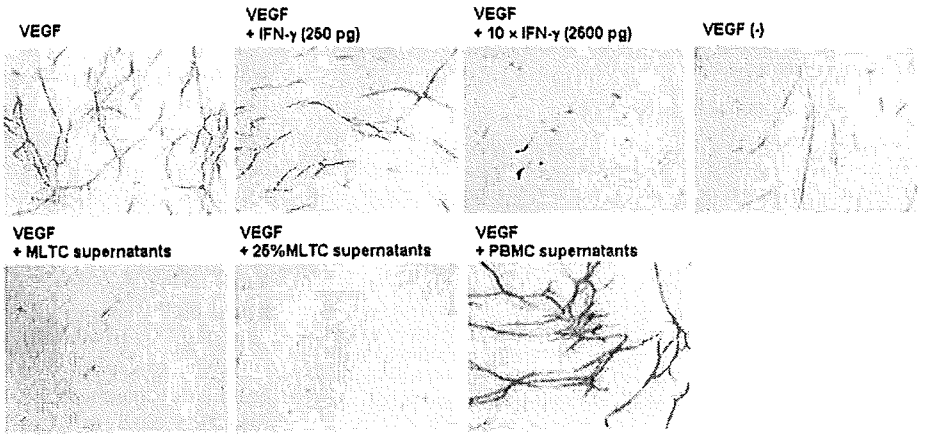


**FIGURE 2.** Inhibition of in vitro angiogenesis by the supernatants of OBP-301-infected oncolytic cells and PBMC. HUVECs were incubated in a medium containing the supernatants of MLTC obtained 72 h after coculture with OBP-301-infected oncolytic cells and PBMC or recombinant IFN-γ in the presence or absence of VEGF (10 ng/ml). The formation of the capillary network was confirmed by staining with anti-human CD31 Ab on day 11. Representative images depicting formation of capillary-like tube structures by HUVECs are shown. Original magnification is at ×40.

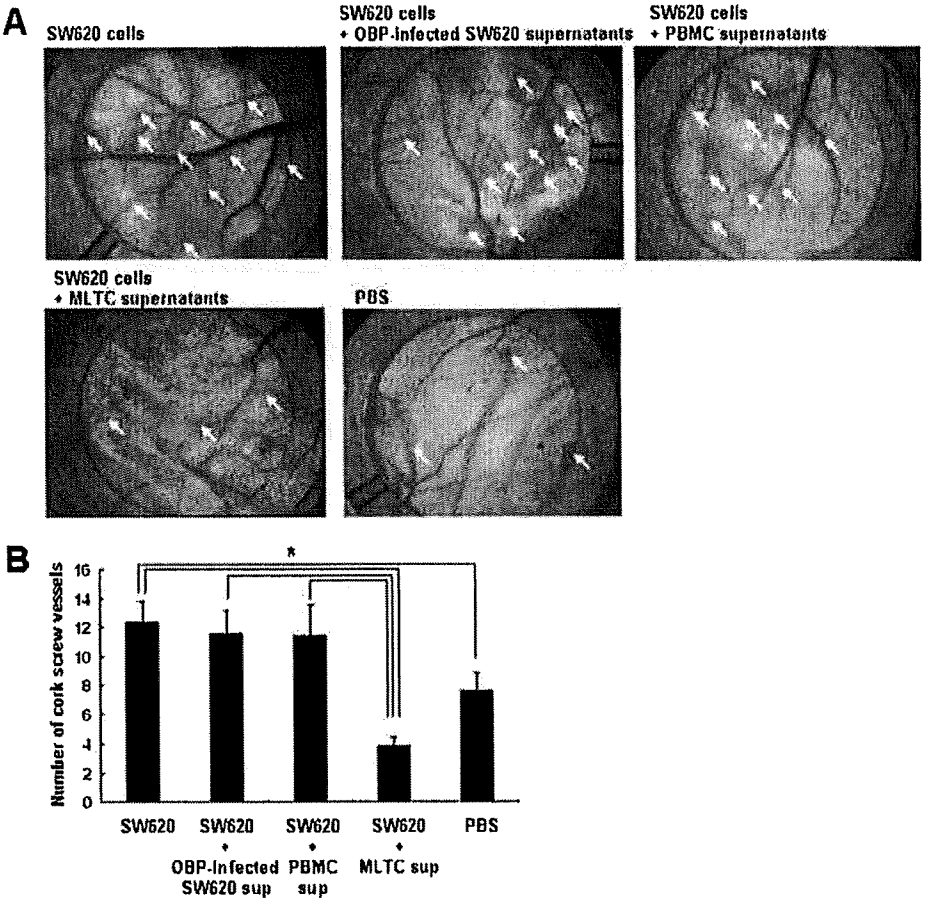


**FIGURE 3.** Inhibition of tumor cell-mediated in vivo angiogenesis by the supernatants of OBP-301-infected oncolytic cells and PBMC. *A*, SW620 human colorectal tumor cells at a density of $2 \times 10^6$ were placed in a diffusion chamber in PBS containing the diluted supernatants of MLTC obtained 72 h after coculture with OBP-301-infected oncolytic cells and PBMC or control mediums, and it was implanted into a dorsal air space produced in BALB/c *nu/nu* mice on day 0. Mice were sacrificed on day 5, and the chamber was removed from the s.c. tissue. A new ring without filters was placed on the same site to mark the position of the chamber. The capillary networks developed inside the rings were photographed to determine the effect of treatments. Representative images of treatment groups are shown. Curled microvessels are shown (arrow). *B*, The number of cork screw vessels was semiquantitatively counted to assess the neovascularization. Data are mean ± SD. *, $p < 0.01$. Similar results were observed in two independent experiments conducted in triplicate.

*Antiangiogenic effect of OBP-301 on syngenic and immunodeficient murine tumor models*

When Colon-26 s.c. tumors implanted in BALB/c mice were injected with PBS, replication-deficient dl312 adenovirus, or OBP-301. Macroscopically, tumors treated with OBP-301 were consistently smaller than those of the other two cohorts of mice 14 days after last virus injection (Fig. 5*A*). Furthermore, a reddish area was noted on the tumor surface on two of six mice treated with OBP-301, indicating virus-induced intratumoral necrosis of tumor cells in vivo.

To better understand the mechanisms underlying the induction of necrosis following OBP-301 treatment, histologic and immunohistochemical analyses were performed on Colon-26 tumors harvested 14 days after last injection. A standard H&E staining demonstrated the presence of many vessels in tumors injected with PBS or dl312. However, OBP-301-treated tumors showed few vessels. In addition, massive tumor cell death and cellular infiltrates at the central portions of the tumors were observed where OBP-301 was injected (Fig. 5*B*). Immunohistochemical staining of tumor sections with the Ab for CD31 Ag, an endothelial cell marker, also revealed that Colon-26 tumors injected with OBP-301 displayed very few and extremely small blood vessels (Fig. 5*C*). In contrast, OBP-301 injection could not apparently reduce the vessel numbers on Colon-26 tumors implanted in SCID mice (Fig. 5*D*). These in vivo studies demonstrated that inhibition of angiogenesis due to the stimulation of host immune system might be an important mechanism of OBP-301-mediated in vivo antitumor effect.

*Contribution of in vivo IFN-γ production to the OBP-301-mediated antiangiogenic effects*

Finally, to determine whether IFN-γ is involved in OBP-301-mediated antiangiogenic effects, in vivo neutralizing experiments were performed by using anti-IFN-γ mAb or isotype-matched control mAb. Angiogenesis was reduced by intratumoral injection of

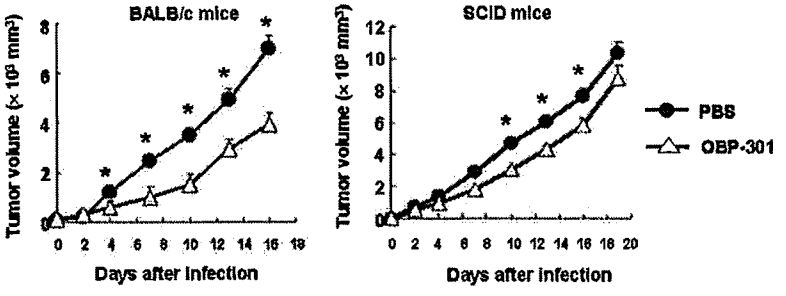


**FIGURE 4.** Antitumor effects of intratumorally injected OBP-301 against Colon-26 murine colon adenocarcinoma tumors in syngenic immunocompetent BALB/c and immunodeficient SCID mice. Colon-26 cells ($2 \times 10^6$ cells/each) were injected s.c. into the right flank of mice. OBP-301 ($1 \times 10^8$ PFU/body) was administered intratumorally for three cycles every 2 days. PBS was used as a control. Six mice were used in each group. Tumor growth was expressed by tumor mean volume ± SD. *, $p < 0.01$.

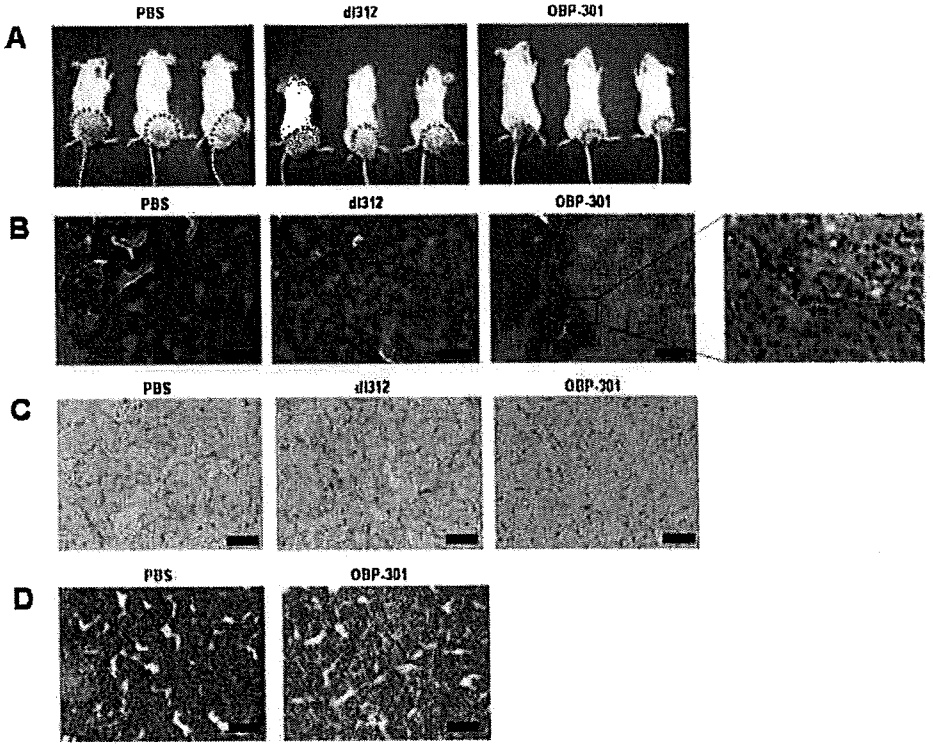


**FIGURE 5.** Macroscopic and histopathological analysis of Colon-26 tumors treated intratumorally with OBP-301. Colon-26 cells ($2 \times 10^6$ cells/each) were injected s.c. into the right flank of syngenic BALB/c mice and SCID mice and OBP-301 ($1 \times 10^8$ PFU/body) was administered intratumorally for three cycles every 2 days as described in Fig. 4. *A*, Macroscopic appearance of Colon-26 tumors on BALB/c mice 14 days after treatment. Note the reddish area on the tumor surface in two mice treated with OBP-301. *B*, Tumor sections were obtained from BALB/c mice 14 days after final administration of OBP-301. Frozen sections of tumors were stained with H&E. Scale bar represents 100 $\mu$m, and magnification is $\times$100. Magnified view of the boxed region in *B* is shown. The area with cellular infiltrates is indicated with the green dotted line. *C*, Blood vessel formation in Colon-26 tumors injected with OBP-301. Frozen sections of the tumors were also probed with an Ab against CD31. Scale bar represents 50 $\mu$m, and magnification is a $\times$200. *D*, Tumor sections were obtained from SCID mice 14 days after final administration of OBP-301. Frozen sections of tumors were stained with H&E. Scale bar represents 100 $\mu$m, and magnification is at $\times$100 magnification.

OBP-301 on Colon-26 tumors; this antiangiogenic effect, however, could be partially inhibited in the presence of anti-IFN-$\gamma$ mAb (Fig. 6). Treatment with control IgG1 had no effect on the antiangiogenic effects of OBP-301. These results suggest that IFN-$\gamma$ may be one of the important factors for OBP-301 to inhibit angiogenesis in vivo.

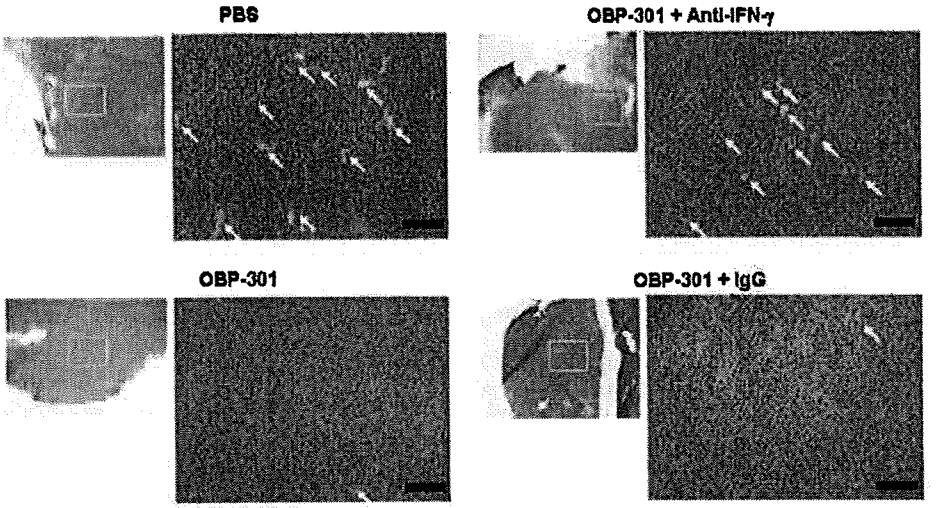


**FIGURE 6.** Effects of anti-IFN-$\gamma$ Abs on angiogenesis in Colon-26 tumors. Colon-26 cells ($2 \times 10^6$ cells/each) were injected s.c. into the right flank of syngenic BALB/c mice and OBP-301 ($1 \times 10^8$ PFU/body) was administered intratumorally for three cycles every 2 days as described in Fig. 4. Mice were administered 200 $\mu$g of anti-IFN-$\gamma$ mAb (XMG1.2) i.p. to neutralize IFN-$\gamma$ 1 day before the first injection of OBP-301 and on days 1 and 3 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG1 or PBS. Frozen sections of tumors obtained 14 days after final administration of OBP-301 were stained with H&E. Magnified view (*right*) of the boxed region (*left*). Microvessels are shown (arrow). Scale bar represents 50 $\mu$m, and magnification is at $\times$200.

## Discussion

The tumor vasculature provides a new and attractive target for cancer therapy because of the reliance of most tumor cells on an adequate vascular supply for their growth and survival. Although the beneficial effects of novel antiangiogenic agents such as bevacizumab have been recently shown (15), regulation of endogenous antiangiogenic mediators may be another approach to inhibit angiogenesis. In the present study, we showed that OBP-301 infection and replication induced cytolysis of tumor cells with subsequent stimulation of host immune cells, which in turn inhibited tumor angiogenesis in vivo. Treatment of established murine colon tumors with intratumoral injection of OBP-301 resulted in a significant antitumor response characterized by extensive necrosis and reduced vascularity.

We reported previously that wild-type *p53* tumor suppressor gene transfer by a replication-deficient adenovirus vector (Advexin) could have antiangiogenic effects. The effects could be through down-regulation of angiogenic factor VEGF and up-regulation of antiangiogenic factor BAI1 because tumor p53 protein is a potent transcriptional factor (16, 17). In contrast, OBP-301 contains no therapeutic genes such as *p53* and, therefore, its infection may not directly influence the angiogenic property of infected tumor cells. However, because viral infection is known to trigger innate and adaptive immune responses presumably through the release of proinflammatory cytokines (18–20), local administration of OBP-301 might affect the tumor microenvironment, thus explaining the potential therapeutic benefit on tumor angiogenesis. In fact, dying tumor cells infected with OBP-301 promoted the production of Th1 cytokines by PBMC such as IFN-γ, which is one of the most potent antiangiogenic factors (21, 22) (Fig. 1). Viral infection itself has been reported to activate dendritic cells to secrete pro- or anti-inflammatory cytokines (23); our preliminary experiments, however, demonstrated that OBP-301 alone had no effect on cytokine production by PBMC (13), indicating that OBP-301 itself may be less infective or stimulatory to PBMC. The result is consistent with our previous finding that OBP-301 attenuated replication as well as cytotoxicity of human normal cells (9, 10). Moreover, OBP-301-infected tumor cells, but not untreated tumor cells, enhanced IFN-γ-inducible proteasome activator PA28 expression in the presence of PBMC (13), indicating that only dying tumor cells could trigger IFN-γ production by PBMC.

IFN-γ has been also known to inhibit tumor angiogenesis through the subsequent stimulation of secondary mediators, including monokine induced by IFN-γ and IFN-inducible protein 10 (24). Indeed, the observation that the supernatants of PBMC cocultured with OBP-301-infected human colorectal cancer cells exhibited a more profound antiangiogenic effect than recombinant IFN-γ (Fig. 2) suggests that other factors in addition to IFN-γ, which may not be related to IFN-γ, play important roles in inhibition of tumor cell-mediated angiogenesis. For example, we also found that oncolytic cells stimulated PBMC to secrete IL-12, which is an inducer of IFN-γ as well as an antiangiogenic factor, into the culture supernatants (13). The supernatants of neither virus-infected tumor cells alone nor PBMC alone were more antiangiogenic compared with those of MLTC in vivo (Fig. 3). Therefore, the interaction of oncolytic cells and PBMC is required to produce antiangiogenic mediators and to inhibit in vivo angiogenesis following OBP-301 treatment. The question what kind of cells produce mediators for antiangiogenic effects is of interest. We reported previously that OBP-301 replication produced the endogenous danger signaling molecule, uric acid, in infected human tumor cells, which in turn stimulated dendritic cells to produce IFN-γ as well as IL-12 into the supernatants (13). The amount of IFN-γ produced by dendritic cells was ~40 pg/ml, although 250 pg/ml IFN-γ was detected in the MLTC supernatants (Fig. 1*B*), indicating that other cell types may contribute to IFN-γ production. Lymphocytes that promote innate immunity (i.e., NK cells) as well as classical $CD4^+$ and $CD8^+$ T cells are also know to produce IFN-γ (25). Thus, dendritic cells represent one of the sources of IFN-γ; however, IL-12 secreted from dendritic cells activated with OBP-301-infected tumor cells might trigger these cells to produce IFN-γ.

To more directly evaluate the antiangiogenic effect of OBP-301, we used a syngenic BALB/c model established by s.c. inoculation of Colon-26 murine colon adenocarcinoma cells. OBP-301 is reported to have high infectivity and the potential to induce cell death in a variety of human cancer cells (9–12), whereas murine cells are relatively refractory to adenovirus infection due to the low expression of the coxsackievirus and adenovirus receptor. We have confirmed previously that telomerase-specific oncolytic adenovirus could infect and replicate in Colon-26 cells (12). Intratumoral administration of OBP-301 significantly inhibited the growth of Colon-26 tumors in syngenic immunocompetent BALB/c mice, although the magnitude of suppression was much less when compared with that in human tumor xenografts (9, 10). The finding that tumor growth suppression by OBP-301 was partially inhibited in immunodeficient SCID mice (Fig. 4) indicates that the host immune system could be partially responsible for the antitumor effect of OBP-301. Histopathologic analysis revealed that the presence of the immune cell infiltrates and the massive necrosis in Colon-26 tumors are exclusively due to the tumor-specific viral replication because dl312-injected tumors showed neither cellular infiltrates nor tissue damages (Fig. 5*B*). In view of the fact that a cellular infiltration could be still observed as late as 14 days after the last OBP-301 injection, immune responses are likely to be induced by oncolytic tumor cells. Furthermore, as expected, tumors injected with OBP-301 formed less blood vessels than mock- or dl312-treated tumors (Fig. 5, *B* and *C*), suggesting that inhibition of angiogenesis by infiltrating cell-secreted mediators partially elicits the antitumor activity of OBP-301. In contrast, antiangiogenic effect of OBP-301 was impaired in SCID mice (Fig. 5*D*), indicating that host immune cells are necessary for this function of OBP-301. Moreover, IFN-γ is considered to be partially responsible for the antiangiogenic effects of OBP-301 because in vivo neutralization of IFN-γ by anti-IFN-γ mAb increased angiogenesis on Colon-26 tumors (Fig. 6).

It remains to be studied whether OBP-301-infected oncolytic cells are capable of inhibiting the growth of distant tumors. Circulating inhibitors of angiogenesis such as angiostatin and endostatin can suppress the growth of remote metastases (26). The observation that none of mice treated with OBP-301 showed signs of viral distress (ruffled fur, weight loss, lethargy, or agitation) as well as histopathologic changes in any organs at autopsy (data not shown) suggests that the cytokine secretion by oncolytic cell-stimulated immune cells might be local rather than systemic. Thus, it is unlikely that locally produced antiangiogenic factors interfere with the distant tumor growth, although the circulating virus itself can infect and replicate in metastatic tumors. This question is being currently investigated in our laboratory.

In conclusion, we provide for the first time evidence that oncolytic virotherapy induces novel antiangiogenic effect by stimulating host immune cells to produce antiangiogenic mediators such as IFN-γ. Our data suggest that the antitumor effect of OBP-301 might be both direct and indirect.

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## Disclosures

The authors have no financial conflict of interest.

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# Telomerase-Specific Virotheranostics for Human Head and Neck Cancer

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**Abstract**

**Purpose:** Long-term outcomes of patients with squamous cell carcinoma of the head and neck (SCCHN) remain unsatisfactory despite advances in combination of treatment modalities. SCCHN is characterized by locoregional spread and it is clinically accessible, making it an attractive target for intratumoral biological therapies.

**Experimental Design:** OBP-301 is a type 5 adenovirus that contains the replication cassette in which the human telomerase reverse transcriptase promoter drives expression of the *E1* genes. OBP-401 contained the replication cassette and the green fluorescent protein (*GFP*) gene. The antitumor effects of OBP-301 were evaluated *in vitro* by the sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate assay and *in vivo* in an orthotopic xenograft model. Virus spread into the lymphatics was also orthotopically assessed by using OBP-401.

**Results:** Intratumoral injection of OBP-301 resulted in the shrinkage of human SCCHN tumors orthotopically implanted into the tongues of BALB/c *nu/nu* mice and significantly recovered weight loss by enabling oral ingestion. The levels of GFP expression following *ex vivo* infection of OBP-401 may be of value as a positive predictive marker for the outcome of telomerase-specific virotherapy. Moreover, whole-body fluorescent imaging revealed that intratumorally injected OBP-401 could visualize the metastatic lymph nodes, indicating the ability of the virus to traffic to the regional lymphatic area and to selectively replicate in neoplastic lesions, resulting in GFP expression and cell death in metastatic lymph nodes.

**Conclusions:** These results illustrate the potential of telomerase-specific oncolytic viruses for a novel therapeutic and diagnostic approach, termed theranostics, for human SCCHN.

Cancer remains a leading cause of death worldwide despite improvements in diagnostic techniques and clinical management (1, 2). An estimated 500,000 patients worldwide are diagnosed with squamous cell carcinoma of the head and neck (SCCHN) annually. This aggressive epithelial malignancy is associated with a high mortality rate and severe morbidity among the long-term survivors (3). Current treatment strategies for advanced SCCHN include surgical resection, radiation, and cytotoxic chemotherapy. Although a combination of these modalities can improve survival, most patients eventually experience disease progression that leads to death; disease progression is often the result of intrinsic or acquired resistance to treatment (4, 5). A lack of specificity for tumor cells is the primary limitation of radiotherapy and chemotherapy. To improve the therapeutic index, there is a need for anticancer agents that selectively target only tumor cells and spare normal cells.

Replication-selective tumor-specific viruses present a novel approach for cancer treatment (6, 7). We reported previously that telomerase-specific replication-competent adenovirus (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase (hTERT) promoter element drives the expression of *E1A* and *E1B* genes linked with an IRES, induced selective E1 expression, and efficiently killed human cancer cells but not normal cells (8–10). We also found that intratumoral injection of telomerase-specific replication-selective adenovirus expressing the green fluorescent protein (*GFP*) gene (OBP-401, TelomeScan) causes viral spread into the regional lymphatic area with subsequent selective replication in

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## Translational Relevance

Despite new therapeutic modalities, long-term outcomes of patients with squamous cell carcinoma of the head and neck (SCCHN) remain unsatisfactory. Thus, the development of efficient treatment methods to enable the reduction of tumors in these patients is clearly imperative. Tumor-targeted oncolytic viruses have the potential to selectively infect target tumor cells, multiply, and cause cell death and release of viral particles, leading to the spread of viral-mediated antitumor effects. We developed a telomerase-specific oncolytic adenovirus OBP-301 (Telomelysin) as well as OBP-401 – expressing *GFP* gene (TelomeScan). Our data showed that telomerase-specific oncolytic viruses can be effective to kill human SCCHN cells *in vitro* and *in vivo* as well as to identify the patients who will likely benefit from virotherapy, suggesting that an oncolytic virus-based approach exhibited desirable features of a novel "virotheranostics," the combination of a diagnostic assay with a therapeutic entity for human SCCHN. This is a preclinical study for the future clinical trials.

metastatic lymph nodes in *nu/nu* mice (11). Although up to 25% of patients with SCCHN develop distant metastasis to the lung, liver, or bone, lymph node metastases are more common in SCCHN patients (12); therefore, locoregional disease control with telomerase-specific oncolytic viruses may be a novel therapeutic strategy that is clinically applicable for the treatment of human SCCHN.

In the present study, we explore the therapeutic as well as diagnostic ability of telomerase-specific oncolytic viruses *in vitro* and *in vivo*. To this end, we adopted an orthotopic head and neck cancer xenograft model by inoculating human SCCHN cells into the tongues of *nu/nu* mice; this model resembles human SCCHN in a number of biological properties (13).

## Materials and Methods

*Cell lines and cell culture.* The human oral squamous carcinoma cell lines SAS-L, SCC-4, SCC-9, HSC-2, HSC-3, and HSC-4 were maintained *in vitro* as monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin (complete medium). The human non-small-cell lung cancer cell line H460 and the human esophageal cancer cell line TE8 were routinely propagated in monolayer culture in RPMI 1640 supplemented with 10% fetal bovine serum. The normal human lung diploid fibroblast cell line WI38 (JCRB0518) was obtained from the Health Science Research Resources Bank (Osaka, Japan) and grown in Eagle's MEM with 10% fetal bovine serum. The normal human lung fibroblast NHLF (TaKaRa Biomedicals) and the normal human embryonic lung fibroblast MRC-5 (RIKEN BioResource Center) were cultured according to the vendors' specifications.

*Adenoviruses.* The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin), in which the hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an IRES, was previously constructed and characterized (8–10). OBP-401 is a telomerase-specific replication-competent adenovirus variant with the replication cassette, and *GFP* gene under the control of the cytomegalovirus promoter was inserted into the E3 region for monitoring viral replication (11, 14). The viruses were purified by ultracentrifugation in cesium chloride step gradients, their titers were determined by a plaque-forming assay using 293 cells, and they were stored at -80°C.

*Cell viability assay.* An sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay was done to assess the viability of tumor cells. Human SCCHN cells (1,000 per well) were seeded onto 96-well plates 18 to 20 h before viral infection. Cells were then infected with OBP-301 at a multiplicity of infection (MOI) of 1, 10, 50, and 100 plaque-forming units (pfu) per cell. Cell viability was determined at the indicated time points by using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

*Fluorescence microplate reader.* Cells were infected with OBP-401 at the indicated MOI values in a 96-well black-bottomed culture plate and further incubated for the indicated time periods. GFP fluorescence was measured by using a fluorescence microplate reader (DS Pharma Biomedical) with excitation/emission at 485 nm/528 nm.

*Animal experiments.* SAS-L and HSC-3 human oral squamous cell carcinoma cells were harvested and suspended at a concentration of $5 \times 10^6$/mL in the medium. To generate an orthotopic head and neck cancer model, 6-wk-old female BALB/c *nu/nu* mice were anesthetized and injected directly with 20 μL of cell suspension at a density of $10^5$ cells. The cells were injected into the right lateral border of the tongue with a 27-gauge needle. When the tumor grew to 2 to 3 mm in diameter ~5 to 7 days later, 20 μL of solution containing $1 \times 10^8$ pfu of OBP-301, OBP-401, or PBS were injected into the tumor. The perpendicular diameter of each tumor was measured every 3 d, and tumor volume was calculated by using the following formula: tumor volume ($mm^3$) = $a \times b^2 \times 0.5$, where $a$ is the longest diameter, $b$ is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. The body weights of mice were monitored and recorded. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University.

*In vivo fluorescence imaging.* *In vivo* GFP fluorescence imaging was acquired by illuminating the animal with a Xenon 150-W lamp. The reemitted fluorescence was collected through a long-pass filter on a Hamamatsu C5810 3-chip color charge-coupled device camera (Hamamatsu Photonics Systems). High-resolution image acquisition was accomplished by using an EPSON PC. Images were processed for contrast and brightness with the use of Adobe Photoshop 4.0.1J software (Adobe). A fluorescence stereomicroscope (SZX7; Olympus) was also used to visualize GFP-positive tissues.

*Statistical analysis.* The statistical significance of the differences in the *in vitro* and *in vivo* antitumor effects of viruses was determined by using the Student's $t$ test (two-tailed). The antitumor effect viruses on orthotopically implanted tumors in nude mice were assessed by plotting survival curves according to the Kaplan-Meier method. $P$ values <0.05 were considered statistically significant.

## Results

*In vitro cytopathic efficacy of OBP-301 on human SCCHN cell lines.* We examined the cytopathic effect of OBP-301, which is an attenuated adenovirus in which the hTERT promoter element drives expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (IRES; Fig. 1A), on various human SCCHN cell lines by the XTT cell viability assay. OBP-301 infection induced cell death in human SCCHN cells in a dose-dependent manner; the sensitivity, however, varied among different cell lines (Fig. 1B). The $ID_{50}$ values calculated from the dose-response curves confirmed that SAS-L cells could be efficiently killed by OBP-301 at an multiplicity of infection (MOI) of <150 ($ID_{50}$ = 148), whereas HSC-3 cells were less sensitive to OBP-301 ($ID_{50}$ = 500; Fig. 1C).