Table III. *Characteristics of patients treated with WT1 peptide with CpG.*

No.	Diagnosis	Gender	Age (years)	HLA	Completion of therapy (times and duration of vaccination)	RECIST (at 8 weeks)	Adverse events (>G3)	Causality
CpG 1	Cervical cancer	Female	39	A2402	Yes (9 times: 9 weeks)	PD	None	
CpG 2	Epithelioid sarcoma	Female	54	A2402	Yes* (47 times: 65 weeks)	SD	None	
CpG 3	Rectal cancer	Male	55	A0206	Yes (9 times: 9 weeks)	PD	None	
CpG 4	Pancreatic cancer	Female	67	A2402	Yes (19 times: 19 weeks)	SD	None	
CpG 5	Colon cancer	Female	55	A2402	Yes (9 times: 9 weeks)	PD	Gastrointestinal bleeding	No
CpG 6	Lung cancer	Female	61	A0206	Yes (15 times: 19 weeks)	SD	None	
CpG 7	Lung cancer	Male	71	A2402	No: poor general condition (6 times)	PD	None	
CpG 8	Papilla cancer	Female	54	A2402	Yes (18 times: 21 weeks)	SD	None	
CpG 9	Ovarian cancer	Female	52	A2402	Yes (16 times: 17 weeks)	SD	None	
CpG 10	Pancreatic cancer	Male	35	A2402	Yes* (32 times: 43 weeks)	SD	None	

PD: Progressive disease; SD: stable disease. *Continuous administration.

Clinical outcome. Clinical outcome data for all patients categorized by immunoenhancing adjuvants are summarized in Tables I-III. For primary analysis, clinical response was assessed according to the RECIST criteria. The disease control rate of cohort 1, 2 and 3 in the initial two months (the clinical trial period) was 20%, 25% and 60%, respectively.

Discussion

In this study, patients with HLA-A*2402, A-*0201 or A-*0206 were immunized by injecting the WT1 peptide, added with GM-CSF or CpG-ODN, intradermally once every week for eight weeks and evaluated the safety and efficacy. As vaccine-related adverse events, grade 1 and 2 injection-site reactions were observed within 24-72 h. The intensity of the skin reaction was augmented by repeated vaccinations, suggesting the reaction was a delayed-type hypersensitivity reaction towards WT1 peptide. It is reasonable to believe that the skin toxicity of vaccine therapy at the injection sites is due to the natural course of the immune activation. Therefore, the treatment was considered to be well-tolerated.

The potential of the WT1 protein as a cancer antigen is of considerable interest. Many cancer antigens are relatively easy to isolate because of advances in tumor and molecular immunology. Nevertheless, determination of the clinical efficacy of these cancer antigens can be achieved only by clinical studies that are very laborious, and moreover, only clinical studies can determine their potential as cancer antigens. It is therefore a laborious and time-consuming work to determine and confirm the clinical usefulness of a given cancer antigen. Recently, 75 representative cancer antigens including WT1 were prioritized (21). The selection and prioritization of these antigens were performed according to the following criteria: (i) therapeutic function, (ii) immunogenicity, (iii) role of the antigen in oncogenicity, (iv) specificity, (v) expression level and percentage of antigen-positive cells, (vi) stem cell expression, (vii) number of patients with antigen-positive cancer, (viii) number of antigenic epitopes, and (ix) cellular location of antigen expression. Although none of the 75 cancer antigens had all the characteristics of the ideal cancer antigen, WT1 was at the top of the ranking. This finding can be expected to promote the development of WT1-targeted cancer immunotherapy.

The cytokine GM-CSF is involved in the recruitment and maturation of antigen-presenting cells and has been incorporated into numerous clinical studies with cancer vaccines to enhance immune responses (22-24). Previous studies have revealed the safety of therapeutic application using WT1 peptides in Montanide adjuvant with GM-CSF in patients with myeloid malignancy (25-27) and mesothelioma (28). The present study also demonstrated that GM-CSF was safe as adjuvant in patients with various types of cancer. However, the disease control rate in the group of patients treated with the WT1 peptide vaccine with GM-CSF (cohort 2) (25%), was only slightly better than or comparable to that of the group treated with the WT1 peptide alone (cohort 1) (20%).

CpG-ODN can be synthesized for therapeutic use and has been evaluated as a vaccine adjuvant in several clinical studies. CpG-ODN acts as a very potent adjuvant in combination with Montanide, and has been shown to promote strong antigen-specific $CD8^+$ T-cell responses in patients with melanoma (29, 30). In addition, intradermal injections of CpG-ODN around the excision site of melanoma activate the plasmacytoid DCs and myeloid DCs, and reduce the number of regulatory T-cells in sentinel lymph nodes (31, 32). Vaccination with NY-ESO-1 peptide in combination with CpG-ODN was reported to successfully induce NY-ESO-1-specific immune responses and revealed clinical benefit by extending survival in patients with NY-ESO-1-positive cancer (33). As established by the seminal

study of Iwahashi *et al.* (34), immunization with two kinds of squamous cell carcinoma-specific peptides, LY6K-177 and TTK-567, in combination with CpG-ODN, successfully elicited antigen-specific $CD8^+$ T-cell responses in patients with advanced esophageal squamous cell carcinoma. In addition, expression of interferon (IFN)-α and its related chemokines were up-regulated and, correspondingly, natural killer (NK) cells were activated. These results suggest that not only tumor-specific acquired immunity, but also innate immunity were enhanced by this vaccination.

CpG-ODN can stimulate both innate immunity and adoptive immune responses through endosomal TLR9, which is expressed in plasmacytoid DCs in humans. Plasmacytoid DCs produce high levels of type I interferons, as well as a variety of other cytokines and chemokines to promote Th1-like immune responses involving other cell types, including additional DC subsets, monocytes, NK cells, and neutrophils (35-37). Therefore, CpG-ODN is considered to play important roles as an adjuvant for cancer vaccines using epitope peptides.

In our study, we have shown that the disease control rate in the group of patients treated with the WT1 peptide vaccine with CpG-ODN (cohort 3) (60%), was much higher than that of the other groups. Recently, Hong *et al.* (38) revealed that idiotypic vaccine combined with CpG-ODN or IFN-α, but not GM-CSF, not only efficiently protected mice from developing myeloma, but also eradicated the already established myeloma. The therapeutic responses were associated with an induction of strong humoral immune responses, including anti-idiotypic antibodies, and cellular immune responses, including idiotype- and myeloma-specific $CD8^+$ CTLs, $CD4^+$ Th1 cells and memory T-cells in mice receiving idiotypic vaccine combined with CpG or IFN-α. Furthermore, idiotypic vaccine, combined with CpG or IFN-α induced idiotype- and tumor-specific memory immune responses that protected surviving mice from tumor reocenitence. Thus, these results clearly show that CpG is a better immune adjuvant than GM-CSF. However, our study was still a phase I trial, and we will determine whether the immune response to WT1 can be induced by this vaccine protocol in the next phase II study.

For decades, investigators have relied on modified WHO criteria (39) or, more recently, RECIST (20) to assess the clinical activity of anticancer agents. These standard criteria were designed to capture effects of cytotoxic agents and depend on tumor shrinkage to demonstrate activity. However, the response patterns seen with immunotherapeutic agents extend beyond those of cytotoxic agents and can manifest, for example, after a period of stable disease in which there is no tumor shrinkage, or after initial tumor burden, an increase in, or the appearance of new lesions (*e.g.* tumor-infiltrating lymphocytes) (40-43). This potential delayed detection of clinical activity on radiographic assessment may reflect the dynamics of the immune system, the time required for T-cell expansion followed by infiltration of the tumor, and a subsequent measurable antitumor effect. For example, our previous trial (8, 44) and other studies (40-43) of clinical cancer vaccines demonstrated that patients with stable or progressive disease may have subsequent tumor regression, or initial mixed responses, with regression in some lesions, while other lesions remain stable or progress.

Such patterns have been noted by many investigators; however, they were inconsistently included in publications or were not systematically captured because of the absence of suitable response criteria, which, in turn, did not allow for their clinical significance to be adequately studied (45). It has become evident that RECIST and WHO criteria may not offer a complete description of the response to immunotherapeutic agents, and therefore either adjusted or new criteria are needed (45).

Cancer immunotherapy is considered to be the fourth cancer therapy after the three major cancer therapies of surgery, chemotherapy and radiotherapy. It is thought that complete eradication of cancer stem cells is essential for the cure of cancer and that only immunotherapy is capable of killing non-dividing, quiescent cancer stem cells. Therefore, ideal and future immunotherapy should be started as soon as possible after the diagnosis of cancer and continued as long as possible, so that surgery, chemotherapy and radiotherapy can be performed under conditions of enhanced cancer immunity.

In conclusion, the addition of GM-CSF or CpG-ODN to a WT1 peptide vaccine, for patients with solid malignancy, was safe and apparently improved the effectiveness of clinical response.

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ORIGINAL ARTICLE

CD138-negative clonogenic cells are plasma cells but not B cells in some multiple myeloma patients

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Clonogenic multiple myeloma (MM) cells reportedly lacked expression of plasma cell marker CD138. It was also shown that CD19$^+$ clonotypic B cells can serve as MM progenitor cells in some patients. However, it is unclear whether CD138-negative clonogenic MM plasma cells are identical to clonotypic CD19$^+$ B cells. We found that *in vitro* MM colony-forming cells were enriched in CD138$^-$CD19$^-$CD38^{++} plasma cells, while CD19$^+$ B cells never formed MM colonies in 16 samples examined in this study. We next used the SCID-rab model, which enables engraftment of human MM *in vivo*. CD138$^-$CD19$^-$CD38^{++} plasma cells engrafted in this model rapidly propagated MM in 3 out of 9 cases, while no engraftment of CD19$^+$ B cells was detected. In 4 out of 9 cases, CD138$^+$ plasma cells propagated MM, although more slowly than CD138$^-$ cells. Finally, we transplanted CD19$^+$ B cells from 13 MM patients into NOD/SCID IL2R$\gamma c^{-/-}$ mice, but MM did not develop. These results suggest that at least in some MM patients CD138-negative clonogenic cells are plasma cells rather than B cells, and that MM plasma cells including CD138$^-$ and CD138$^+$ cells have the potential to propagate MM clones *in vivo* in the absence of CD19$^+$ B cells.

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Keywords: multiple myeloma; progenitor cells; CD138

INTRODUCTION

Multiple myeloma (MM) is characterized by the clonal expansion of malignant plasma cells.[1,2] The immunoglobulin gene sequences in MM plasma cells are somatically hyper-mutated and remain constant throughout the clinical course, suggesting that the disease arises from a post-germinal center B cell or a more differentiated cell.[3–5] Previous studies have found that MM patients harbor phenotypic B cells expressing the immunoglobulin gene sequence and the idiotype unique to the individual myeloma clone.[6–9] These findings imply that clonotypic B cells may be involved in the disease process but offer no definitive proof that B cells in fact correspond to the proliferating tumor compartment.

Clonogenic MM cells are thought to be responsible for disease progression[10,11] so that it is important to identify and target them. The first successful *in vitro* system capable of growing human MM colonies was described by Hamburger and Salmon.[10] They showed that the clonogenic frequency of clinical myeloma specimens ranged from 0.001 to 0.1% of BM cells from MM patients. In a later study utilizing methylcellulose media supplemented with lymphocyte conditioned media as growth factors, clonogenic MM progenitor cells were found in BM cells lacking expression of the plasma cell marker CD138.[11–15] It was further reported that rituximab inhibited MM colony formation[11] and that CD20$^+$ B cells from some MM patients could produce MM plasma cells in a 3-D culture *in vitro*,[16] which suggests that CD138$^-$ clonogenic MM cells might be B cells. However, it is still unclear whether clonogeic MM cells are B cells or plasma cells, because some CD38^{++} MM plasma cells lack CD138 expression.[17]

CD19$^+$ B cells isolated from MM patients could reportedly generate MM disease upon transplantation into NOD/SCID mice,[11,12,18,19] indicating that clonotypic CD19$^+$ B cells served as MM progenitor cells in these MM patients. However, B cell depletion by means of rituximab in MM patients was not clinically effective in most cases, at least for short periods, in which plasma cells did not express CD20.[20] It is therefore still unclear whether CD19$^+$ or CD20$^+$ clonogenic MM progenitor cells are responsible for disease progression and maintenance. Studies using the SCID-hu or rab models, which are SCID mice implanted with human fetal or rabbit bone fragments, respectively, to create a humanized or human-like microenvironment, suggested that malignant plasma cells have tumorigenic properties.[21–23]

For the development of effective treatment, it is important to know whether clonogenic cells in MM are B cells or plasma cells. In the study presented here, we aimed to clarify whether CD138$^-$ clonogenic MM cells are B cells or plasma cells by using an *in vitro* colony assay and two types of xeno-transplant assays.

MATERIALS AND METHODS

Patient samples

BM cells from MM patients were collected from iliac bone after the patients' informed consent had been obtained. Mononuclear cells were purified using Ficoll Paque (GE Healthcare, Piscataway, NJ, USA) and analyzed. Cord blood cells were obtained from the Keihan Cord Blood Bank (Osaka, Japan).

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A total of 50 patients diagnosed with multiple myeloma were used in this study, 16 of whom were subjected to *in vitro* clonogenic assay. Twelve patients were used for the SCID-rab experiments with un-fractionated BM cells, and the samples of nine patients were subjected to SCID-rab experiments with fractionated BM cells. Finally, Samples from 13 patients were used for transplants into NOG mice.

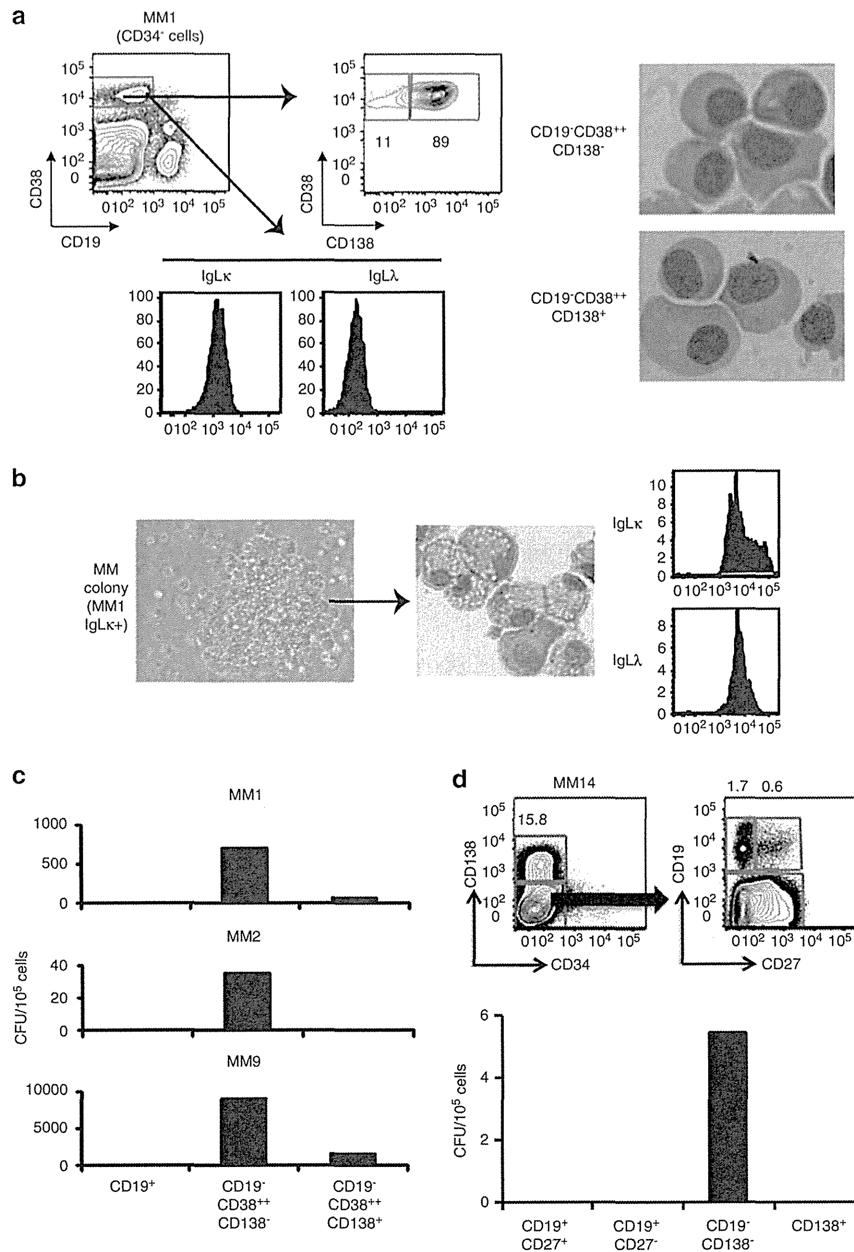

Figure 1. Clonogenic MM progenitor cells were enriched in the $CD19^{-}CD38^{++}CD138^{-}$ plasma cells. (**a**) FACS analysis of BM cells from an MM patient (MM1). $CD19^{-}CD38^{++}$ MM plasma cells were divided into two populations according to CD138 expression. Results of May-Giemsa staining of FACS-sorted $CD19^{-}CD38^{++}CD138^{-}$ and $CD19^{-}CD38^{++}CD138^{+}$ cells are also shown. (**b**) An MM colony derived from $CD19^{-}CD38^{++}CD138^{-}$ BM cells. May-Giemsa staining of cytospin specimens of MM colonies and FACS analysis for IgLκ/λ expression in cells from MM colonies are also shown. (**c**) *In vitro* colony assay with FACS-sorted $CD34^{-}CD19^{+}$, $CD34^{-}CD19^{-}CD38^{++}CD138^{-}$ and $CD34^{-}CD19^{-}CD38^{++}CD138^{+}$ BM cells from MM samples. (**d**) FACS analysis of the expression of CD34, CD138, CD19 and CD27 on BM cells from an MM patient. The results of an *in vitro* colony assay with FACS-sorted cells are also shown. All fractions were $CD34^{-}$.

Figure 2. Both $CD138^{-}$ and $CD138^{+}$ plasma cells, but not $CD19^{+}$ B cells, could engraft and propagate MM clones in the SCID-rab model (**a**) FACS analysis of rabbit BM transplanted with whole BM cells from an MM patient 12 weeks after transplant (Exp. 12, Table 1). MCD45, hCD19 and hCD38 denote mouse CD45, human CD19 and human CD38. Human $CD38^{++}$ cells were further analyzed for the expression of cytoplasmic IgLκ/λ or CD138. May-Giemsa staining of FACS-sorted $hCD38^{++}$ cells is also shown. (**b**) Transplantation of purified $CD138^{-}CD34^{-}$ or $CD138^{+}$ cells from MM BM cells into SCID-rab recipients (Exp.2, Table 2). Concentration of human IgLκ in serum at 12 weeks post transplant and the results of analysis of BM cells at 12 weeks ($CD138^{-}$) or 24 weeks ($CD138^{+}$) are shown.

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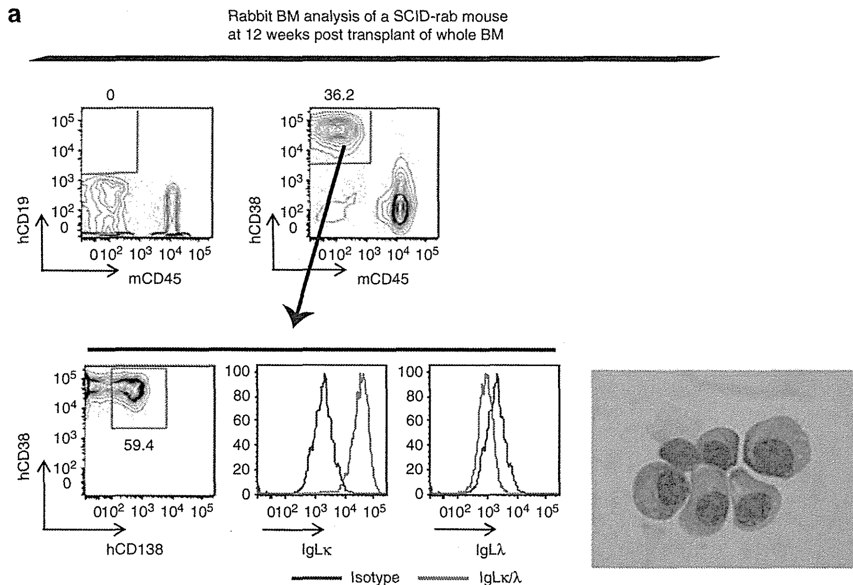
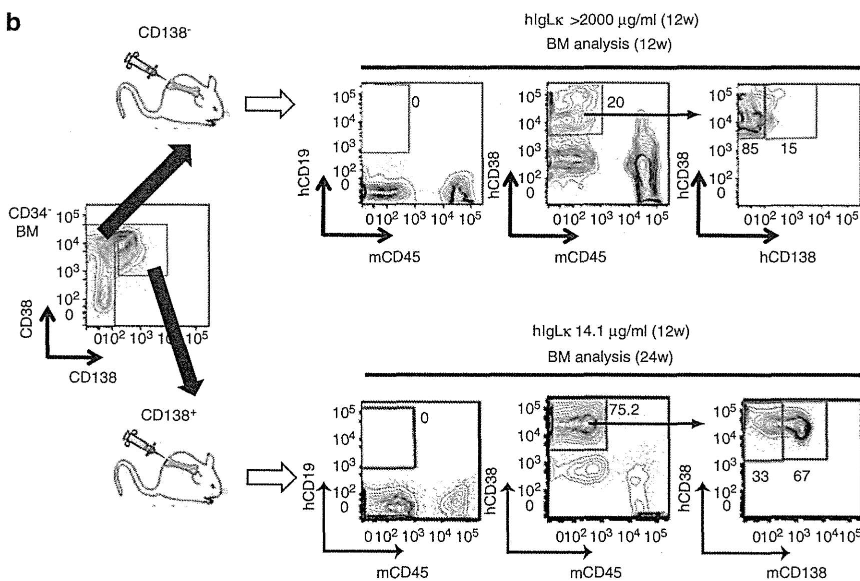
Table 1. SCID-rab experiments with whole BM cells from MM patients

Experiment	*Cell no.*	*% of CD38^{++} plasma cells*	*No. of plasma cells*	*Engraftment*[a]	*Serum human M protein in recipient mice (μg/ml)(10–16w)*
Exp 1	1×10^6	4.6	4.6×10^4	+	λ 324
Exp 2	3×10^6	16.6	5×10^5	−	
Exp 3	3.3×10^6	47.0	1.6×10^6	−	
Exp 4	5×10^6	4.8	2.4×10^5	+	κ 239
Exp 5	1×10^6	11.6	1.2×10^5	−	
Exp 6	3×10^6	6.3	1.9×10^5	−	
Exp 7	5×10^6	57.5	2.9×10^6	+	κ 104
Exp 8	5×10^6	75.0	3.8×10^6	−	
Exp 9	3×10^7	38.3	1.1×10^7	+	λ 25
Exp 10	4×10^6	4.0	1.6×10^5	−	
Exp 11	2.5×10^6	10.1	2.5×10^5	−	
Exp 12	1×10^7	52.8	5.3×10^6	+	N.A.[b]

[a]Engraftment was monitored by measuring IgLκ/λ and finally determined by FACS analysis of rabbit BM cells 12 or more weeks post transplant. [b]Engraftment of MM cells was examined only by FACS analysis





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Flow cytometry and cell sorting

Single cell suspensions from BM were treated with ACK solution (150 mM NH_4Cl, 10 mM $KHCO_3$) for 3 min on ice to lyse erythrocytes, then washed with PBS containing 2% FCS, incubated with 10% human AB serum for 20 min to prevent nonspecific antibody binding, and finally stained with fluorochrome-conjugated CD138 (MI15), CD38 (HB7), CD34 (8G12) and CD19 (H1B19) mAbs (BD Pharmingen, San Jose, CA, USA) on ice for 30 min. After washing, the cells were re-suspended in 1 µg/ml propidium iodide. Analysis and cell sorting were performed on FACS Aria (BD Biosciences, San Jose, CA, USA). The BD Cytofix/Cytoperm kit (BD Pharmingen) and phycoerythrin-conjugated anti-IgLκ(G20-193) or IgLλ(JDC-12) (BD Pharmingen) for staining cytoplasmic immunoglobulin were used according to the manufacturer's instructions.

Colony forming assay

Methylcellulose culture assays were performed in Methocult M3223 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 10% lymphocyte conditioned media, which was generated by culturing human peripheral blood mononuclear cells in RPMI1640 supplemented with 10%fetal bovine serum and 2.5 µg/ml PHA. Colonies were counted and scored on culture days 14–21.

SCID-rab model

SCID-rab mice were constructed as previously described.[23] The Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine approved the experimental procedures and protocols. Six- to eight-week-old CB.17/Icr-SCID mice were obtained from Crea Japan (Kanagawa, Japan) and 4-week-old New Zealand rabbits from Kitayama Rabesu (Nagano, Japan). The femurs and tibias from the rabbits were cut into two pieces with the proximal and distal ends kept closed. One piece of bone was inserted subcutaneously through a small (5–mm) incision, which was then closed with sterile surgical staples. Four to eight weeks after, BM cells from MM patients were injected directly into the implanted rabbit bone. The mice were periodically bled from the tail vein and changes in levels of circulating human immunoglobulin light chain (hIgL) of the M-protein isotype were used as an indicator of MM growth. Serum human immunoglobulin was measured by means of ELISA using the human kappa/lambda ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). To enrich $CD138^-CD34^-$ or $CD138^+$ cells, CD138-microbeads and CD34-microbeads kits (Miltenyi Biotech, Auburn, CA, USA) were used according to the manufacture's instruction.

Transplantation into NOG mice

Intra-BM transplantation was performed as previously reported.[24] Seven-week-old female NOD/Shi-scid, IL-2 Rγnull (NOG) mice (Central Institute for Experimental Animals, Kawasaki, Japan) irradiated with 200 cGy 4-24 h before transplantation were injected into the tibia with FACS-sorted PB or BM cells from the MM patients. Transplantation into newborn NOG mice was performed within 72 h after birth. The mice were irradiated with 100 cGy 4-24 h before transplantation and injected with sorted cells via the anterior facial vein.[25] Development of MM was monitored by measuring human immunoglobulin light chain (IgL)κ or λ by means of ELISA. Twelve or more weeks after transplantation, the mice were sacrificed and BM cells were collected from the tibias and femurs and analyzed by means of FACS. Cells were stained with human CD45 (HI30)-APC, CD138-PE, CD38-FITC, CD19-Cy7APC (BD Pharmingen) and mouse CD45 (30-F11)-Cy7PE (eBioscience, San Diego, CA, USA), followed by analysis using FACS.

RESULTS

Clonogenic cells are enriched in $CD19^-CD38^{++}CD138^-$ plasma cells of some MM patients

MM plasma cells expressing a monotypic immunoglobulin light chain, could be identified as $CD34^-CD19^-CD38^{++}$ cells by FACS analysis in the BM samples of most MM patients (Figure 1a). $CD34^-CD19^-CD38^{++}$ MM plasma cells were separated into $CD138^-$ and $CD138^+$ cells (Figure 1a). $CD34^-CD19^+$ B cells, $CD34^-CD19^-CD38^{++}CD138^-$ plasma cells, or $CD34^-CD19^-CD38^{++}CD138^+$ plasma cells were FACS-sorted and subjected to colony assay. An *in vitro* colony assay was performed in methylcellulose medium supplemented with lymphocyte conditioned media, as previously reported.[11] Formation of MM colonies consisting of plasma cells (Figure 1b) was detected in 3 out of 13 MM samples. In those 3 MM samples, the frequencies of colony formation in the $CD34^-CD19^-CD38^{++}CD138^-$ plasma cells were 700, 35, and 9053 colonies per 10^5 cells, while those in $CD34^-CD19^-CD38^{++}CD138^+$ plasma cells were much lower (Figure 1c) and $CD34^-CD19^+$ B cells did not form any MM colonies. These results indicate that clonogenic cells were found in BM cells lacking the expression of a plasma cell marker CD138, but only in $CD138^-CD19^-CD38^{++}$ plasma cells, not in $CD19^+$ B cells.

We further investigated whether colony-forming cells could be detected in $CD19^+CD27^+$ B cells, which are reportedly enriched with clonogenic cells.[12] Samples from 3 patients were examined, and MM colony-forming cells were detected in one. In that sample, MM colonies were formed not from $CD19^+CD27^+$ B cells, but from $CD19^-CD138^-$ cells (Figure 1d).

In the SCID-rab model, only $CD38^{++}$ MM plasma cells engrafted and expanded *in vivo* without engraftment of $CD19^+$ B cells

SCID-rab mice were constructed as previously reported.[23] A rabbit bone fragment was inserted under the skin of a SCID mouse more than 4 weeks before transplantation of MM cells. First, whole BM cells from MM patients were transplanted and engraftment of MM cells was monitored by measuring human IgLκ and λ in serum of

Table 2. SCID-rab experiments with $CD138^-$ or $CD138^+$ BM cells from MM patients

	Population	Cell no.	Serum human M protein (µg/ml) in recipient mice (12–24 w posttransplant)
Exp1	$CD138^-$	1.2×10^6	—
	$CD138^+$	1.6×10^6	—
Exp2	$CD138^-$	1×10^7	>2000
	$CD138^+$	1×10^7	14
Exp2-2[a]	$CD138^-$	2×10^6	>2000
	$CD138^+$	2×10^6	95
Exp3	$CD138^-$	4×10^6	—
	$CD138^+$	2×10^5	313
Exp4	$CD138^-$	2.6×10^6	>2000
	$CD138^+$	4×10^5	—
Exp5	$CD138^-$	1.6×10^6	—
	$CD138^+$	2×10^5	11
Exp6	$CD138^-$	2×10^6	>2000
	$CD138^+$	2×10^6	81
Exp7	$CD138^-$	4×10^6	—
	$CD138^+$	2×10^5	—
Exp8	$CD138^-$	2×10^6	—
Exp9	$CD138^-$	2×10^6	—
	$CD138^+$	2×10^4	—

[a]Secondary transplantation.

Figure 3. Neither B cells nor plasma cells from MM samples engrafted in NOG mice. (**a**) Analysis of cytoplasmic immunoglobulin light chain (cIgL) κ or λ expression in $CD38^{++}$ plasma cells or in $CD19^+$ cells from the MM BM sample used for exp. 6 in Table 3. Findings of FACS analysis of BM cells of NOG mice transplanted with $CD19^+$ B cells from the patient's BM (exp. 6-2 in Table 3). Corresponding data for an NOG mouse transplanted with cord blood-derived $CD34^+$ cells is shown for reference (exp. CB, Table3). Analyses were performed 12 weeks post-transplant. (**b**) Analysis of cytoplasmic immunoglobulin light chain (cIgL) κ or λ expression in $CD38^{++}$ plasma cells, or $CD19^+$ B cells from MM BM sample used for exp. 13 shown in Table 3. FACS analysis findings of NOG mice transplanted with $CD3^-$ BM cells from the patient sample 12 weeks after transplant. Expression of cytoplasmic IgLκ and λ in $CD38^{++}CD138^+$ cells was also analyzed to determine whether they were clonal MM cells.

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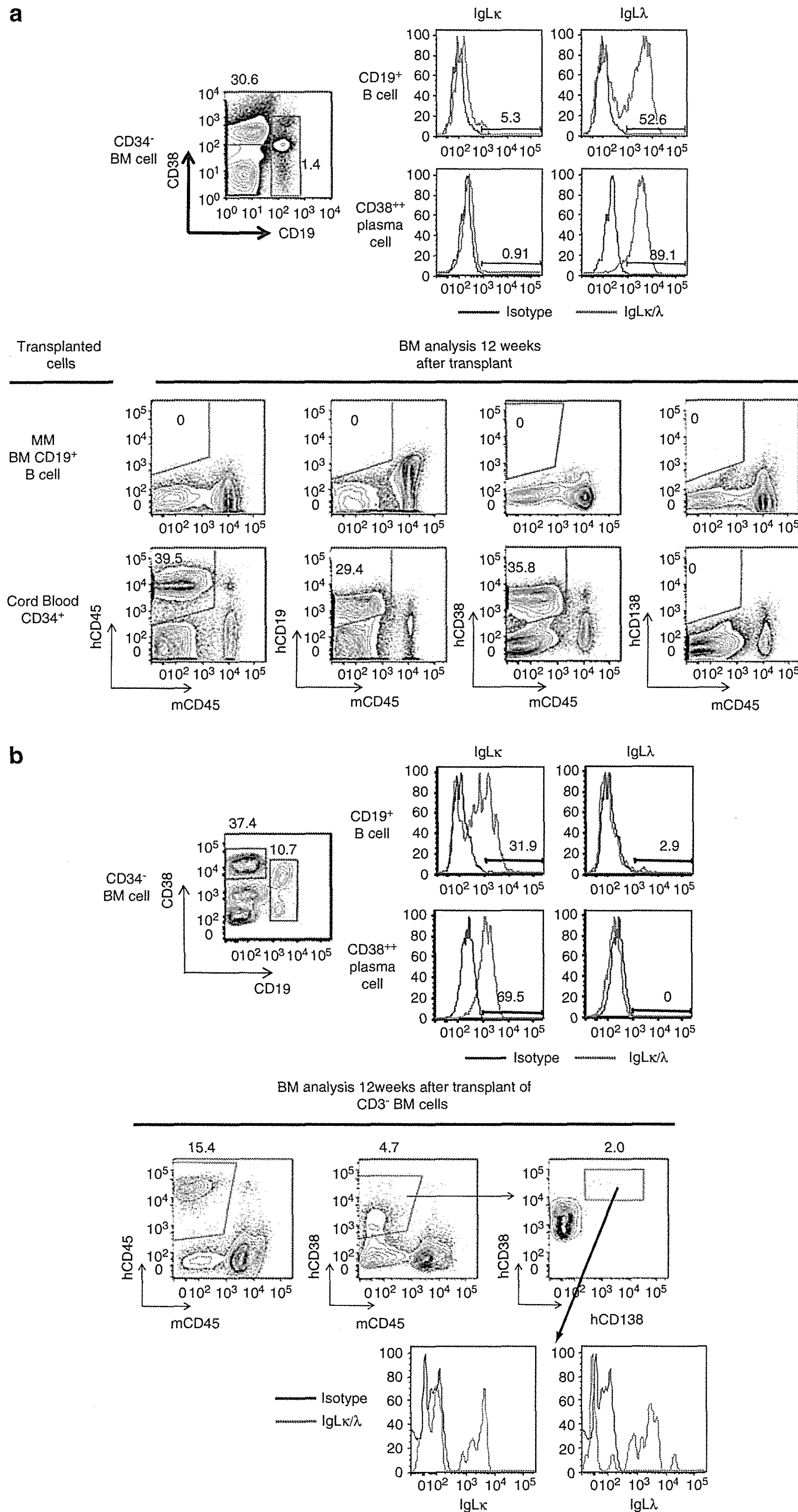
the recipient mice. Engraftment and expansion of MM cells were observed in 5 out of 12 cases (Table 1). Rabbit BM was analyzed 12 weeks or more after transplant to determine whether engraftment of not only MM plasma cells but also CD19^{+} B cells had taken place. Robust engraftment of human CD38^{++} MM plasma cells expressing the monotypic immunoglobulin light chain and



containing both $CD138^-$ and $CD138^+$ cells was detected in the rabbit BM, but no human $CD19^+$ B cells were detected (Figure 2a). These results indicate that $CD19^-CD38^{++}$ plasma cells could engraft and expand at least for several months without engraftment of $CD19^+$ B cells.

Both $CD138^-$ and $CD138^+$ plasma cells could engraft and propagate MM clones in SCID-rab model

Next, we transplanted purified $CD138^-$ or $CD138^+$ BM cells into SCID-rab mice to test whether proliferating cell compartments were present in the $CD138^-$ population. Transplants with 9 MM samples were performed and in 3 of the samples, rapid increase of either human IgLκ or λ was observed in serum of the mice transplanted with $CD138^-$ cells (Table 2). In the rabbit BM, $CD38^{++}$ MM plasma cells including $CD138^-$ and $CD138^+$ cells, but not $CD19^+$ cells, were detected (Figure 2b). In 4 of 8 cases $CD138^+$ plasma cells also engrafted and expanded, although more slowly than $CD138^-$ BM cells (Table 2, Figure 2b). In addition, $CD138^-$ cells from the SCID-rab mice that had initially been engrafted with $CD138^-$ BM cells could be secondarily transplanted to another SCID-rab recipient and propagate MM disease more rapidly than $CD138^+$ cells (Table 2, exp. 2-2). These results indicate that $CD138^-$ plasma cells of some MM patients have the potential to propagate MM disease rapidly in the SCID-rab model, while $CD138^+$ plasma cells can also engraft and propagate MM slowly.

$CD19^+$ B cells from MM patients did not engraft to NOD/Shi-scid,IL-2Rγnull (NOG) mice

To examine whether $CD19^+$ B cell fractions contain MM-initiating cells upon transplantation to immune-deficient mice, $CD19^+$ B cells were FACS-sorted from PB of 5 MM patients and BM cells from 4 MM patients including one patient whose $CD19^+$ B cells exclusively expressed IgL λ (Figure 3a), and transplanted directly into BM of NOD/Shi-scid,IL-2Rγnull (NOG) mice or intravenously to new born pups of NOG mice (Table 3, exp. 1-9). Engraftment of human MM cells was monitored by measuring human immunoglobulin light chain (IgL) κ and λ in serum of the recipient mice, but no human IgL was detected at any time. We also analyzed BM of the recipient mice 12–20 weeks after transplant, but no human $CD19^+$ or $CD38^{++}$ cells were detected (Table 3, Figure 3a). $CD19^-CD38^{++}$ plasma cells from the MM BM samples were also transplanted into NOG mice, but did not engraft (Table 3, exp. 6–9). In contrast, robust engraftment of human cells was observed upon transplantation of cord blood-derived $CD34^+$ cells (Table 3, exp. CB, Figure 3a). In three experiments (Table 3, exp. 10–12), $CD3^-CD34^-CD138^-$ cells were transplanted into BM of NOG mice, but did not engraft. Finally, CD3-depleted BM cells from an MM patient whose $CD19^+$ B cells exclusively expressed IgLκ (Figure 3b) were transplanted intravenously into a newborn NOG mouse (Table 3, exp. 13). When the BM cells were analyzed 12 weeks after transplant, significant engraftment of human $CD45^+$ cells was observed because $CD3^-$ BM cells contained many $CD34^+$ hematopoietic stem and progenitor cells. Small numbers of human $CD38^{++}CD138^+$ plasma cells were also detected, but analysis of their IgL κ and λ expression showed that they were not clonal MM plasma cells (Figure 3b). This result suggests that normal human hematopoietic cells, but not MM cells, engrafted in the recipient mice.

Table 3. Transplantation of cells from MM patients into NOG mice

Experiment	BM or PB	Population	Transplant method[a]	Cell no.	Engraftment
Exp 1	PB	$CD19^+$	iBMT	3.5×10^4	–
Exp 2	PB	$CD19^+$	iBMT	3.0×10^5	–
Exp 3	PB	$CD19^+$	iBMT	1.5×10^5	–
Exp 4	PB	$CD19^+$	iBMT	1.6×10^5	–
Exp 5	PB	$CD19^+$	iBMT	7.5×10^5	–
Exp 6-1	BM	$CD19^+$	iBMT	2.5×10^4	–
	BM	$CD19^-CD38^{++}$	iBMT	5×10^5	–
Exp 6-2	BM	$CD19^+$	Newborn	1×10^4	–
	BM	$CD19^-CD38^{++}$	Newborn	1.3×10^5	–
Exp 7	BM	$CD19^+$	Newborn	4×10^3	–
	BM	$CD19^-CD38^{++}$	Newborn	5×10^4	–
Exp 8	BM	$CD19^+$	iBMT	1×10^5	–
	BM	$CD19^-CD38^{++}$	iBMT	1×10^5	–
Exp 9	BM	$CD19^+$	iBMT	4.5×10^4	–
	BM	$CD19^-CD38^{++}$	iBMT	5×10^4	–
Exp 10	BM	$CD3^-CD34^-CD138^-$	iBMT	4×10^5	–
Exp 11	BM	$CD3\text{-}CD34^-CD138^-$-	iBMT	5×10^5	–
Exp 12	BM	$CD3\text{-}CD34^-CD138^-$	iBMT	3×10^5	–
Exp 13	BM	$CD3^-$	Newborn	5×10^6	–[b]
Exp-CB	CB	$CD3^-CD34^+$	Newborn	1×10^5	+

[a]iBMT denotes intra-BM transplantation, and new born denotes transplantation to new born pups. [b]Engraftment of MM plasma cells was not detected, but normal hematopoietic cells engrafted robustly.

DISCUSSION

In this study, we showed that *in vitro* clonogenic cells that were detected in some MM patients lacked expression of the plasma cell marker CD138,[11,13,14] and that they were $CD19^-CD38^{++}CD138^-$ plasma cells, not $CD19^+$ B cells. Consistent with our results, it has been recently reported that $CD138^-CD38^{++}$plasma cells contain more cycling cells compared to $CD138^+$ plasma cells.[17] An *in vitro* colony assay of $CD138^-CD19^-CD34^-$ cells showed colony formation only in 4/16 (25%) patients which is a lower ratio than the one previously reported (88%) by Matsui *et al.*[11] even though our method for *in vitro* colony assay was the same. Only cells that proliferate extensively in response to stimulation by lymphocyte-conditioned medium (LCM) can be detected with the clonogenic assay used in our study. When the survival of clonogenic MM cells depends on factors other than the soluble factors contained in LCM, for example attachment to stromal cells, clonogenic MM cells cannot be detected with the assay used in our study. On the other hand, cells from aggressive or advanced MM cases may be more independent of several cell extrinsic factors and efficiently produce colonies. Heterogeneity of MM patients should thus be the reason for the differences in the frequencies of MM colony formation between our study and the one by Matsui *et al.*[11]

The SCID-rab experiments showed that highly proliferative myeloma cells were present in the CD138-negative fraction in some patients, but those cells were MM plasma cells, not B cells. Consistent with the findings of previous studies,[22,23] we also found that MM developed in the SCID-hu/rab mice transplanted with $CD138^+$ plasma cells. MM plasma cells thus have the potential to propagate and maintain MM clones, at least for several months, in the absence of clonotypic B cells. This may explain why B cell depletion by rituximab was not clinically effective, at least in the short run, for most MM patients.

Interestingly, $CD138^-CD38^{++}$ plasma cells were detected in the SCID-rab mice transplanted with $CD138^+$ plasma cells. This suggests that CD138 expression on MM plasma cells may be reversible, although we cannot exclude the possibility of minor contamination of $CD138^-$ cells in the purified $CD138^+$ cells. The significance of CD138 expression on clonogenic MM cells thus needs to be carefully interpreted. It was reported that interaction with bone marrow stromal cells induced expression of CD138 in MM plasma cells,[26] suggesting that changes in CD138 expression depend on the microenvironment. In addition,

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Jakubikova *et al*. recently reported that clonogenic side populations in MM cell lines were not enriched in the CD138$^{low/+}$ but not in the CD138^{-} population, although it is not clear whether clonogeic side populations in primary MM cells are also enriched in CD138$^{low/+}$ cells.[27]

CD19^{+} B cells in some MM patients generated MM disease upon transplantation into NOD/SCID mice,[11,12,18,19] indicating that CD19^{+} B cells of some MM patients definitely contain MM progenitor cells. In our experiments, however, we could not find any MM patients whose CD19^{+} B cells induced MM disease upon transplantation into NOG mice. However, this does not necessarily mean that CD19^{+} clonotypic B cells cannot be MM progenitor cells in MM patients. It should be noted that there are many difficulties involved in the engraftment of human cells in xeno-graft models. For example, mouse IL6, which is one of the major growth factors for plasma cells, does not transduce its signals through human IL6 receptors, and probably other factors lack inter-species cross reactivity between human and mice. This means that MM progenitor cells can be detected in xeno-graft assays only when they can survive independently of human IL6 or other human factors. Thus, CD19^{+} B cells from advanced MM patients may be independent of several survival factors and effectively engraft and propagate MM disease upon transplant into immuno-deficient mice. In addition, signals from B cell receptors (BCRs) on clonotypic CD19^{+} B cells need to be taken into consideration. When BCRs of clonotypic CD19^{+} B cells show very high affinity to xeno-antigen in mice, they may be depleted and cannot survive in xeno-graft models. Thus, assuming that CD19^{+} MM progenitor cells are present, they can be detected in xeno-graft assays only when their BCRs are suitable for survival in mice. We can therefore not deny the possibility that CD19^{+} B cells have potential as MM progenitor cells, even if CD19^{+} B cells do not engraft in immunodeficient mice.

Taken together, our findings show that CD138^{-} clonogeic cells are plasma cells and not B cells in some MM patients. Furthermore, we suggest that MM plasma cells, which include CD138^{-} and CD138^{+} cells, have potential to propagate and maintain MM clones for at least several months without the need for CD19^{+} clonotypic B cells. Our findings also indicate that clonogenic MM plasma cells should be considered important therapeutic targets. The Hedgehog signaling pathway was reported to be a promising candidate as a therapeutic target against clonogenic MM cells.[15] In addition, since clonogenic MM plasma cells mainly reside in the BM microenvironment, it is also important to understand the mechanisms involved in how the BM microenvironment supports clonogenic MM plasma cells and targets them. The Notch signaling pathway may be a good candidate for such a target.[28]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CD166/Activated leukocyte cell adhesion molecule is expressed on glioblastoma progenitor cells and involved in the regulation of tumor cell invasion

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For improvement of prognosis for glioblastoma patients, which remains poor, identification and targeting of glioblastoma progenitor cells are crucial. In this study, we found that the Cluster of Differentiation (CD)166/activated leukocyte cell adhesion molecule (ALCAM) was highly expressed on $CD133^+$ glioblastoma progenitor cells. $ALCAM^+CD133^+$ cells were highly enriched with tumor sphere-initiating cells in vitro. Among gliomas with isocitrate dehydrogenase-1/R132H mutation, the frequencies of $ALCAM^+$ cells were significantly higher for glioblastomas than for World Health Organization grade II or III gliomas. The function of ALCAM in glioblastoma was then investigated. An in vitro invasion assay showed that transfection of ALCAM small interfering RNA or small hairpin RNA into glioblastoma cells significantly increased cell invasion without affecting cell proliferation. A soluble isoform of ALCAM (sALCAM) was also expressed in all glioblastoma samples and at levels that correlated well with ALCAM expression levels. In vitro invasion of glioblastoma cells was significantly enhanced by administration of purified sALCAM. Furthermore, overexpression of sALCAM in U87MG glioblastoma cells promoted tumor progression in i.c. transplants into immune-deficient mice. In summary, we were able to show that ALCAM constitutes a novel glioblastoma progenitor cell marker. We could also demonstrate that ALCAM and its soluble isoform are involved in the regulation of glioblastoma invasion and progression.

Keywords: activated leukocyte cell adhesion molecule, cancer stem cell, CD166, glioblastoma, invasion.

Glioblastoma is one of the most frequently occurring malignancies in the CNS. Despite intensive treatment, including surgery, radiation, and chemotherapy, the prognosis for glioblastoma is still very poor, and the median survival time is only 12–15 months.[1] One major reason for the extremely poor prognosis is that glioblastoma progenitor cells possessing tumor-initiating ability[2,3] are resistant to radiation and chemotherapy.[4] Glioblastoma progenitor cells reportedly exist in the Cluster of Differentiation (CD)133^+ glioblastoma cell population.[2] While CD133 is the most promising marker for the identification of glioblastoma progenitor cells, additional cell surface markers for glioblastoma progenitor cells are needed for more efficient enrichment of these cells and identification of their location in a microenvironment. Recently, stage-specific embryonic antigen–1,[5] A2B5,[6] neural cell adhesion molecule L1,[7] and integrin alpha-6[8] have been mentioned as candidates for novel glioblastoma stem/progenitor cell markers.

One main source of the high malignancy of glioblastoma is the invasion of isolated tumor cells into the surrounding parenchyma.[9] It is therefore important to clarify the molecular mechanism of this strong invasiveness of glioblastoma cells, and several molecules, such as cadherin,[10] neural cell adhesion molecule,[11] and integrin,[12] are reportedly involved in glioblastoma invasion.

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The CD166/activated leukocyte cell adhesion molecule (ALCAM) is a member of the immunoglobulin superfamily and is widely expressed in various tissues, such as neurons, fibroblasts, endothelial cells, and keratinocytes.[13–15] ALCAM is involved in neurogenesis,[13] angiogenesis, hematopoiesis,[16] leukocyte trafficking,[17] and hematopoietic stem cell maintenance in bone marrow niches.[18] It is reported to be a cell surface marker for mesenchymal stem cells[19,20] and hematopoietic progenitor cells.[16,21] It is also expressed in several kinds of cancer and is reportedly a marker for cancer stem cells in colon cancer[22] and prostate cancer.[23] However, there have been no reports about ALCAM expression on glioblastoma cells. On the other hand, functional roles of ALCAM have been investigated in several kinds of cancer,[24] especially in metastatic melanoma, in which it functions as a cell surface sensor for cell density and controls the transition from local cell proliferation to tissue invasion.[25,26] Moreover, ALCAM was found to be required for promoting cell invasion because of its efficient triggering of the activation of the metalloproteinase cascade in response to extensive cell-to-cell and cell-to-matrix contacts.[25]

The soluble isoform of ALCAM (sALCAM) was isolated as an alternative short ALCAM transcript comprising only the first 3 exons.[27] Since the sALCAM protein possesses the immunoglobulin domain D1, which is required for homophilic ALCAM binding, sALCAM impairs cell-to-cell interaction through homophilic ALCAM binding,[27] and as a result affects the coordination of local tumor growth, invasion, and metastasis.[28] It was also reported that sALCAM attenuates melanoma invasion.[28]

In this study, we examined whether ALCAM could serve as a progenitor cell marker for glioblastoma, while the clinical significance of ALCAM as an indicator of the histological grade or as a prognostic factor was also investigated. In addition, we investigated the functional roles of ALCAM and sALCAM in glioblastoma.

Materials and Methods

Glioma Samples and Clinical Data

For fluorescence activated cell sorter (FACS) analysis, 12 glioblastoma samples from patients who had undergone surgery at Osaka University Hospital between 2007 and 2008 were analyzed. For immunohistochemical analysis, formalin-fixed paraffin-embedded (FFPE) glioma samples from patients who had undergone surgery at Osaka University Hospital between 2003 and 2010 were analyzed. For Kaplan–Meier analysis, we obtained clinical data to estimate progression-free survival (PFS) and overall survival (OS) from our database or medical records of glioblastoma patients who had undergone maximal surgical resection and chemoradiation therapy between 2005 and 2010 at Osaka University Hospital.

This study was approved by the institutional review board of Osaka University School of Medicine. The details of the study were explained to glioma patients before they underwent surgery at Osaka University Hospital. When written agreement had been obtained from a patient for the use of the resected tissue for this research, a part of the tissue was subjected to the analyses in this study.

FACS Analysis

Glioblastoma samples were first minced with a scalpel and then dissociated using a neural cell dissociation kit containing papain (Miltenyi Biotec), according to the manufacturer's instructions. Single-cell suspensions generated from the glioblastoma samples were stained with biotin-conjugated anti-CD133 monoclonal antibody (mAb) (AC133; Miltenyi Biotec), phycoerythrin-conjugated anti-ALCAM mAb (3A6; BD Pharmingen), CD45-allophycocyanin (APC) (BD Pharmingen), CD31-APC (eBioscience), and then with streptavidin–fluorescein isothiocyanate (BD Pharmingen) or Cy7PE (eBioscience). The stained suspensions were then analyzed on FACSAria flow cytometer (Becton Dickinson).

Tumor-Sphere Formation Assay

Five hundred FACS-sorted cells were seeded in 96-well plates and cultured in a serum-free medium supplemented with 20 ng/mL of epidermal growth factor (R&D Systems), 20 ng/mL of basic fibroblast growth factor (Peprotech), and 20 ng/mL of leukemia inhibitory factor (Millipore) in 5% CO_2. Cells were nourished every 2 days by refreshing half of the medium. The tumor spheres were counted 14 days after the seeding.

Quantitative PCR

Total RNA was extracted from the glioma cell line and primary glioblastoma samples using Trizol (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNA was generated using Moloney murine leukemia virus reverse transcriptase (Promega) and then subjected to quantitative PCR with SYBR (Synergy Brands) green in an Applied Biosystems 7900HT system. To measure ALCAM or sALCAM expression, the following primers were used: for ALCAM: CGTGAATTCCACCAAGAAGGAGGAGGA for sense primers and TCTGTCTTTGTATTCTGGT ACATCG for antisense primers; for sALCAM: AGAC AGATTGAACCTCTCTCAGAAAAC for sense primers and GCTGCAGACTACTTACTGAACACC for antisense primers.

Knockdown of ALCAM Expression by siRNA and shRNA

Anti-ALCAM small interfering (si)RNA, short hairpin (sh)RNA, or control RNA was transfected to U87MG and U251 cells (American Type Culture Collection) using Lipofectamine RNAimax reagent (Invitrogen). Sequences of siRNAs specific for ALCAM were:

siRNA1: UCUACAAUGAGAGUCAAUGACUCUC and GAGAGUCAUUGACUCUCAUUGUAGA, siRNA2: A GUAAUUGUCCACUGAAUGGCUGGC and GCCAG CCAUUCAGUGGACAAUUACU. Stealth RNA interference negative control (Invitrogen) was used as the negative control siRNA. Two days after transfection, cells were subjected to FACS analysis for detection of ALCAM expression and to a Matrigel invasion assay. The cell proliferation assay was started soon after siRNA transduction.

To establish cell lines in which ALCAM expression was stably knocked down, we used a Mission shRNA (Sigma Aldrich) lentivirus carrying an shRNA sequence against ALCAM (CCGGCAGCCATGATAATAGGT CATACTCGAGTATGACCTATTATCATGGCTGTTT TTG). Lentivirus was produced by transfection of the lentiviral vector with the gag-pol–expressing vector and the vector expressing a vesicular stomatitis virus-glycoprotein envelope (both were kindly donated by Hiroyuki Miyoshi). Viruses were concentrated by centrifugation with PEG-it (System Bioscience). U87MG and U251 glioblastoma cells were infected with lentivirus carrying ALCAM-shRNA. Knockdown of ALCAM was confirmed by FACS analysis.

Generation of sALCAM Isoform-Expressing Glioblastoma Cells

The expression vector carrying flag-tagged sALCAM cDNA (sALCAM-p3XFLAG) (Sigma Aldrich)[27] was kindly donated by Koji Ikeda MD of Kyoto Prefectural University of Medicine. U87MG glioblastoma cells were transduced with flag-tagged sALCAM cDNA by means of electroporation. Stable transfectants were selected by culturing cells in medium supplemented with 500 μg/mL of G418 (Roche). The sALCAM-Flag protein was detected by western blotting using anti-Flag M2 antibody (Sigma Aldrich) and anti-mouse immunoglobulin G-alkaline phosphatase (Santa Cruz Biotechnology). The sALCAM-Flag protein was purified from U87MG expressing sALCAM-Flag cells using anti-Flag affinity gel (Sigma Aldrich) according to the manufacturer's instructions.

Invasion Assay

The invasiveness of U87MG and U251 cells was assayed with a modified Boyden Chamber Matrigel method[29] using the Biocoat Matrigel invasion chamber (Becton Dickinson Bioscience) according to the manufacturer's instructions. Cells were washed with phosphate buffered saline (PBS) and harvested using a cell dissociation buffer (Invitrogen), after which 2.5×10^4 cells in serum-free Dulbecco's modified Eagle's medium (DMEM) were seeded onto Matrigel-coated filters. DMEM containing 10% fetal bovine serum was added to the lower compartment, and cells were incubated for 48 h. After removal of the cells that remained in the top chamber, the top surface of each membrane was cleared of cells with a cotton swab. Cells that had penetrated to the bottom side of the membrane were then fixed in buffered formalin, stained with a Diff-Quik Stain Set (Wako), and counted.

Gelatin Zymography

The same number of cells (5×10^5) were placed in 100 μL of serum-free medium (DMEM/F12 Ham's) and incubated for 24 h. Supernatant from each well was subjected to gelatin zymography with zymogram gel (Tris-glycine; Invitrogen) according to the manufacturer's instructions. The gels were then stained with Coomasie brilliant blue R-250.

Intracranial Xenograft Model

Newborn Rag2$^{-/-}\gamma c^{-/-}$ mice (kindly donated by Irving Weissman, MD, of Stanford University) were used as recipients. Pups were anesthetized on ice, and glioblastoma cells (2×10^5) in 2 μL of PBS were injected into the right lateral ventricle with a stereotactic injector (Stoelting).

Immunohistochemical Analysis

For double immunostaining, indirect immunoalkaline phosphatase and immunoperoxidase methods were used. Fresh frozen glioblastoma tissue sections (6 μm) were fixed in pure acetone for 10 min and then in a formol-calcium solution for 1 min after rehydration in PBS. After washing in PBS and incubation with a blocking solution (Block Ace; DS Pharma Biomedical) for 10 min, the sections were incubated with the first mAb, anti-CD166 (3A6; Abcam), for 1 h at room temperature. Each subsequent step was followed by washing 3 times with PBS for 2 min. Bound mAb was detected with an alkaline phosphatase-labeled second antibody for 20 min, and the sections were fixed further with 1% glutaraldehyde (Nacalai Tesque) in PBS for 30 s. The labeled cells were then colored red with Alkaline Phosphatase Substrate Kit I (Vector red; Vector Laboratories). The sections were then incubated with the biotin-labeled second mAb, anti-CD31 (eBioscience), reacted with streptavidin peroxidase (N-Histofine; Nichirei), and colored brown with 3,3′-diaminobenzidine (DAB) hydrochloride (Histofine Simple Stain DAB; Nichirei). Levamisole (Sigma Chemical) and hydrogen peroxide solution (Nacalai Tesque) were used to inhibit endogenous alkaline phosphatase and peroxidase activity, respectively. The sections were counterstained with Lillie-Mayer's hematoxylin solution (Wako) and mounted in Aquatex (Merck).

FFPE glioma samples were also stained with anti-ALCAM mAb (3A6; Abcam) and anti-IDH1-R132H mAb (H09; Dianova). The histofine simple stain MAX-PO (Multi; Nichirei) was used as a secondary antibody. For visualization, the specimens were reacted with 3,3′-DAB tetrahydrochloride (Dojindo).

Statistical Analysis

Student's *t*-test was used to determine statistical significance for the in vitro experiments. For the analysis of clinical data and in vivo experiments, Kaplan–Meier analysis and the Wilcoxon test were used. For all analyses, differences were defined as statistically significant at $P < .05$.

Results

ALCAM Is a Glioblastoma Progenitor Cell Marker

ALCAM expression on $CD31^{-}CD45^{-}CD133^{+}$ glioblastoma cells ($CD133^{+}$ glioblastoma cells) or $CD31^{-}CD45^{-}CD133^{-}$ glioblastoma cells ($CD133^{-}$ glioblastoma cells) was subjected to FACS analysis. Ratios of $ALCAM^{+}$ cells were significantly ($P < .05$, $n = 12$) higher in the $CD133^{+}$ glioblastoma cell population ($37.0 \pm 10.1\%$ [1.9%–95.4%]) than in the $CD133^{-}$ cell population ($17.4 \pm 6.2\%$ [0%–57.6%]) (Fig. 1A). $CD133^{+}$ glioblastoma cells could be separated into an $ALCAM^{+}$ and an $ALCAM^{-}$ population. To examine whether $ALCAM^{+}CD133^{+}$ glioblastoma cells were enriched with glioblastoma progenitor cells, a tumor-sphere formation assay was performed with fluorescence activated cell sorted $ALCAM^{+}CD133^{+}$ or $ALCAM^{-}CD133^{+}$ glioblastoma cells. Five glioblastoma samples were examined, and cells that formed tumor spheres accounted for 4.6 ± 0.4 per 500 cells in the $ALCAM^{+}CD133^{+}$ fraction, and for 0.6 ± 0.6 per 500 cells in the $ALCAM^{-}CD133^{+}$ fraction ($P < .05$, Fig. 1B). These results indicate that ALCAM in combination with CD133 can be used as a glioblastoma progenitor cell marker. Furthermore, in a glioblastoma sample that contained no $CD133^{+}$ cells, only $ALCAM^{+}$ cells formed tumor spheres (Fig. 1C), showing that ALCAM is a potential marker for glioblastoma progenitor cells even in $CD133^{-}$ glioblastoma samples. Immunohistochemical analysis with anti-ALCAM mAb identified distinct $ALCAM^{+}CD31^{-}$ glioblastoma cells, whereas endothelial cells were $ALCAM^{+}CD31^{+}$ (Fig. 1D).

Frequency of $ALCAM^{+}$ Cells Correlates with Histological Grade in IDH1-R132H Mutation-Positive Glioma and Prognosis for IDH1-R132H Mutation-Negative Primary Glioblastoma Patients

It is now known that World Health Organization (WHO) grade II gliomas are almost uniformly characterized by isocitrate dehydrogenase (IDH)-1 mutations, while most primary glioblastomas are IDH1 wild type.[30–33] In addition, the presence of IDH1 mutation reportedly correlates well with positive staining for anti-IDH1-R132H mAb in immunohistochemical studies.[34,35] $IDH1\text{-}R132H^{+}$ glioma samples (Fig. 2A) were examined for ALCAM expression. For this analysis, we used 16 WHO grade II glioma samples (10 diffuse astrocytomas, 3 oligoastrocytomas, and 3 oligodendrogliomas), 10 WHO grade III glioma samples (6 anaplastic astrocytomas, 3 anaplastic oligoastrocytomas, and 1 anaplastic oligodendroglioma), and 9 glioblastoma samples. The percentages of $ALCAM^{+}$ cells were 27.7 ± 9.1, 29.4 ± 20.2, and $48.7 \pm 8.9\%$ for WHO grades II, III, and IV, respectively (Fig. 2B). The frequency of $ALCAM^{+}$ cells was significantly ($P < .05$) higher in glioblastomas than in WHO grade II or III gliomas.

We also examined whether the frequencies of $ALCAM^{+}$ cells correlated with the prognosis for primary glioblastoma (IDH1-R132H $mutation^{-}$) patients. PFS and OS were compared for glioblastoma patients with a high percentage and those with a low percentage of $ALCAM^{+}$ cells. At a cutoff value of 60% $ALCAM^{+}$ cells, median PFS for the patients with a high percentage of $ALCAM^{+}$ cells (115.75 ± 67.41 days, $n = 12$) was significantly ($P < .05$) higher than for their low-percentage counterparts (447.97 ± 308.36 days, $n = 27$) (Fig. 2C). The corresponding median OS was 275.77 ± 151.27 and 731.61 ± 300.69 days ($P < .05$) (Fig. 2C). However, at a cutoff value of 50% or less of $ALCAM^{+}$ cells, the difference in PFS or OS was not statistically significant (data not shown).

ALCAM Is Involved in the Regulation of Glioblastoma Cell Invasion

The functional roles of ALCAM in glioblastoma were investigated next. Two sequences of siRNA specific for ALCAM were used for knocking down ALCAM in glioblastoma cells. A comparison between cell growth of anti-ALCAM siRNA-transfected and negative control siRNA-transfected U87MG glioblastoma cells (Fig. 3A) showed that the reduction in ALCAM expression had no effect on cell growth (Fig. 3B). We then used a modified Boyden Chamber Matrigel assay to examine the effects of ALCAM knockdown on U87MG and U251 glioblastoma cell invasion. The numbers of cells that reached the bottom of the filters through the Matrigel were 23.3 ± 6.8, 66.7 ± 12.9, and 5.7 ± 1.9 of the ALCAM siRNA1, ALCAM siRNA2, and negative control siRNA-transfected U87MG cells, respectively, and were 221.2 ± 9.3 and 50.2 ± 8.4 of the ALCAM shRNA- and negative control-transfected U251 cells, respectively (Fig. 3C), indicating that downregulation of ALCAM expression of glioblastoma cells significantly ($P < .05$) enhanced tumor cell invasion. Conditioned media from cultures of ALCAM siRNA- or negative control siRNA-transfected U87MG cells were analyzed with gelatin zymography. Conversion of promatrix metalloproteinase (MMP)-2 to active MMP-2 was observed in both of the conditioned media, while there was no difference in the quantity of active MMP-2 between ALCAM siRNA-transfected U87MG and control siRNA-transfected cells (Fig. 3D).

The soluble isoform of ALCAM (sALCAM) expressed in glioblastoma cells enhances cell invasion in vitro and promotes tumor progression in vivo.

Endogenous expression levels of ALCAM and sALCAM in primary glioblastoma samples were examined

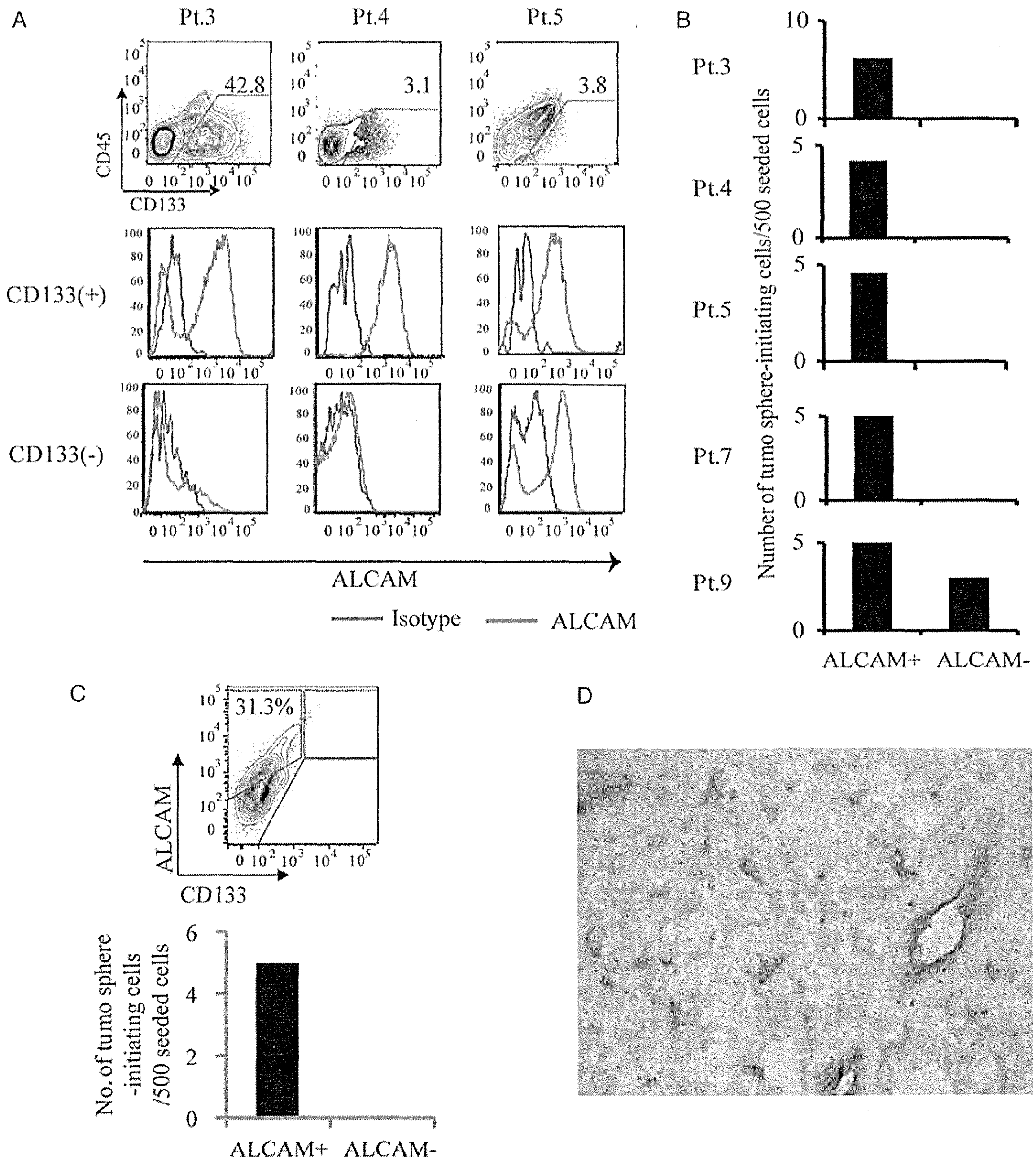

Fig. 1. ALCAM is a glioblastoma progenitor cell marker. (A) Flow cytometric analyses of $CD45^-CD31^-$ cells from glioblastoma samples. Three representative cases are shown. $CD45^-CD31^-$ cells were separated into $CD133^+$ and $CD133^-$cell populations and then analyzed for ALCAM expression. (B) Tumor-sphere formation assays using FACS-sorted $CD133^+ALCAM^+$ cells or $CD133^+ALCAM^-$ glioblastoma cells. (C) Tumor-sphere assays using $CD133^-$ $ALCAM^+$ cells or $CD133^-$ $ALCAM^-$ cells in a glioblastoma sample that contained no $CD133^+$ cells. (D) Immunohistochemical staining of ALCAM and CD31 expression on glioblastoma samples. Red (vector red): ALCAM; brown (DAB):CD31.

by using quantitative real-time PCR (Fig. 4A). All primary glioblastoma samples expressed sALCAM. In addition, sALCAM expression levels in glioblastoma samples correlated well with ALCAM expression levels (Fig. 4A).

To examine the functional role of sALCAM in glioblastoma cells, U87MG cells transduced with sALCAM-Flag or an empty vector (U87MG-sALCAM or U87MG-mock) were generated (Fig. 4B). There was no difference in cell proliferation between U87MG-mock and U87MG-sALCAM cells (Fig. 4C). We next used a modified Boyden Chamber Matrigel assay to examine whether sALCAM was involved in

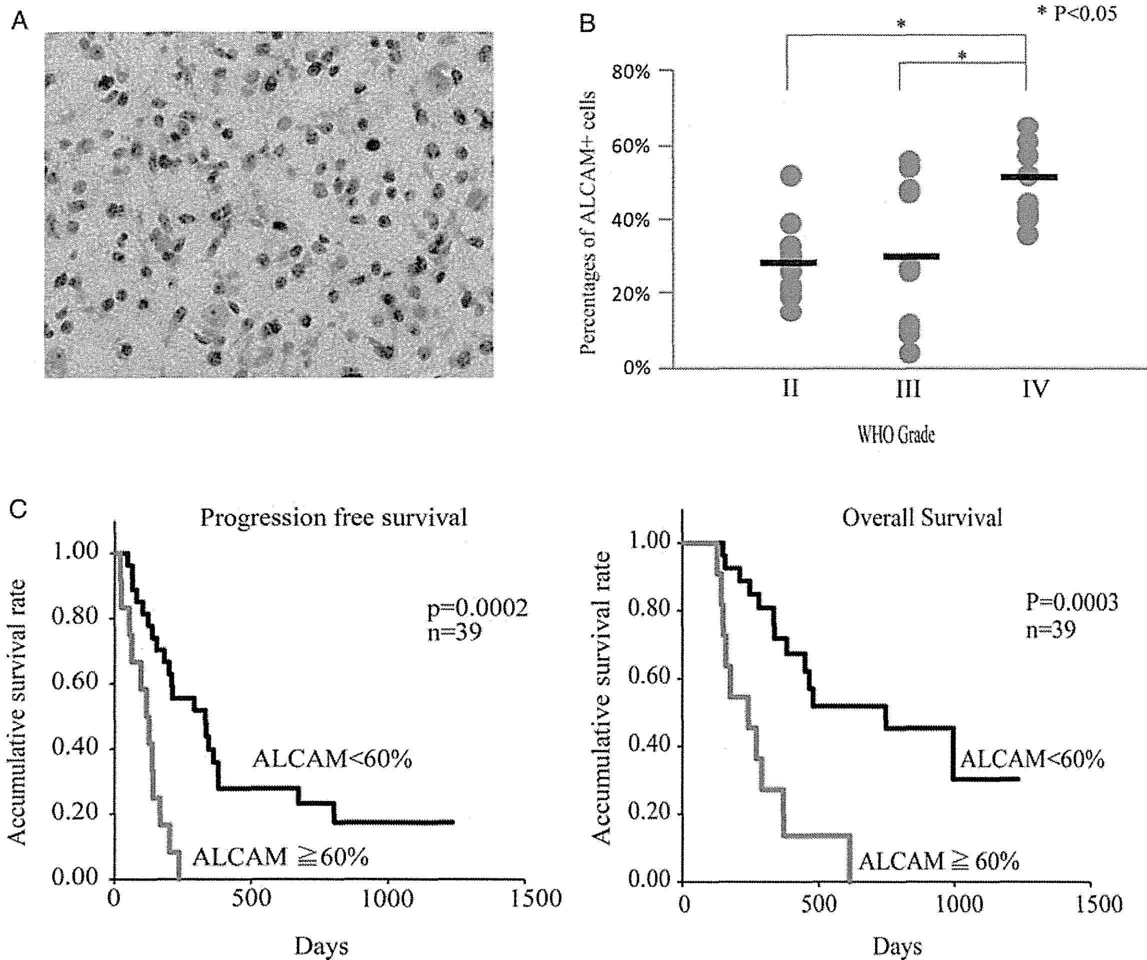

Fig. 2. ALCAM expression correlates with histological grade of glioma and prognosis for glioblastoma patients. (A) Immunohistochemical staining of anti-IDH1-R132H mAb in astrocytoma samples. (B) Percentages of $ALCAM^+$ cells in IDH1-R132H mutation+ WHO grades II–IV glioma specimens. The horizontal bar shows the average percentages of $ALCAM^+$ glioblastoma cells. (C) Plots for progression-free survival and overall survival of primary glioblastoma patients with high percentages (≧60%) and of those with low percentages (<60%) of $ALCAM^+$ cells.

glioblastoma cell invasion. The numbers of cells that reached the bottom of the filters through the Matrigel were 30.8 ± 13.2, 27.0 ± 4.8, and 5.8 ± 1.8 of the U87MG-sALCAM clone 1, U87MG-sALCAM clone 2, and U87MG-mock cells, respectively. These results indicate that sALCAM expression in glioblastoma cells significantly ($P < .05$) enhances tumor cell invasion (Fig. 4D). Conditioned media from cultures of U87MG-sALCAM or U87MG-mock cells were also analyzed by means of gelatin zymography. Conversion of pro-MMP-2 to active MMP-2 was observed in both of the conditioned media, while there was no difference in the amount of active MMP-2 between ALCAM siRNA-transfected U87MG and control siRNA-transfected cells.

Western blotting with anti-Flag mAb led to the detection of sALCAM-Flag protein in the culture supernatant of U87MG-sALCAM cells (Fig. 4E). We used the anti-Flag affinity gel to purify the sALCAM-Flag protein (Fig. 4E) and then examined with an in vitro invasion assay whether this purified sALCAM protein promoted the invasion of glioblastoma cells. We found that the ability of invasion of U87MG and U251 cells was significantly ($P < .05$) increased by the addition of purified sALCAM, with the effect depending on the quantity added (Fig. 4F). This result makes it clear that sALCAM promotes the invasion of glioblastoma cells.

Next, we examined the effect of sALCAM on glioblastoma progression in vivo. Two clones of U87MG-sALCAM or U87MG-mock cells were injected i.c. into the right ventricle of the newborn pups of $Rag2^{-/-}\gamma c^{-/-}$ mice. Two independent clones of the U87MG-sALCAM cells were examined. Difference in the survival curve was significant ($P < .05$) for mice injected with sALCAM-expressing U87MG cells (both clone 1 and clone 2) and those with U87MG-mock cells, but was not significant for sALCAM-expressing clones 1 and 2 (Fig. 5A). All of the mice ($n = 8$)

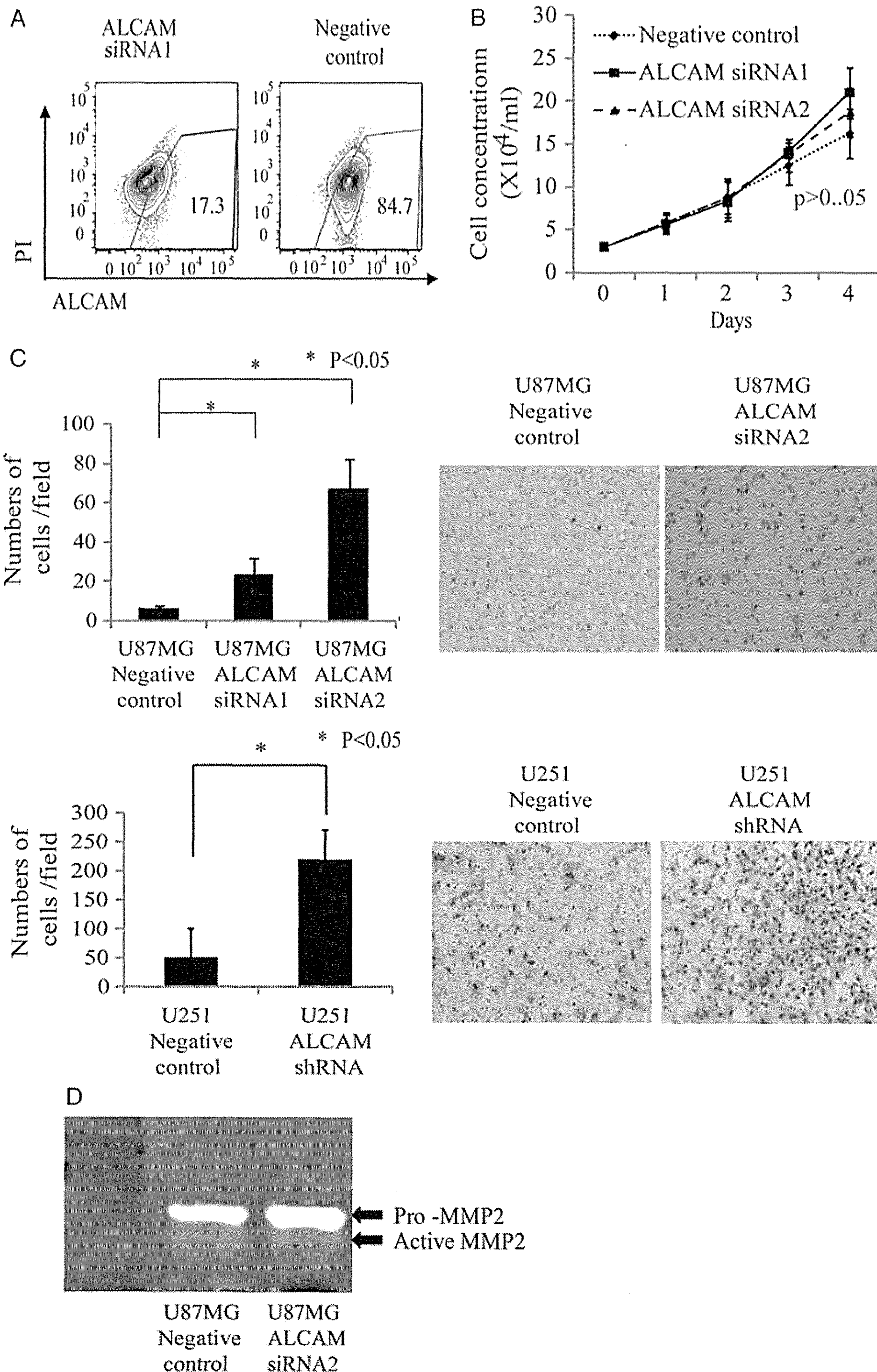

Fig. 3. Downregulation of ALCAM expression on glioblastoma cells promotes cell invasion. (A) FACS analysis of ALCAM expression on anti-ALCAM siRNA–transfected or negative control siRNA–transfected U87MG cells. (B) Comparison of cell proliferation between anti-ALCAM siRNA–transfected and negative control siRNA–transfected U87MG cells. (C) Matrigel invasion assay with anti-ALCAM siRNA–transfected, anti-ALCAM shRNA–transfected, or negative control–transfected U87MG and U251 cells. Bar graph shows numbers of cells having migrated through Matrigel layer. Representative pictures of cells having penetrated to the bottom side of the membrane. (D) Gelatin zymography using medium conditioned by anti-ALCAM siRNA–transfected or negative control siRNA–transfected U87MG cells.

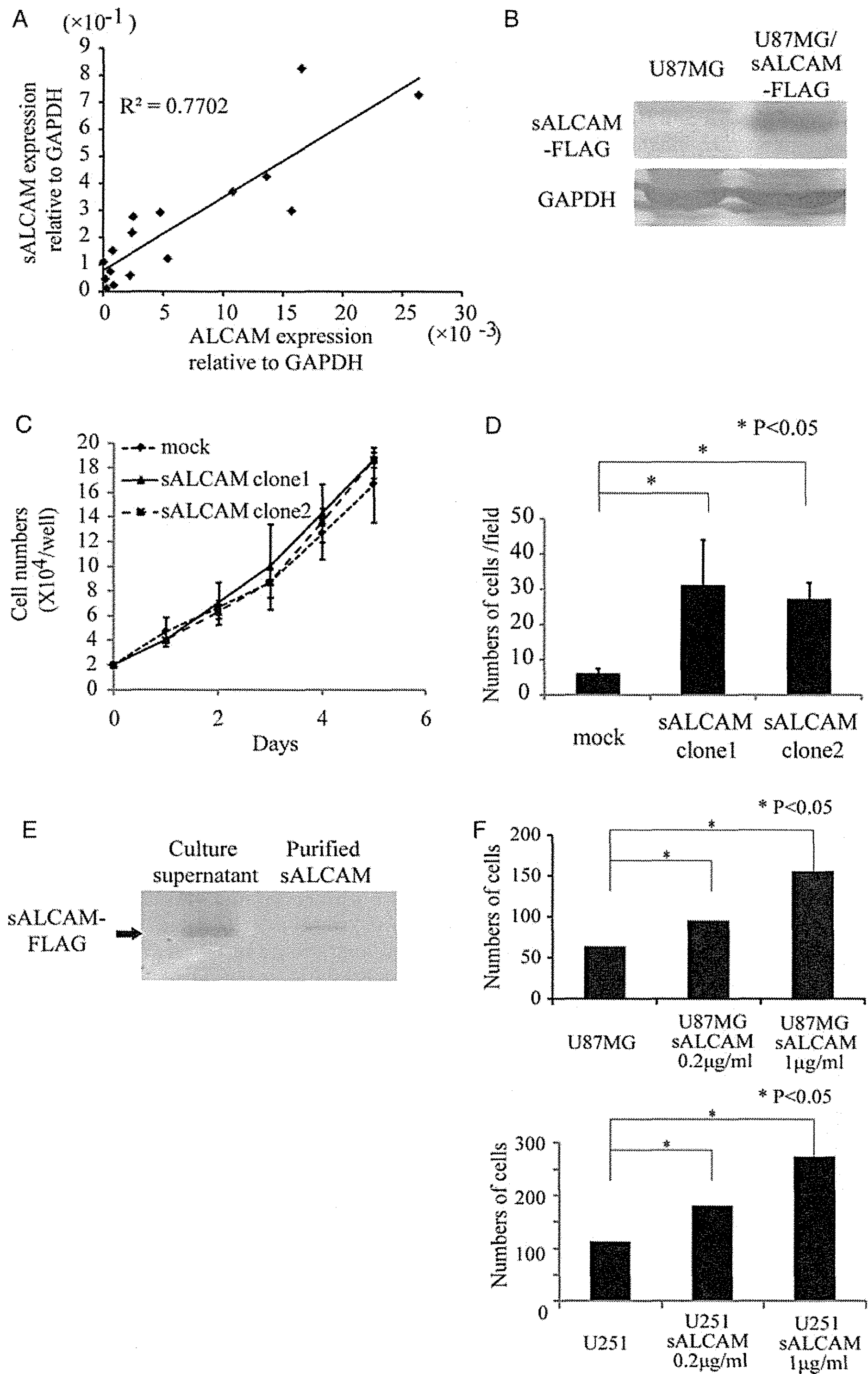

Fig. 4. A soluble isoform of ALCAM is expressed in glioblastoma cells and promotes cell invasion. (A) Correlation between ALCAM mRNA expression and sALCAM mRNA expression in glioblastoma samples. (B) Detection by western blot with anti-FLAG mAb of sALCAM-FLAG protein in cell lysate of sALCAM-expressing U87MG cells. (C) Comparison of cell proliferation between empty vector-transduced U87MG cells (U87MG-mock) and sALCAM-expressing U87MG (U87MG-sALCAM) cells. Two independent clones of U87MG-sALCAM cells were used for the experiments. (D) Matrigel invasion assay with U87MG-mock or U87MG-sALCAM cells. Numbers of cells having passed through the Matrigel layer are shown. (E) Detection by western blot with anti-FLAG mAb of sALCAM-FLAG protein in culture supernatant of U87MG-sALCAM cells and purified sALCAM-FLAG. (F) Matrigel invasion assay with U87MG and U251 cells. Purified sALCAM (0.2 μg/mL, 1 μg/mL) was added to the upper chamber of the transwells.

transplanted with the U87MG-sALCAM cells died of glioblastoma development within 35 days after tumor injection, while none of the mice transplanted with the U87MG-mock cells had developed glioblastoma by posttransplant day 35 (Fig. 5A and B), thus demonstrating that sALCAM significantly enhances tumor progression in vivo.

Discussion

In this study we showed that ALCAM$^+$ CD133$^+$ glioblastoma cells are enriched with tumor sphere-initiating cells, indicating that ALCAM is a novel glioblastoma progenitor cell marker. Some researchers have reported that glioblastoma stem-like cells can be derived from CD133$^-$ cells,[6,36,37] and we also found that some glioblastoma samples contained no CD133$^+$ cells. For such a CD133$^-$ glioblastoma sample, ALCAM is also useful for the identification of glioblastoma progenitor cells. Furthermore, immunohistochemical analysis with anti-ALCAM mAb is effective for the identification of glioblastoma progenitor cells in tumor specimens.

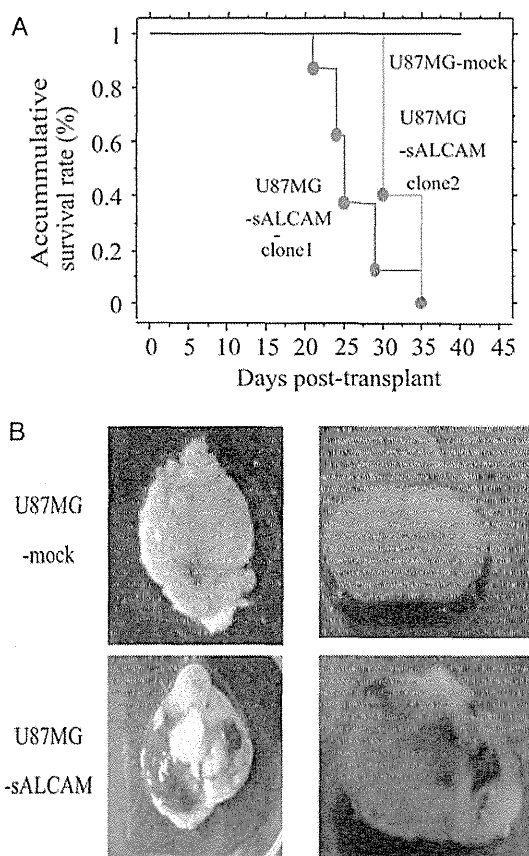

Fig. 5. Expression of sALCAM in U87MG glioblastoma cells promotes tumor progression in vivo. (A) Newborn pups of Rag2$^{-/-}$$\gamma$c$^{-/-}$ mice were injected with U87MG-sALCAM or U87MG-mock cells. Two independent clones of U87MG-sALCAM cells were used for the experiments. Kaplan–Meier survival curve for each group is shown. (B) Representative photos of the brain of the mouse transplanted with U87MG-sALCAM cells and of the one transplanted with control U87MG cells.

While previous studies reported that ALCAM could be used as a prognostic factor for several types of cancers, their conclusions differed. Some studies concluded that high levels of ALCAM expression were related to poor prognosis for breast cancer,[38] colorectal cancer,[39] pancreatic cancer,[40] and melanoma.[41] On the other hand, other studies came to the conclusion that high ALCAM expression was a favorable prognostic factor for prostate cancer,[42] breast cancer,[43,44] and epithelial ovarian cancer.[45] This is probably because, as discussed below, the function of ALCAM varies depending on the cell type and the microenvironment surrounding tumor cells. Our results showed that the frequencies of ALCAM$^+$ cells in primary glioblastomas correlated significantly with both PFS and OS using an arbitrary cutoff value. We also examined other cutoff values of $\leq$50%, but the difference in survival was not significant statistically, which might be due to small patient numbers and/or the difficulties in matching other prognostic factors between the 2 groups. These suggested that the frequency of ALCAM$^+$ cells is a candidate prognostic marker for glioblastoma, but its significance needs to be tested in further studies with greater numbers of patients using multivariate analysis.

Knockdown of ALCAM expression in glioblastoma cells resulted in promotion of tumor cell invasion without affecting cell proliferation. This finding is compatible with previously reported results for a metastatic melanoma cell line. In the case of melanoma, interference with endogenous ALCAM by the expression of an amino terminal-truncated ALCAM protein increased cell migration and invasive growth in vitro,[26] while in a metastatic melanoma cell line, downregulation of ALCAM expression by siRNA inhibited MMP-2 activation.[25] However, no such effect on MMP-2 was observed in the glioblastoma cells used in our experiments. The function of ALCAM may thus vary depending on the cell type and/or microenvironment.

Van Kilsdonk et al.[28] showed that the soluble isoform of ALCAM attenuated the invasion of melanoma cell lines in vitro and also in reconstructed skin. This finding led us to hypothesize that sALCAM also attenuates the invasion of glioblastoma cells, and sALCAM was in fact highly expressed and secreted from glioblastoma cells. However, in contrast to our supposition, overexpression of sALCAM in glioblastoma cells enhanced tumor cell invasion in vitro and tumor progression in vivo. Another study found that sALCAM also promoted cell migration in endothelial cells in vitro.[27] The functions of sALCAM may therefore also vary depending on the cell type and microenvironment.

In conclusion, ALCAM is expressed in glioblastoma progenitor cells. Frequencies of ALCAM-expressing cells may correlate with disease progression of glioma and prognosis of glioblastoma patients. Furthermore, we showed that not only membrane-bound ALCAM