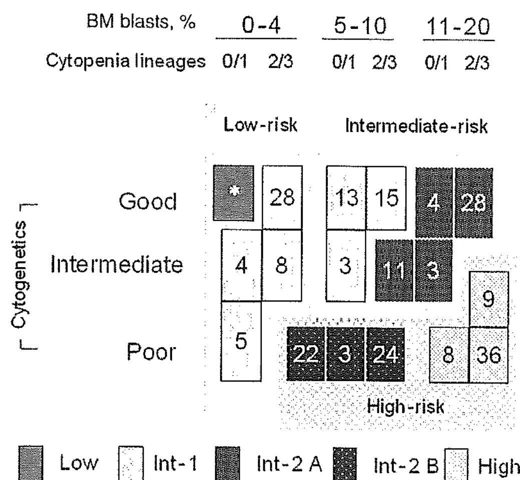

Figure 5. We propose 3 possible risk groups based on International Prognostic Scoring System (IPSS) subclassification. Our proposition may be reflected by the effectiveness of chemotherapy. The high-risk group consists of the intermediate 2B (Int-2B) category and the High category of myelodysplastic syndrome by the World Health Organization classification (WHO-MDS). The intermediate-risk group includes the Int-2 category and patients with more than 5% bone marrow (BM) blasts in the Int-1 category. The low-risk group consists of patients with less than 5% BM blasts and should be treated with noncytotoxic agents.

than that of patients treated with low-dose therapy or supportive care. Intensive therapy may be indicated in young patients. Although we did not analyze PS or the infectious history of patients, we usually chose low-dose therapy or supportive care for patients with poor PS or infection problems. Although we were unable to perform a central review for hematologic profiles in this study, it may well be that low-dose therapy or supportive care was chosen for patients with marked dysplasia. Another important issue is consideration of treatment after initial therapy that affects survival. In this study, however, we have only information concerning initial therapy for each patient. Furthermore, patients for whom cytogenetic data were not successfully obtained may have been excluded from the study. We also have to consider selection bias in the number of patients in each institute. Prospective studies are needed to clarify these possible biases.

MDS cases frequently are considered low risk or high risk rather than one of the 4 IPSS categories. The IPSS Low and Int-1 categories usually are included in the low-risk group, whereas the Int-2 and High categories are considered the high-risk group. On the basis of the data from the present study, we propose 3 groups: low, intermediate, and high risk. The purposes of our proposition are to obtain maximum therapeutic effectiveness and to give chemotherapy only to patients in whom a favorable outcome will be obtained. In our proposed system, the low-risk group includes all patients in the IPSS Low category and those with less than 5% BM blasts in the Int-1 category. These patients can be treated with noncytotoxic agents. The intermediate-risk group, consisting of patients with 5% or more BM blasts and those in the Int-2A category, can be expected to have a favorable outcome when treated with chemotherapy. Finally, patients with WHO-MDS in the Int-2B category and the High category belong to the high-risk group, in which we cannot expect a favorable outcome even with chemotherapy. Stem cell transplantation, if possible, may be a plausible treatment of MDS patients in this category. There is a lack of both consensus and understanding concerning cases included in the FAB criteria for MDS but not in the WHO criteria. Although heterogeneous outcomes can be obtained in such cases, it would be problematic to propose that chemotherapy should not be performed in cases with more than 20% blasts. Further study is necessary to find an appropriate therapeutic strategy for patients in this category.

In general, chemotherapy is still one of the major therapeutic options, because acute leukemia develops in approximately 30% of patients. The indications for chemotherapy must be reconsidered. We propose subdividing MDS patients into 3 groups on the basis of the IPSS. An additional prospective, large-scale study is needed to clarify the usefulness of the possible new risk groups and to determine the therapeutic strategy in the treatment of MDS.

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SHORT COMMUNICATION

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Oral cryotherapy for the prevention of high-dose melphalan-induced stomatitis in allogeneic hematopoietic stem cell transplant recipients

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Abstract *Goals:* The purpose of this study was to evaluate the efficacy of oral cryotherapy to prevent high-dose melphalan-induced stomatitis. *Patients and methods:* Eighteen consecutive recipients of allogeneic hematopoietic stem cell transplant conditioned with high-dose melphalan (140 mg/m^2) in combination with fludarabine alone or with fludarabine and additional chemotherapy or radiation were enrolled. The severity of stomatitis was graded according to the National Cancer Institute Common Toxicity Criteria. Patients were kept on oral cryotherapy using ice chips and ice-cold water shortly before, during, and for additional 90 min after completion of melphalan administration. *Results:* Only two of 18 patients (11.1%) developed grade 2 or 3 stomatitis while six of seven patients in the historical control developed it (85.7%; P=0.001). *Conclusion:* These results suggested that oral cryotherapy could effectively prevent stomatitis caused by high-dose melphalan, and we recommend that it should be incorporated into the conditioning regimen with high-dose melphalan.

Keywords High-dose melphalan · Stomatitis · Oral cryotherapy · Hematopoietic stem cell transplant

Introduction

High-dose melphalan is frequently used in the conditioning regimen for both autologous and allogeneic hematopoietic stem cell transplantation (HSCT) alone or in combination with other chemotherapeutic agents or total body irradiation (TBI). Recently, high-dose melphalan combined with fludarabine has increasingly been used as a reduced-intensity conditioning regimen in the setting of allogeneic HSCT [2, 4, 6, 7].

Oral mucositis, or stomatitis, is one of the major dose-limiting toxicities of high-dose melphalan and contributes significantly to peritransplant morbidity and sometimes to secondary systemic infections. Oral cryotherapy has proven to be effective to reduce the incidence and severity of stomatitis caused by 5-fluorouracil (5-FU) [1, 8, 10]. Like that of 5-FU, the pharmacokinetics of high-dose melphalan demonstrates a short plasma half-life, which suggest that oral cryotherapy only during the periadministration period could ameliorate the subsequent stomatitis caused by melphalan. In this study, we evaluated the efficacy of oral cryotherapy for the prevention of high-dose melphalan-induced stomatitis in recipients of allogeneic HSCT who underwent reduced-intensity conditioning.

Patients and methods

Patients and treatment procedures

Patients with hematologic malignancies who underwent allogeneic HSCT in Keio University Hospital with a reduced-intensity conditioning regimen consisting of fludarabine and high-dose melphalan (140 mg/m^2) were consecutively included. Patients who had received the same conditioning regimen for allogeneic HSCT without oral

cryotherapy before this study was started served as a historical control.

Basically, patients intravenously received fludarabine at a dose of 25 mg/m^2 daily for 5 days (days −6 to −2) and melphalan at a total dose of 70 mg/m^2 per day for 15 min for 2 days (days −3 and −2). In the study group, five patients received additional chemotherapy or radiation as conditioning. For the prophylaxis of acute graft-versus-host disease (GVHD), patients received cyclosporine A (CSA) or tacrolimus with or without short-term methotrexate. Administration of lenograstim at a dose of 5 μg/kg was initiated 1 day after HSCT.

Oral cryotherapy

After informed consent had been obtained, patients received oral cryotherapy on each day of melphalan administration. Patients were instructed to put ice chips and ice-cold water in their mouths 15 min before, during, and for additional 90 min after the melphalan infusion. Patients were advised to continuously swirl ice chips around in their mouths and to gargle with and swallow ice-cold water every 10–20 min throughout oral cryotherapy.

Grading of stomatitis

For grading of stomatitis, the National Cancer Institute Common Toxicity Criteria (NCI-CTC) were used. Grading of stomatitis was as follows: Grade 0: none; grade 1: painless ulcers, erythema, and/or mild soreness in the absence of lesions; grade 2: painful erythema, edema, or ulcers, but can swallow; grade 3: painful erythema, edema, or ulcers preventing swallowing or requiring hydration or parenteral (or enteral) nutritional support; grade 4: severe ulceration requiring prophylactic intubation or resulting in documented aspiration pneumonia. The graded degree of stomatitis was evaluated every day from the day of HSCT to day 28, and the maximum grade of each patient was considered as his or her grade.

Statistical analysis

The difference in the incidence of stomatitis was determined by comparing the two groups by using Fisher's exact test.

Results

Patient characteristics

Eighteen patients were enrolled, and their characteristics are shown in Table 1. In addition to high-dose melphalan and fludarabine, five patients received chemotherapy or radiation such as TBI (400 cGy in three), craniospinal irradiation (240 cGy in one), or high-dose cytarabine (3 g/m^2 every 12 h over 2 days in one) as a conditioning regimen.

Table 1 Patient characteristics. *Flu* fludarabine, *Mel* melphalan, *TBI* total body irradiation, *HDCA* high-dose cytarabine, *CSI* craniospinal irradiation, *GVHD* graft-versus-host disease, *CSA* cyclosporin A, *sMTX* short-term methotrexate

	Study group (n=18)	Control (n=7)
Gender (male/female)	10/8	3/4
Median age	47.5	49
Diagnosis		
Acute leukemia	7	3
Non-Hodgkin's lymphoma	6	3
Myelodysplastic syndrome	2	1
Multiple myeloma	2	0
Chronic myelogenous leukemia	1	0
Preparative regimen		
Flu + Mel	13	7
Flu + Mel + TBI	3	0
Flu + Mel + HDCA	>1	0
Flu + Mel + CSI	1	0
Stem cell source		
Bone marrow (related/unrelated)	7/6	0/2
Related peripheral blood	0	5
Unrelated cord blood	5	0
Acute GVHD prophylaxis		
CSA or tacrolimus + sMTX	15	6
CSA or tacrolimus alone	3	1

Incidence and severity of stomatitis

Only two of 18 patients (11.1%) receiving oral cryotherapy developed moderate-to-severe stomatitis (grade 2 or 3 in NCI-CTC) while six of seven (85.7%) subjects in the historical control developed it (P=0.001). Two patients who developed moderate-to-severe stomatitis were conditioned with fludarabine and high-dose melphalan without additional chemotherapy or radiation. No episode of stomatitis of grade 4 was seen among patients in the study group or in the historical control.

Tolerability of oral cryotherapy

Oral cryotherapy was basically well tolerated. Seven patients (39%) complained of chills, and four patients (22%) had nausea during oral cryotherapy. However, none of these events resulted in the discontinuation of oral cryotherapy, except in one case in which oral cryotherapy was

discontinued at 60 min after melphalan infusion because of nausea.

Discussion

Stomatitis is one of the prominent features of toxicity of high-dose melphalan ($\geq$140 mg/m^2). The incidence and severity of stomatitis depend on the dose of melphalan and probably on the coadministration of other chemotherapeutic agents. Moderate-to-severe stomatitis interferes with nutrition and quality of life and frequently leads to secondary infection in HSCT recipients. Therefore, effective prophylaxis of stomatitis is required as a measure of patient care and, finally, for the improvement of treatment outcome.

Oral cryotherapy for the prevention of stomatitis has already been established in the setting of 5-FU administration [1, 8, 10, 11]. Oral cryotherapy during the periadministration period is only effective for chemotherapeutic agents with short plasma life time. Because of melphalan's short plasma half-life, two groups evaluated the efficacy of oral cryotherapy to eliminate stomatitis caused by high-dose melphalan in an autologous HSCT setting [3, 9]. Dumonet et al. first reported the efficacy of oral cryotherapy in recipients of autologous HSCT conditioned with various regimens, including high-dose melphalan alone or in combination with total TBI, and BEAM chemotherapy [3]. In their study, oral cryotherapy eliminated the development of severe stomatitis in patients receiving high-dose melphalan alone while it was less effective in patients receiving BEAM or combination therapy with TBI. Meloni et al. also reported the efficacy of oral cryotherapy in preventing stomatitis by using ice pops to ameliorate the uncomfortable symptoms due to oral cryotherapy [9].

With the recent emergence of reduced-intensity regimens, high-dose melphalan has become recognized as one of the important agents, particularly in combination with fludarabine, in the conditioning regimens for allogeneic HSCT. However, moderate-to-severe stomatitis was observed in 60–80% of patients undergoing this regimen [2, 6, 7]. In this study, the incidence of moderate-to-severe stomatitis in patients receiving high-dose melphalan-based reduced-intensity conditioning regimens in conjunction with oral cryotherapy was only 11.1%. In spite of the small number of patients and their heterogeneity, the incidence of stomatitis was significantly lower than that of the historical control in which the incidence (85.7%) was similar to that reported in previous studies [2, 6, 7]. Furthermore, five of the patients received additional chemotherapy or radiation which could be associated with stomatitis. However, these additional therapies were only given to the patients receiving oral cryotherapy, and the reduction in the incidence of stomatitis was successfully achieved even in five patients receiving additional chemotherapy or radiation.

The mechanisms of the efficacy of oral cryotherapy in eliminating stomatitis have not been elucidated, and hypothesis includes local vasoconstriction causing less exposure to melphalan, temperature-dependent reduction of melphalan's cytotoxicity, reduced production of chemical meiators, and mucous membrane stability.

In this study, patients were asked to continue oral cryotherapy for 90 min after completion of melphalan infusion. The time course of this procedure was determined according to melphalan's plasma half-life after high-dose administration. The pharmacokinetics of high-dose melphalan demonstrated a biphasic plasma half-life, a distribution phase t1/2, 6–16 min, and an elimination phase t1/2, 40–83 min (Gouyette et al. [5] and an internal report of GlaxoSmithKline Inc.). It is strongly suggested that its distribution phase, because the concentration is higher during the distribution phase, plays a more crucial role in causing stomatitis than does the elimination phase. Therefore, the optimal time for oral cryotherapy could be shortened which may lead to elimination of unpleasant symptoms (e.g., chills or nausea) associated with this procedure.

These findings suggested that oral cryotherapy could be an effective and reasonable therapy for the prevention of development of stomatitis caused by high-dose melphalan in an allogeneic HSCT setting. Future randomized trials are required to further evaluate its efficacy and also to determine the appropriate time course.

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Possible involvement of allogeneic antigens recognised by donor-derived CD4$^+$ cytotoxic T cells in selective GVL effects after stem cell transplantation of patients with haematological malignancy

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Summary

Cytotoxic T lymphocyte (CTL) lines specific for allogeneic antigens were generated by *in vitro* stimulation of donor-derived peripheral blood mononuclear cells obtained from patients who received human leucocyte antigen (HLA)-matched allogeneic haematopoietic stem cell transplantation (HSCT). One of the allogeneic antigen-specific CD4$^+$ CTL lines, CTL-A, generated from a patient with T cell acute lymphoblastic leukaemia, recognised HLA-DPB1*0501-positive Epstein–Barr virus-immortalised human B cell line (EBV-B cells), phytohaemagglutinin blasts and leukaemia cells, but not interferon-γ (IFN-γ) treated HLA-DPB1*0501-positive fibroblasts, indicating that this CD4$^+$ T-cell line recognised a minor histocompatibility antigen (mHa) that is preferentially expressed in haematopoietic cells in an HLA-DPB1*0501-restricted manner. The other CD4$^+$ CTL line, CTL-B, generated from a patient with chronic myeloid leukaemia, recognised mismatched HLA-DQB1*0303 on EBV-B cells and phytohaemagglutinin (PHA) blasts. Interestingly, this CTL line did not recognise IFN-γ-treated recipient's skin fibroblasts, as HLA-DQ was merely upregulated even after IFN-γ stimulation in non-haematopoietic cells including fibroblasts, endothelial cells and hepatocytes. These results suggest that these CD4 positive CTLs, specific for mismatch HLA-DQ and mHa that are preferentially expressed on haematopoietic cells, may play an important role in induction of selective graft-*versus*-leukaemia effect without development of graft-*versus*-host disease after allogeneic HSCT.

Keywords: minor histocompatibility antigens, CD4$^+$ cytotoxic T cells, stem cell transplantation, graft-*versus* leukaemia effect.

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Following allogeneic haematopoietic stem cell transplantation (HSCT), graft-*versus*-leukaemia (GVL) effects by donor lymphocytes contribute to the elimination of residual leukaemia cells. T cells appear to play a major role in GVL activity, because T-cell depletion from stem cell grafts results in a higher rate of leukaemic relapse, and donor leucocyte infusion (DLI) or non-myeloablative allogeneic HSCT results in the successful elimination of leukaemia cells (Falkenburg *et al*, 2003). Although the target antigens involved in GVL responses have not been fully characterised, several antigens have previously been suggested as targets for T cells. They include allogeneic antigens, such as mismatched major histocompatibility complex (MHC) or minor histocompatibility antigens (mHas) (Perreault *et al*, 1998). mHas are allogeneic antigens consisting of a complex of MHC and polymorphic peptides that differ between recipients and donors, and are recognised by T cells. The difference is caused by single nucleotide polymorphisms (SNPs) that result in single amino acid changes. In contrast to the relatively weak immunogenecity of self-tumour antigens, allogeneic antigens have the potential to induce strong immune responses that are sufficient to eliminate tumour cells in patients (Goulmy, 2004; Mutis *et al*, 1999). However, they may also cause severe graft-*versus*-host disease (GVHD). Thus, it is important to develop methods

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that selectively induce GVL against allogeneic antigens without causing GVHD.

One of the strategies for selective GVL induction is to identify allogeneic antigens that are preferentially expressed on haematopoietic cells. Because normal haematopoietic cells are substituted by donor blood cells and residual leukaemia cells are of recipient origin after allogeneic HSCT, allogeneic antigens preferentially expressed on residual leukaemia cells are interesting targets for selective GVL induction. T cells recognise a complex of MHC and antigenic peptides, thus the selective expression of either MHC or antigenic peptides should be considered. Because MHC class II is constitutively expressed in haematopoietic cells, including leukaemia cells, but not in most non-haematopoietic cells, mHas that are restricted by MHC class II, or mismatched MHC class II, could be preferentially expressed on residual leukaemia cells. Most of the previously identified mHas are MHC class I-restricted, and all of three identified MHC class II-restricted mHas are derived from the Y chromosome (Bleakley & Riddell, 2004). Therefore, it is interesting to investigate additional MHC class II-restricted allogeneic antigens recognised by $CD4^+$ T cells for understanding the mechanisms of selective GVL induction.

In this study, we investigated two allogeneic antigen specific $CD4^+$ CTLs and their target antigens, established from patients with long-term complete remission (CR) after allogeneic HSCT. One CTL line appeared to recognise a novel mHa that was preferentially expressed in blood cells including leukaemia cells in HLA-DPB1*0501-restricted manner. The other CTL line recognised mismatched HLA-DQB1*0303, which showed preferential expression in blood cells. These antigens may be involved in the selective GVL induction without GVHD in these transplanted patients, and are attractive targets for immunotherapy against haematological malignancies.

Materials and methods

Profile of patients and samples

Fifteen Japanese patients with various haematological malignancies, who underwent allogeneic HSCT from human leucocyte antigen (HLA)-matched donors through the Keio bone marrow transplantation (BMT) programme, were studied (Table I). Nine of 15 patients developed grades I–II acute GVHD after the transplantation. The grading of GVHD was performed according to Przepiorka *et al* (1995). The donor and recipients' HLA types were determined using serological typing for HLA-A, HLA-B, HLA-C, and HLA-DR, and polymerase chain reaction (PCR)-based genetic typing for HLA-DPB1, HLA-DQB1, and HLA-DRB1. Peripheral blood was obtained from donors and patients before and after transplantation. Mononuclear cells were isolated from peripheral blood and bone marrow using the Ficoll graduation method. Samples from patients were collected after obtaining informed consent.

Patient 5 (HLA-A11/24, B7/67, Cw7, DRB1*0101/1602, DPB1*0402/0501) was diagnosed as T-cell acute lymphoblastic leukaemia (T-ALL). This patient received an allogeneic BMT from an unrelated HLA-matched donor during first CR after chemotherapy. He developed grade II acute GVHD in the skin and chronic GVHD in the liver, which resolved upon treatment, and has been in CR for 81 months since the BMT. Patient 6 was diagnosed with chronic myeloid leukaemia (CML). After interferon (IFN)-α treatment, he underwent allogeneic BMT from an unrelated HLA-matched donor on the Keio BMT programme. Prior to BMT, donor and recipients' HLA types were determined using a serological typing for HLA-A, HLA-B, HLA-C, and HLA-DR. PCR-based genetic

Table I. Characteristics of 15 patients received allogeneic BMT.

Patient	Sex (R/D)	Diagnosis	Donor type	GVHD prophylaxis	Acute GVHD grade	CTL induction
1	M/F	CML	MUD	CSA, MTX	I	ni
2	M/M	CML	SIB	CSA, MTX	None	ni
3	F/M	ALL	SIB	CSA, MTX	II	ni
4	F/F	AML	MUD	FK506, MTX	None	ni
5 (A)	M/M	ALL	MUD	FK506, MTX	II	CD4
6 (B)	M/M	CML	MUD	FK506, MTX	II	CD4
7	F/M	CML	SIB	CSA, MTX	None	ni
8	F/M	CML	SIB	CSA, MTX	None	ni
9	M/M	AML	MUD	FK506, MTX	II	CD4, CD8
10	F/M	AML	SIB	CSA, MTX	None	ni
11	F/M	MM	SIB	CSA, MTX	II	CD8
12	F/F	ALL	SIB	CSA, MTX	II	ni
13	M/F	CML	SIB	CSA, MTX	II	ni
14	M/M	CML	MUD	FK506, MTX	None	ni
15	F/F	CML	SIB	CSA, MTX	I	CD4, CD4+ 8

M, male; F, female; R, recipient; D, donor; CML, chronic myeloid leukaemia; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; MM, multiple myeloma; MUD, matched unrelated donor; SIB, sibling; CSA, cyclosporine A; MTX, methotrexate; ni, allo-specific CTL was not induced; CD4, allo-specific $CD4^+$ CTL; CD8, allo-specific $CD8^+$ CTL; CD4 + CD8; mixture of allo-specific $CD4^+$ CTL and $CD8^+$ CTL

typing for HLA-DPB1, HLA-DQB1, and HLA-DRB1 were conducted later. Following the BMT, he developed grade II acute GVHD in skin and gastrointestinal tract, which was successfully treated with steroid. He also developed chronic GVHD of liver and eyes, which improved without increasing dose of cyclosporine. He has been in CR for 82 months after the BMT.

Cell lines used in this study

Epstein–Barr virus-immortalised human B (EBV-B) cell lines were established by incubating peripheral blood mononuclear cells (PBMC) from patients before HSCT, their donors, or various unrelated individuals in media supplemented with EBV containing supernatants from B95 to B98 cells in the presence of cyclosporine A. Phytohaemagglutinin (PHA) blasts were generated by stimulating PBMC with 1 mg/ml of PHA-M for 7 d. Fibroblasts were cultured from recipients' skin in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, St. Louis, MO, USA) with 10% fetal calf serum (FCS). Leukaemia and lymphoma cells were obtained from the bone marrow of patients, frozen and stored in liquid nitrogen, and used after a quick thaw and washing. HLA-DPB1*0501- and HLA-DPB1*0402-positive EBV-B cells were kindly provided by Dr M. Kuwana, Keio University, Tokyo, Japan. Melanocytes were kindly provided by Dr Honjou, Morinaga Institute of Biological Science, and cultured in serum-free MM-4 medium (Morinaga, Yokohama, Japan). Human umbilical cord venous endothelial cells (HUVEC) were purchased from Cambrex company (East Rutherford, NJ, USA), and cultured in EBM-2 medium (Cambrex). Hepatocytes (Cambrex) were cultured in HCM medium (Cambrex).

Generation of allogeneic antigen-specific T-cell lines

Allogeneic antigen-specific T-cell lines were generated by stimulating 1×10^6 donor-derived PBMC obtained from patients following HSCT. 1×10^6 of recipients' pretransplant PBMC were irradiated with 40 Gy in Iscove's Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% pooled human serum and 100 IU/ml interleukin-2 (IL-2). In seven cases (patients 9–15), the $CD4^+$ or $CD8^+$ cells were separated using magnetic-activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and used as responder cells. On day 7 in culture, 1×10^6 viable cells were restimulated with 1×10^6 irradiated pretransplant PBMC from the patient. On days 14 and 21, 1×10^6 viable cells were restimulated with 5×10^5 irradiated recipient-derived EBV-B cells.

IFN-γ release assay and cytotoxicity assay

The recognition of various cells by T cells was evaluated with IFN-γ release assay and standard ^{51}Cr release cytotoxicity assay. IFN-γ release assay was performed as previously described (Kawakami *et al*, 1994). Briefly, 5×10^4 CTL were cultured with 1×10^5 stimulator cells in 200 μl of Iscove's DMEM with 10% human serum and 25 U/ml IL-2. Following 24 h of culture, the supernatants were collected and the level of IFN-γ was determined by enzyme-linked immunosorbent assay. Antibody blocking was performed as follows: stimulator cells or target cells were incubated with anti-HLA class I (w6/32), anti-class II (IVA12), anti-HLA-DP (B7/21; Becton Dickinson, Mountain View, CA, USA), anti-HLA-DQ (SK10; Becton Dickinson), anti-HLA-DR (L243) or isotype-matched control monoclonal antibodies (MoAbs) for 30 min at 4°C before mixing them with the T cells.

A standard ^{51}Cr release assay with some modifications was used to evaluate the cytolytic activity of T cells (Kawakami *et al*, 1992). Briefly, target cells were labelled with 3·7 MBq ^{51}Cr for 2 h, then washed three times and mixed with effector T cells in 96-well U-bottom plates. The plates were spun and incubated for 4 h. Then, 100 μl of supernatant per well was harvested and mixed with scintillation liquid. The radioactivity was measured using a Top counter (Packard, CA, USA). The per cent specific lysis was calculated using the values of the experimental, spontaneous, and total release of ^{51}Cr.

Analysis of cell-surface antigens by flow cytometry

The expression of cell-surface antigens was evaluated by indirect immunofluorescence methods by fluorescence-activated cell sorting (FACS) (FACSCalibur; Becton Dickinson). T cells were analysed for the expression of CD3, CD4 and CD8 using fluorescein isothiocyanate (FITC)-conjugated anti-CD3, FITC-conjugated anti-CD4 and phycoerythrin (PE)-conjugated anti-CD8 antibodies (Ancell). EBV-B cells, PHA blasts, fibroblasts, hepatocytes, HUVEC and leukaemia cells from patients were analysed for the expression of HLA class I and class II using anti-HLA class I (w6/32), anti-class II (IVA12), anti-HLA-DP (B7/21; Becton Dickinson), anti-HLA-DQ (SK10; Becton Dickinson), and anti-HLA-DR (L243 ascites) antibodies.

Results

Generation rate of allogeneic antigen-specific T cells from patients who received allogeneic HSCTs

Allogeneic antigen-specific T cell lines that recognised recipient-derived target cells, but not donor-derived cells were generated from samples of five patients (patients 5, 6, 9, 11 and 15). FACS analysis revealed that four of these five T cell lines contained predominantly $CD4^+$ T cells (>95%). All $CD4^+$ T cells that preferentially responded to recipient-derived cells were induced in the patients who received sex-matched, related or unrelated transplants. Two of the $CD4^+$-dominant T-cell lines, CTL-A (patient 5) and CTL-B (patient 6), were characterised further in this study. Both patients developed

grade II acute GVHD of the skin and patient 5 developed limited chronic GVHD of the liver; all were successfully treated. They have both maintained CR for >80 months following allogeneic BMT.

MHC class II restricted recognition of a minor histocompatibility antigen expressed on haematopoietic cells by CD4^{+} CTL-A

The CD4-dominant T-cell line, CTL-A, generated from patient 5 showed significantly higher IFN-γ release when cultured with recipient-derived EBV-B cells and PHA blasts than with donor-derived EBV-B cells and PHA blasts. They did not respond to IFN-γ treated autologous skin fibroblasts that expressed HLA-DR and DP (Fig 1A). Cytotoxic assay showed that CTL-A lysed recipient-derived EBV-B cells, but not donor-derived EBV-B cells or K562 cells (Fig 1B). The IFN-γ release by CTL-A was inhibited by anti-HLA-DP,DR Ab IVA12, anti-HLA-DP Ab, and anti-HLA-DR Ab, but not by HLA-MHC class I Ab W6/32 or anti-HLA-DQ Ab, indicating that this CTL contained both HLA-DP-restricted and HLA-DR-restricted T cell clones that recognised the recipient's EBV-B cells (Fig 1C). These results may indicate that the CD4^{+} CTL-A recognises mHas that are preferentially expressed in haematopoietic cells, including EBV-B cells and PHA blasts in an MHC class II (HLA-DR and HLA-DP) restricted manner, although further studies including the molecular identification of the antigens are necessary for the evaluation of their tissue expression.

Distribution of the HLA-DP-restricted mHa recognised by CTL-A in the Japanese population

To determine the HLA restriction and the distribution of the mHa recognised by CTL-A in the Japanese population, we analysed the recognition by CTL-A of a panel of EBV-B cells that expressed various HLA types. As shown in Table II, CTL-A recognised 11 of 13 HLA-DPB1*0501-positive EBV-B cells, including the recipient's cells, whereas it recognised none of seven HLA-DPB1*0501-negative target cells or two HLA-DPB1*0501-positive EBV-B cells, including the donor's cells. No correlation was found between the recognition of CTL and HLA-DPB1*0402, HLA-DRB1*0101, or HLA-DRB1*1602. These results indicate that the CTL-A recognises the frequently (11/13, 85%) expressed, HLA-DPB1*0501-restricted mHa, in the Japanese population.

Recognition of leukaemia cells by the mHa-specific CTL-A

To evaluate the expression of the mHa on leukaemia cells, the recognition of various leukaemia and lymphoma cells derived from patients with acute myeloid leukaemia (AML), CML, adult T-cell leukaemia (ATL) and non-Hodgkin lymphoma, was evaluated by assaying IFN-γ release. As shown (Fig 2), four of five HLA-DPB1*0501-positive leukaemia and lymphoma cells were recognised by CTL-A, indicating frequent expression of the mHa on various leukaemia and lymphoma cells. One of the AML cells was not recognised, suggesting that the mHa was not expressed on the cell surface. These results

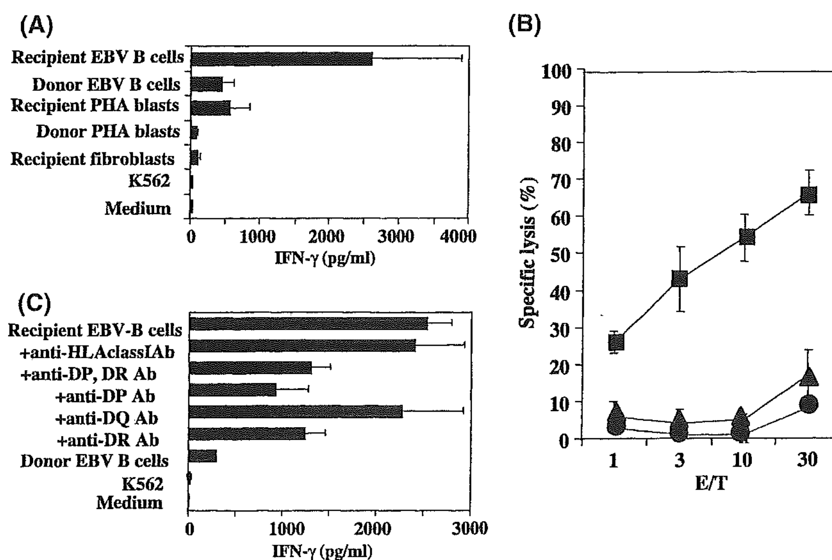

Fig 1. Recognition of recipient-derived EBV-B cells and PHA blasts by CTL-A. Recognition by CTL-A of donor and recipient-derived cells was evaluated using an IFN-γ release assay (A) and a ^{51}Cr release cytotoxicity assay (B). CTL-A released IFN-γ at a significantly higher level against recipient-derived EBV-B cells and PHA blasts. This cell line did not respond to IFN-γ-treated fibroblasts from the recipient. CTL-A also lysed recipient-derived EBV-B cells (■), but not donor-derived EBV-B cells (▲) or K562 cells (●). Specific recognition of the recipient-derived EBV-B cells by CTL-A was inhibited by anti-HLA-DP and DR (IVA12), DP, and DR antibodies, but not by anti-MHC class I (W6/32), anti-HLA-DQ antibodies, and isotype Ig (data not shown) indicating that CTL-A contained both HLA-DP- and HLA-DR-restricted T-cell clones (C). The results are representative of three individual experiments.

Table II. Recognition of mHa on EBV-B cells by CTL-A in a HLA-DPB1*0501 restricted manner.

EBV-LCL	DPB1	DQB1	DRB1	IFN-γ (pg/ml)
Patient	0501/0402	0501/0502	0101/1602	1558
Donor	0501/0402	0501/0502	0101/1602	16
P1	0501/–	0501/–	0101/0802	2363
P2	0501/0201	0601/0604	1302/0803	2263
P3	0501/–	0501/0601	0101/0803	1693
P4	0501/1401	0303/0401	0405/0901	1855
P5	0501/–	0601/–	0405/0803	1408
P6	0501/–	0601/0303	0803/0901	1315
P7	0501/0201	0303/0301	0401/0901	1007
P8	0501/1301	0602/0303	1501/0901	1174
P9	0501/1401	0301/0303	1201/–	804
P10	0501/0201	0601/0303	0803/0901	1338
P11	0501/0201	0601/0604	1302/0803	63
P12	0101/0901	0201/0601	1405/1502	124
P13	0401/0402	0501/0604	0101/1302	52
P14	0402/–	0301/0501	0101/0101	54
P15	0201/0402	0303/0402	0405/0901	0
P16	0201/–	0303/–	0901/–	89
P17	0201/0402	0604/0303	1302/0901	0
P18	0401/0402	0604/0301	1201/1302	0

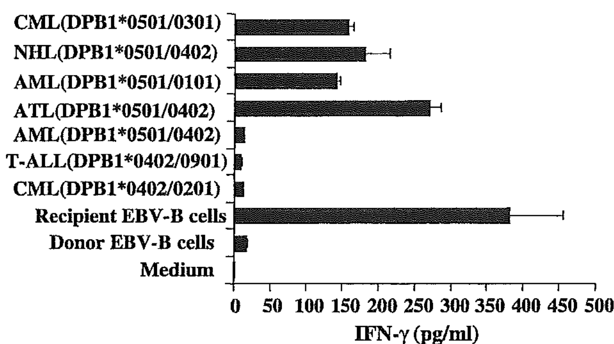

Fig 2. Recognition of various leukaemia and lymphoma cells by CTL-A. Recognition by CTL-A of leukaemia and lymphoma cells derived from patients with AML, CML, ATL, T-ALL and non-Hodgkin lymphoma (NHL), was evaluated using the IFN-γ release assay. Four of five leukaemia and lymphoma cells from HLA-DPB1*0501-positive patients were recognised by CTL-A. The result shows one representative experiment from two independent experiments.

may suggest that the T-cell response to this mHa might be involved in the GVL effect that maintained the CR status of patient 5.

Recognition of mismatched HLA-DQ by CTL-B

The $CD4^+$ CTL line, CTL-B, was generated from a CML patient (patient 6) following the treatment of acute GVHD. Similar to CTL-A, CTL-B, showed significantly higher IFN-γ release when cultured with recipient-derived EBV-B cells and PHA blasts than with donor-derived EBV-B cells and PHA blasts. It did not respond to IFN-γ-treated autologous skin fibroblasts (Fig 3A). The cytotoxicity assay showed that his CTL lysed recipient-derived EBV-B cells, but did not lyse donor-derived EBV-B cells or K562 cells (Fig 3B). The IFN-γ release by this CTL was inhibited by anti-HLA-DQ Ab, but not by HLA-MHC class I Ab W6/32 or anti-HLA-DP, DR Ab, indicating that this CTL contained a CTL clone recognising the recipient's EBV-B cells in an HLA-DQ restricted manner (Fig 3C).

To determine the precise HLA restriction, we first performed PCR-based typing of HLA-DQB1. It was revealed that the recipient had HLA-DQB1*0303/0401, and HLA-DQB1*0303 was absent in donor cells (HLA-DQB1*0402/0401). Therefore, mismatched HLA-DQB1*0303 was suggested to be a target antigen for CTL-B. To confirm the allogeneic response to the mismatched HLA-DQB1*0303 by CTL-B, the CTL response against EBV-B cells derived from patient's family was first examined. As shown in Fig 4A, CTL-B recognised both the mother's (HLA-DQB1*0303/0401) and the father's (HLA-DQB1*0303/0601) EBV-B cells. A panel of EBV-B cells, either HLA-DQB1*0303-positive or negative, was then tested, and all five HLA-DQB1*0303-positive EBV-B cells, but none of five HLA-DQB1*0303-negative EBV-B cells including donor cells (HLA-DQB1 0401/0402), were recognised by the CTL (Fig 4B), indicating that CTL-B recognised a mismatched HLA-DQBI*0303.

Differential expression of HLA-DQ in haematopoietic and non-haematopoietic cells

Expression of MHC class II on leukaemia cells was first examined. Although two leukaemia cell lines (HL60 and U937), but not IM9, showed relatively lower expression of HLA-DQ than HLA-DR and HLA-DP (Fig 5A), nine of 11 fresh leukaemia blasts constitutively expressed HLA-DP and HLA-DQ. The representative results of the constitutive expression of HLA-DR, HLA-DP, and HLA-DQ on CD34-positive blasts from AML and ALL are shown in Fig 5B.

As IFN-γ-treated, MHC class II positive recipient's skin fibroblasts was not recognised by CTL-B, we further detected cell surface expression of HLA-DP, DQ and DR on non-haematopoietic cells, including fibroblasts. As shown in Fig 6A, in contrast to the high upregulation of HLA-DR and HLA-DP, HLA-DQ was not induced on fibroblasts by IFN-γ treatment. Other tissue cells, including melanocytes, hepatocytes and HUVEC were then examined; none of these non-haematopoietic cells tested expressed HLA-DQ as highly as HLA-DP or HLA-DR by IFN-γ treatment (Fig 6B). These results indicated that CTL specific for mismatched HLA-DQ may induce selective GVL effects without induction of GVHD, and might contribute to the favourable prognosis in patient 6.

Discussion

Allogeneic HSCT, including non-myeloablative allogeneic HSCT and DLI, have recently been considered as immuno-

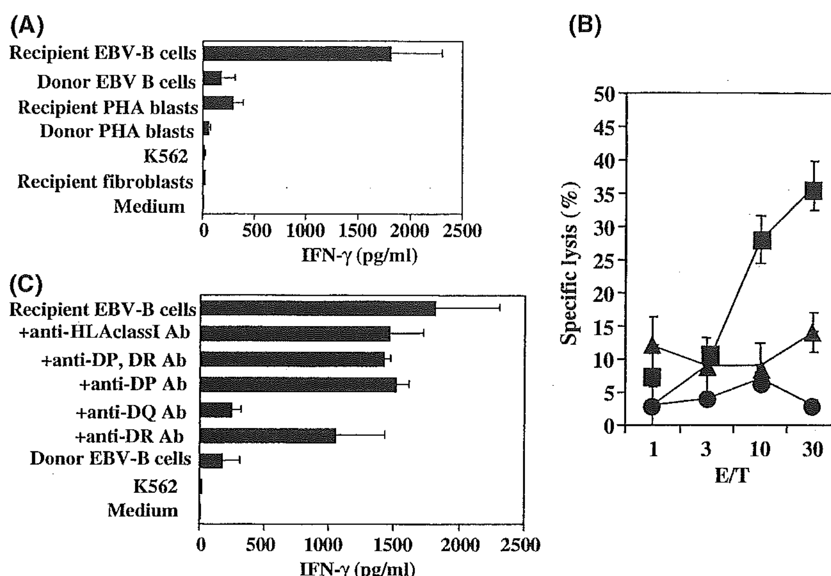

Fig 3. Specific recognition of EBV-B cells and PHA blasts established from the recipient by CTL-B. Recognition of donor and recipient-derived cells by CTL-B was evaluated by IFN-γ release assay (A) and cytotoxicity assay (B). CTL-B released IFN-γ significantly higher when cultured with recipient-derived EBV-B cells and PHA blasts than with donor-derived EBV-B cells and PHA blasts. It did not respond to IFN-γ treated recipient's fibroblasts. This CTL line also lysed recipient-derived EBV-B cells (■), but not donor-derived EBV-B cells (▲) or K562 cells (●). Specific recognition of the recipient-derived EBV-B cells was inhibited only by anti-HLA-DQ antibody, but not inhibited by anti-MHC class I (W6/32) and anti-HLA-DP, DR antibodies (C). The results are representative of three individual experiments.

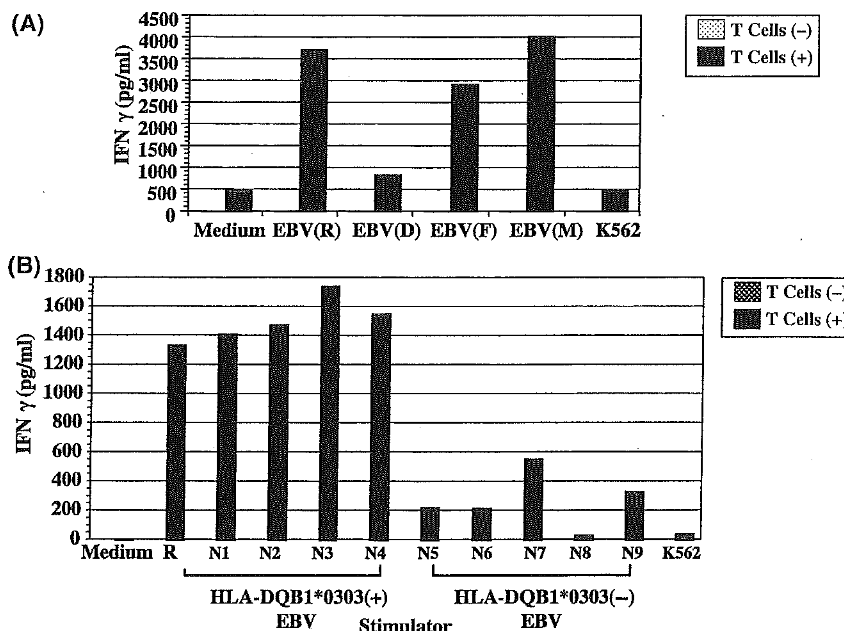

Fig 4. Recognition of allogeneic-derived EBV-B cells by CTL-B. Recognition by CTL-B of recipient's family-derived EBV-B cells was evaluated using IFN-γ release assay (A). All of the family-derived cells were recognised by CTL-B. R; recipient; D; donor; F; father; M; mother. Recognition by CTL-B of allogeneic EBV-B cells was evaluated using IFN-γ release assay (B). All of five HLA-DQB1*0303-positive EBV-B cells were recognised by CTL-B. On the contrary, none of the HLA-DQB1*0303-negative EBV-B cells were recognised.

therapies for patients with haematological malignancies. For developing effective immunotherapy, it is important to identify target antigens recognised by immune cells. Many tumour antigens recognised by T cells have recently been isolated for many cancers, particularly for melanoma (Kawakami & Rosenberg, 1997). Tumour antigens have also been identified in haematological malignancies, such as proteinase 3, WT-1, mutated *BCR-ABL* products, PRAME (preferentially expressed antigen in melanoma) and EBV proteins (Kawakami, 2003). In addition, allogeneic antigens, including

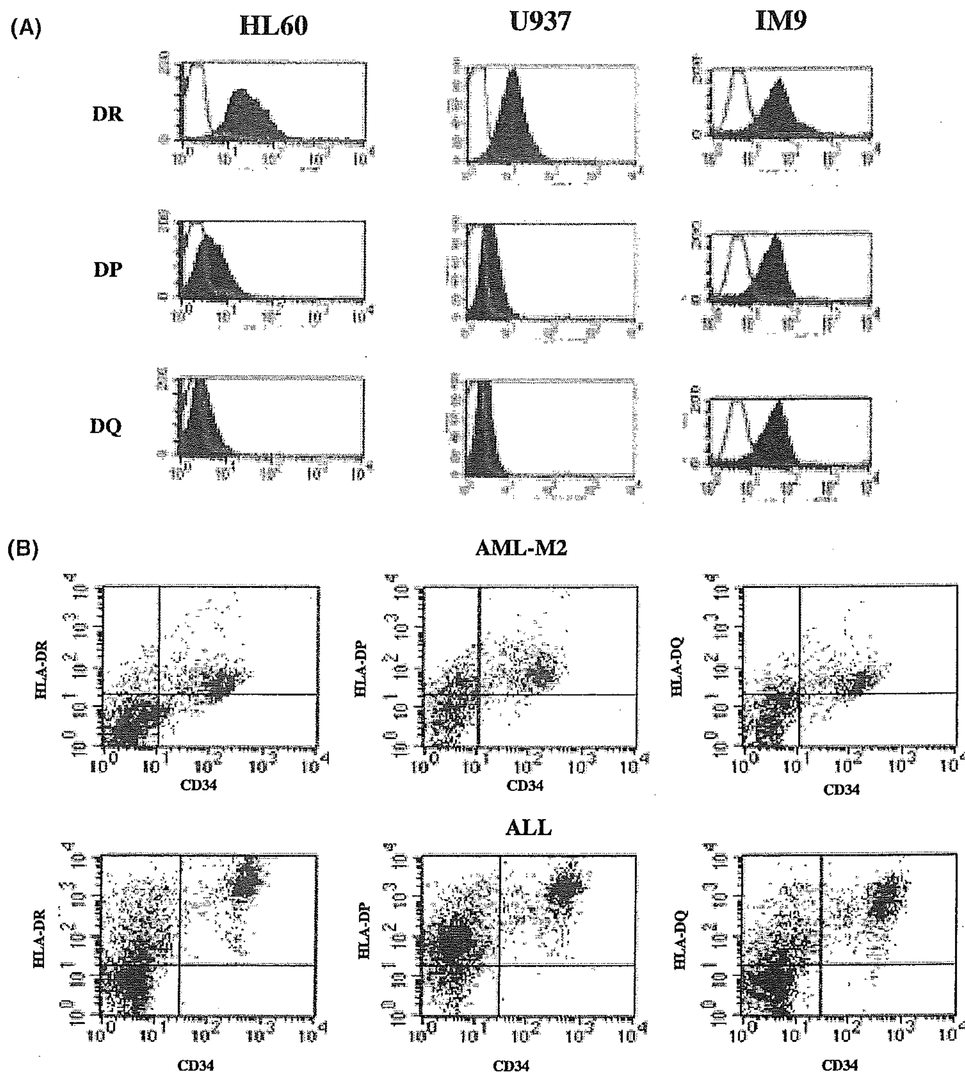

Fig 5. MHC class II expression in leukaemic cell lines and primary leukaemia cells. Leukaemic cell lines, HL60, U937, AND IM9 were stained with anti-HLA-DP, DR, or DQ antibodies (A) (thin line; isotype control). Eleven primary leukaemic cells were stained with anti-CD34 and anti-HLA-DP, DQ, or DR antibodies (B). Two representative data was shown.

mismatched MHC and mHas, are interesting target antigens in the treatment of haematological malignancies following allogeneic HSCT, because they may induce strong immune responses compared with self tumour antigens. However, they may also cause severe GVHD, and methods for selective GVL induction remain to be developed (Simpson *et al*, 2001).

In this study, we succeeded in generating allogeneic antigen-specific T-cell lines in five of 15 allogeneic SCT patients with various haematological malignancies. The success rate of allogeneic antigen-specific T-cell induction appeared to be lower than that previously reported in Caucasians (Warren *et al*, 1998). This difference may be explained by the relative genetic homogeneity in the Japanese population. Interestingly, all of the four CD4$^+$ CTL specific for recipient-derived cells were induced from patients who received the sex-matched HSCT, although the type of donors (sibling or unrelated) or leukaemia did not seem to relate to the CD4$^+$ CTL induction. These observations suggest that the antigens recognised by CD4$^+$ CTL appear to be common antigens, not related to H–Y antigens.

We further characterised two of these five CTL lines. The predominantly CD4$^+$ CTL line A (CTL-A) contained HLA-DPB1*0501-restricted CTL against mHa, which may be expressed frequently and preferentially on blood cells in the Japanese population. This mHa was expressed in various leukaemia and lymphoma cells, including AML, CML, ATL, and non-Hodgkin lymphoma. HLA-DPB1*0501 is the most frequently expressed HLA-DPB1 allele among Japanese (64·0%), and the phenotypic frequency is higher than in Caucasians (12·5%) and Blacks (10·7%). These results may indicate that this T-cell response might be involved in the selective GVL effects in this patient with long-term CR. The other CD4$^+$ T-cell line, CTL-B, recognised mismatched HLA-DQ. In contrast to the constitutive expression of HLA-DQ in

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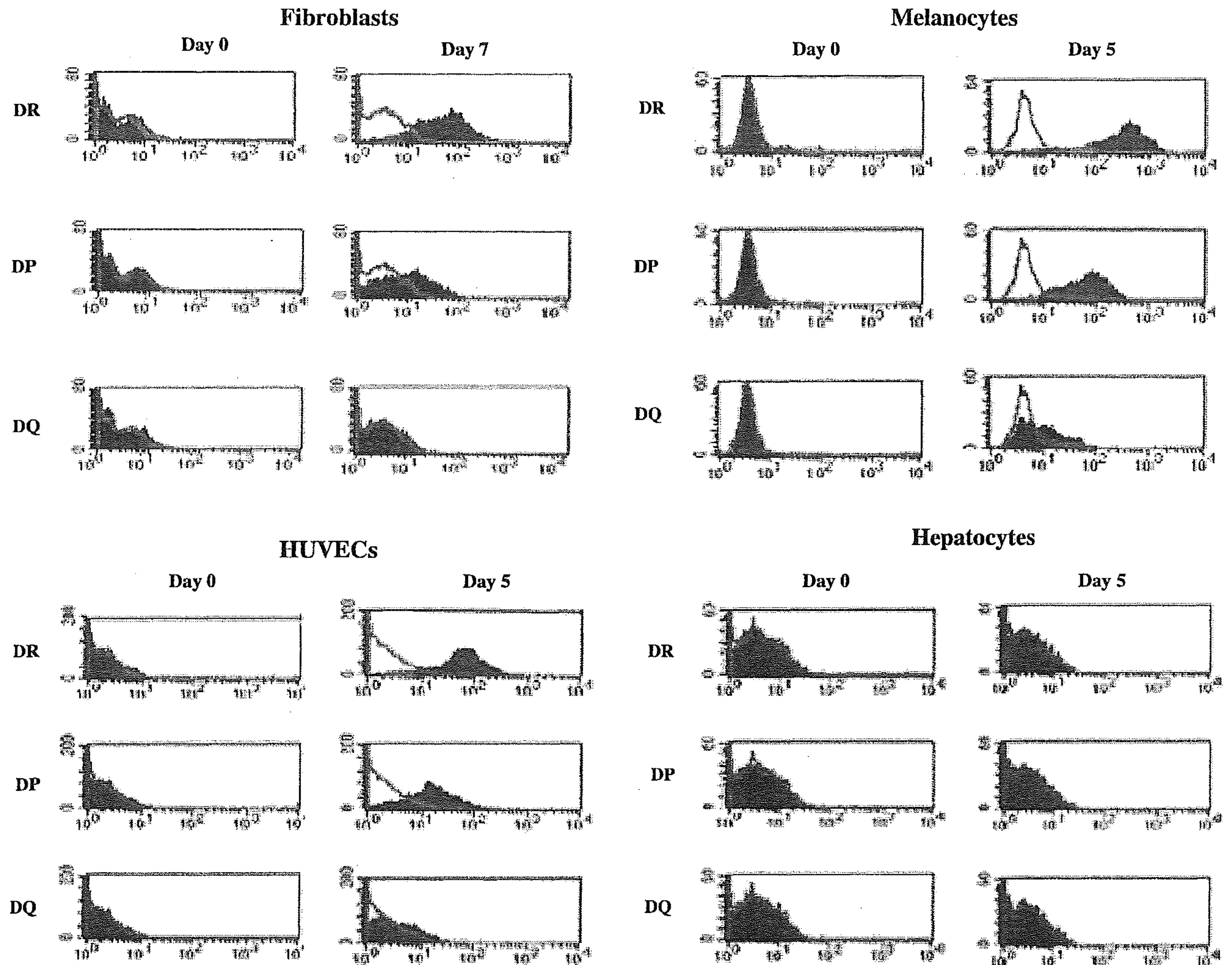

Fig 6. Expression of HLA-class II on fibroblast or other normal cells. Skin fibroblasts, melanocytes, HUVEC and hepatocytes were incubated with IFN-γ for 24 h and assessed for the expression of HLA-DP, DQ, and DR on day 0, 5, or 7. Thin line; isotype control.

many leukaemia cells, it was found to be expressed much less in non-haematopoietic cells including fibroblasts, melanocytes, hepatocytes, and endothelial cells even after IFN-γ treatment, indicating that this CTL may also be involved in the selective GVL effects in this patient with long-term CR.

Induction of selective GVL effects, such as controlling numbers of transferred T cells, and administration of CD8 T-cell-depleted populations, has been attempted for the treatment of haematological malignancies (Cornelissen *et al*, 2003; Alyea *et al*, 2004). However, further strategies are required to improve the selective GVL induction. For this purpose, it is important to select target antigens preferentially expressed on leukaemia cells. In this regard, mHa and MHC have recently been considered as potential targets for T cells involved in GVL effects following allogeneic HSCT. Several mHas recognised by $CD8^+$ CTL and $CD4^+$ CTL have already been identified as peptides either by biochemical isolation methods using HPLC and mass spectrometry or by genetic isolation methods using cDNA expression cloning (Bleakley & Riddell, 2004). Some of the identified mHas, such as HA-1, presented by HLA-A*0201, are specifically expressed in haematopoietic cells including leukaemic cells. These mHas may be useful for mounting immune responses to eliminate residual leukaemia cells. Adoptive transfer of $CD8^+$ T cells specific for such mHas is being conducted (Hambach & Goulmy, 2005).

In this study, we focused on allogeneic antigen-specific $CD4^+$ T cells, because allogeneic antigens have been considered to be more immunogeneic than self-antigens. One CTL line recognises an mHa that appears to be preferentially expressed in haematopoietic cells including leukaemic cells. Recently, three mHas recognised by $CD4^+$ T cells were identified, all of which are products from male-specific genes (Vogt *et al*, 2002; Spierings *et al*, 2003; Zorn *et al*, 2004).

Therefore, the mHa discussed in this study seems to be a novel mHa, because the CTL line was generated from the male patient whose donor was also a male. The other CD4$^+$ T-cells recognised mismatched HLA-DQB1*0303, which was not expressed in target organs of GVHD, such as skin fibroblasts or hepatocytes.

The role of CD4$^+$ T cells in GVHD and GVL responses has not yet been fully understood. It has been reported that adoptive transfer of a CD8-positive T-cell-depleted graft caused less GVHD without an increase of leukaemic relapse (Alyea *et al*, 2004), suggesting the role of CD4$^+$ cells in GVL rather than in GVHD, although CD4$^+$ T cells may cause GVHD when activated by residual recipient cells, including dendritic cells and macrophages in a relatively early phase of HSCT. MHC class II is not constitutively expressed in most somatic cells other than haematopoietic cells, thus MHC class II-restricted CD4$^+$ T cells preferentially recognise leukaemia cells. As normal haematopoietic cells are reconstituted by donor-derived cells, it is considered that mHa-specific CD4$^+$ T cells specifically recognise residual leukaemia cells after allogeneic HSCT. In addition, our data suggest that the mHa recognised by CTL-A is expressed preferentially in haematopoietic cells. The patient from whom this CTL line was generated has remained in CR for 81 months after the allogeneic HSCT. Therefore, this mHa is an attractive antigen for the induction of selective GVL effects against residual leukaemic cells in the treatment of various haematological malignancies. Molecular identification of the mHa remains to be performed for further characterisation and possible clinical use. CTL-B was also induced from a patient with long-term CR, and so these allogeneic reactions, specific to haematopoietic cells, might contribute to GVL effect. The exact role of these antigens in selective GVL effects has to be evaluated with many patients' samples in the future.

In summary, we have generated two allogeneic antigen-specific CD4$^+$ CTL lines from leukaemic patients who received allogeneic HSCT. One may recognise mHa, that is preferentially and frequently expressed on leukaemia cells in the context of popular HLA-DPB1*0501, and the other recognises mismatched HLA-DQB1*0303 preferentially expressed on haematopoietic cells. The preferential expression of these antigens or MHC on haematopoietic cells, including leukaemic cells, and presence of the specific CD4$^+$ T cells in the patients after allogeneic HSCT may indicate that MHC class II-restricted T-cell responses to these allogeneic antigens are involved in selective GVL effects that might lead to the favourable prognosis of these patients.

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Negative regulation of platelet function by a secreted cell repulsive protein, semaphorin 3A

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Semaphorin 3A (Sema3A) is a secreted disulfide-bound homodimeric molecule that induces growth cone collapse and repulsion of axon growth in the nervous system. Recently, it has been demonstrated that Sema3A is produced by endothelial cells and inhibits integrin function in an autocrine fashion. In this study, we investigated the effects of Sema3A on platelet function by using 2 distinct human Sema3A chimera proteins. We detected expression of functional Sema3A receptors in platelets and dose-dependent and saturable binding of Sema3A to platelets. Sema3A dose-dependently inhibited activation of integrin αIIbβ3 by all agonists examined including adenosine diphosphate (ADP), thrombin, convulxin, phorbol 12-myristate 13-acetate, and A23187. Sema3A inhibited not only platelet aggregation induced by thrombin or collagen but also platelet adhesion and spreading on immobilized fibrinogen. Moreover, Sema3A impaired αIIbβ3-independent spreading on glass coverslips and aggregation-independent granular secretion. Sema3A inhibited agonist-induced elevation of filamentous action (F-actin) contents, phosphorylation of cofilin, and Rac1 activation. In contrast, Sema3A did not affect the levels of cyclic nucleotides or agonist-induced increase of intracellular Ca^{2+} concentrations. Thus, the extensive inhibition of platelet function by Sema3A appears to be mediated, at least in part, through impairment of agonist-induced Rac1-dependent actin rearrangement. (Blood. 2005;106:913-921)

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Introduction

Platelets play a crucial role not only in a hemostatic plug formation but also in a pathologic thrombus formation, particularly within atherosclerotic arteries subjected to high shear stress.[1,2] As an initial step in thrombogenesis, platelets adhere to altered vascular surfaces or exposed subendothelial extracellular matrices, then become activated and aggregate each other. These processes are primarily mediated by platelet surface glycoproteins such as GPIb-IX-V, integrin α2β1, GPVI, and integrin αIIbβ3.[3,4] Especially, integrin αIIbβ3 plays an essential part in aggregate formation and adhesive spreading of platelets during hemostasis.[5-7] Pathways that inhibit platelet function are as important as those that activate them. Endothelial cells produce 2 well-documented inhibitors of platelet activation and aggregation, prostaglandin I_2 (PGI_2) and nitric oxide (NO).[8] PGI_2 binds to a specific Gs-coupled receptor, thereby activating adenylate cyclase and cyclic adenosine monophosphate (cAMP)–dependent protein kinase or protein kinase A (PKA). NO activates soluble guanylate cyclase and cyclic guanosine monophosphate (cGMP)–dependent kinase or PKG. Ecto–adenosine diphosphatase (ADPase, CD39) located on the luminal surface of endothelial cells also inhibits platelet aggregation by decreasing the local concentration of ADP. Thus, endothelial dysfunction or damage promotes a prothrombotic state and may be involved in the pathogenesis of cardiovascular disorders, including atherosclerosis, diabetes mellitus, essential hypertension, hypercholesterolemia, and hyperhomocysteinemia.[8]

The semaphorin family comprises soluble and membrane-bound proteins that are defined by the presence of a conserved 500–amino acid semaphorin domain at their amino termini.[9] Class 3 semaphorins are secreted disulfide-bound homodimeric molecules, and Sema3A, a prototypic class 3 semaphorin, causes growth cone collapse and provides chemorepulsive guidance for migrating axons.[10-12] Cell surface receptor for Sema3A consists of a complex of 2 distinct transmembrane receptors, neuropilin-1 and plexin A (A1-A3).[10-13] Neuropilin-1 provides a binding site of Sema3A, while plexin A transduces the Sema3A signals into the cells through its cytoplasmic domain.[10-13] Although the intracellular signaling pathways evoked by Sema3A binding are not fully understood, plexins should interact with signaling molecules to regulate actin reorganization, since growth cone collapse is accompanied by rapid reorganization of the actin filaments normally present in lamellipodia or filopodia.[11,12] In this context, a Rho family small G-protein, Rac, has been identified as a potential regulator of semaphorin-dependent actin cytoskeletal dynamics.[11,12]

Although Sema3A function on neural development is studied intensively, its function in other organs is poorly understood. The fact that semaphorins are expressed in many different tissues suggests that they also play a role in systems other than

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the nervous system.[12] Indeed, in addition to neural abnormalities, mice lacking a functional *Sema3A* gene have abnormalities in their heart and visceral tissues, suggesting that Sema3A signaling might be indispensable for normal development in several organs.[14,15] Very recently, Serini et al reported that semaphorins are also involved in angiogenesis.[16] They showed that endothelial cells generate chemorepulsive autocrine signals of class 3 semaphorins that localize at nascent adhesive sites in spreading endothelial cells.[16] Interestingly, Sema3A inhibits the integrin-mediated adhesion to extracellular matrix and impedes their directional motility, which could explain the aberrant vascularization that is observed in Sema3A-deficient mice.[16] Others also showed that plexin signaling negatively regulates integrin-based adhesive complexes, which leads to the inhibition of cell adhesion, lamellipodia formation, and cell migration.[17]

Since integrin $\alpha IIb\beta 3$ is essential for platelet function and endothelial cells express Sema3A, we sought to investigate the effects of Sema3A on platelet function. In this study, we demonstrate that Sema3A binds to platelets and inhibits $\alpha IIb\beta 3$ activation extensively. Sema3A also inhibits platelet aggregate formation and platelet adhesion and spreading on immobilized fibrinogen. Moreover, Sema3A inhibits $\alpha IIb\beta 3$-independent spreading on glass coverslips and aggregation-independent granular secretion. Further investigation of signaling pathways demonstrates that Sema3A markedly impairs agonist-induced Rac1-dependent actin rearrangement.

Materials and methods

Reagents

Recombinant human Sema3A fused to human Fc fragment (Sema3A/Fc) was obtained from R&D Systems (Minneapolis, MN). A construct consisting of the human *Sema3A* cDNA fused to the catalytic domain of human placental alkaline phosphatase (*AP*) cDNA was prepared as previously described using the pAP-tag2 expression vector (GenHunter, Nashville, TN).[10] The plasmid was transfected to 293T cells by Lipofectamine2000 (Invitrogen, Carlsbad, CA), and recombinant Sema3A/AP was purified from cultured medium using anti–human AP monoclonal antibody–conjugated sepharose beads (clone 8B6) and dialyzed against phosphate-buffered saline (PBS). Human IgG (hIgG) and human placental AP were used for controls of Sema3A/Fc and Sema3A/AP, respectively. Purity of Sema3A/Fc and Sema3A/AP was confirmed by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (SilverSNAP Stain Kit; Pierce, Rockford, IL). Convulxin was kindly provided by Dr M. Moroi (Department of Protein Biochemistry, Institute of Life Science, Kurume University, Fukuoka, Japan). Fibrinogen was purchased from Calbiochem (San Diego, CA) and was labeled with fluorescein isothiocyanate (FITC), as previously described.[18] Type I collagen was obtained from MC Medical (Tokyo, Japan). A hybridoma producing IV.3, a mouse monoclonal antibody specific for human Fcγ-RIIA (CD32), was obtained from American Type Culture Collection (Rockville, MD) and IV.3 Fab fragments were generated as described previously.[19] All other reagents were purchased from Sigma (St Louis, MO), unless otherwise indicated.

Platelet preparation

Washed platelets were prepared as described previously.[20] In brief, 6 vol freshly drawn venous blood from healthy volunteers was mixed with 1 vol acid-citrate-dextrose and centrifuged at 250*g* for 10 minutes to obtain platelet-rich plasma (PRP). After a 5-minute incubation with 1 μM prostaglandin E_1 (PGE_1) and 1 U/mL apyrase, the PRP was centrifuged at 750*g* for 10 minutes, washed once with citrate buffer containing 1 μM PGE_1 and 1 U/mL apyrase, and resuspended in an appropriate buffer. Washed platelets were rested for 30 minutes at 37°C before use in any experiments. In all experiments using Sema3A/Fc, platelet FcγRIIA receptor was blocked by preincubation with 20 μg/mL IV.3 Fab.

Platelets for RNA extraction were prepared as described previously.[21] In brief, to remove the contaminated leukocytes, PRP was passed through a leukocyte removal filter (Sepacell PL-5A; Asahi Medical, Tokyo, Japan), which can remove more than 99.9% of contaminated leukocytes.[21]

Detection of binding of Sema3A to platelets and Sema3A receptors in platelets

For detection of binding of Sema3A/Fc to platelets, 5×10^5 washed platelets in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 3.3 mM NaH_2PO_4, 3.8 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 0.1% glucose, 0.1% bovine serum albumin [BSA], pH 7.4) were incubated with various concentrations of Sema3A/Fc for 30 minutes at room temperature and washed once with citrate buffer. Then, platelets were resuspended in PBS with FITC-labeled anti–human Fc for 20 minutes, followed by flow cytometric analysis. For detection of the binding of Sema3A/AP, 5×10^6 platelets were incubated with various concentrations of Sema3A/AP for 30 minutes at room temperature. After washing with citrate buffer, AP activity was measured using disodium phenylphosphate as a substrate (Sanko Jun-yaku, Tokyo, Japan). The number of Sema3A binding sites was estimated by the maximum AP activity of Sema3A/AP obtained from standard AP activity. In some experiments, platelets were first incubated with 125 μg/mL Sema3A/Fc or hIgG for 10 minutes. After washing, platelets were incubated with 10 μg/mL Sema3A/AP for another 30 minutes, and AP activity was measured.

Western blotting and flow cytometry of neuropilin-1 were performed with mouse anti–neuropilin-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously.[22,23] Horseradish peroxidase (HRP)–conjugated anti–mouse IgG (New England Biolabs, Beverly, MA) and Alexa488-conjugated anti–mouse IgG (Molecular Probes, Eugene, OR) were used as secondary antibodies for Western blotting and flow cytometry, respectively. Reverse transcriptase–polymerase chain reaction (RT-PCR) for detection of plexin-A1, -A2, and -A3 was performed as described.[16] In brief, RNA was extracted by a Trizol reagent (Invitrogen), and cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). RT products were amplified in a PCR reaction with a Taq polymerase (Takara ExTaq; Takara Bio, Shiga, Japan). Primer sequences and PCR conditions were described previously.[16]

Activation of $\alpha IIb\beta 3$ by various agonists

Activation state of $\alpha IIb\beta 3$ was monitored by binding of a ligand-mimetic antibody, PAC-1, or soluble fibrinogen under flow cytometric analysis as described previously.[21,22,24] In brief, 5×10^5 platelets in Walsh buffer were preincubated with Sema3A/Fc or Sema3A/AP for 10 minutes, followed by incubation with agonists and FITC-conjugated PAC-1 (BD Biosciences, Franklin Lakes, NJ) or FITC-fibrinogen for 20 minutes at room temperature. Then, platelets were diluted to 500 μL with Walsh buffer and analyzed immediately on flow cytometry (FACScan; BD Japan, Tokyo, Japan).

Platelet aggregation study

Platelet aggregation was monitored using a platelet aggregometer (model 313M; MC Medical) at 37°C with a stirring rate at 1000 rpm, as previously described.[20] In brief, Sema3A/AP- or AP-treated platelets were suspended in modified Tyrode buffer containing 1 mM $MgCl_2$ at the concentration of $2 \times 10^5/\mu L$. After addition of $CaCl_2$ at the final concentration of 1 mM and incubation for one minute at 37°C, aggregation was initiated by addition of agonists.

Platelet granular secretion

Granular secretion was monitored by FITC-CD62P (Immunotech, Marseille, France) and phycoerythrin-conjugated CD63 (Immunotech) binding to platelets under flow cytometry as described previously.[25]

Adhesion to immobilized fibrinogen or glass coverslips

Adhesion of platelets to immobilized fibrinogen was assessed as described previously.[26] In brief, a 96-well polystyrene plate (Greiner Japan, Tokyo, Japan) was coated with fibrinogen at the various concentrations in PBS for 16 hours at 4°C. Platelets (1.25×10^6) in Tyrode buffer (137 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 0.1% glucose, 0.1% BSA, pH 7.4) were incubated with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature, and then they were placed on each well followed by incubation for one hour at room temperature. After washing 3 times with PBS to remove nonadherent platelets, adhered platelets were quantified by measuring endogenous cellular acid phosphate activity.[27] Relative adhesion to the maximum binding was calculated by dividing the acid phosphatase activity of adherent platelets by that of nontreated platelets adhered on the 10 μg/mL fibrinogen.

Morphologic study of adhered platelets was performed as described previously.[28] In brief, glass coverslips were coated with 20 μg/mL fibrinogen for 16 hours at 4°C, and then washed with PBS. After incubation with Sema3A/Fc or hIgG, 2×10^6 platelets in Tyrode buffer were incubated on the fibrinogen-coated coverslips for 45 minutes at 37°C or on the nontreated coverslips for 10 minutes at room temperature. Nonadherent platelets were washed away and adherent cells were stained with tetramethylrhodamine B isothiocyanate–conjugated phalloidin. Platelet spreading was observed under a florescence microscope (PROVIS AX-80; Olympus, Tokyo, Japan).

Quantification of F-actin contents

Filamentous actin (F-actin) content was analyzed by flow cytometry with bodipy-phallacidin as described previously.[28] In brief, after incubation with 20 μg/mL Sema3A/Fc or hIgG, platelets in Walsh buffer were stimulated with a 30-second incubation with 30 μM protease-activated receptor 1 (PAR1)–thrombin receptor-activating peptide (TRAP) or 0.5 U/mL thrombin at 37°C. Then, platelets were fixed with 4 vol of 2.6% glutaraldehyde in 5.3 mM EDTA (ethylenediaminetetraacetic acid) for 2 hours at 37°C. After washing twice with PBS, the platelets were resuspended to half their initial volume and incubated at 37°C either with 3.3 μM bodipy-phallacidin (Molecular Probes) or bodipy-phallacidin in the presence of a 300-fold molar excess of unlabeled phallacidin. After 30 minutes, the platelets were washed twice with PBS and platelet fluorescence was analyzed in the fluorescence intensity 1 (FL1) channel of the flow cytometer. Specific phallacidin binding was obtained by subtraction of mean fluorescence intensity of FL1 with unlabeled phallacidin from that of FL1 without unlabeled phallacidin.

Detection of phosphorylation of cofilin and activated Rac1

After incubation with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature, 1×10^7 platelets in Walsh buffer were incubated with 0.5 U/mL thrombin for the indicated times at 37°C without stirring. Then, cells were lysed with SDS sample buffer with 5% β-mercaptoethanol (β-ME). Proteins were resolved on a 15% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA). Phosphorylated cofilin was detected by using anti–phospho-cofilin antibody (Cell Signaling Technology, Beverly, MA). After stripping the membrane with a stripping buffer (Restore Western Blot Stripping Buffer; Pierce), the membrane was rehybridzed with anticofilin antibody (BD Biosciences). Optical density of the bands was measured by National Institutes of Health (NIH) Image software (Bethesda, MD). After calibrating the density of phosphorylated cofilin with that of total cofilin, relative increase of phosphorylated cofilin against that of IgG-treated platelets without agonist stimulation was calculated.

Detection of activated Rac1 was performed using a kit of pull-down assay according to the manufacturer's directions (EZ-Detect Rac1 Activation Kit; Pierce). In brief, Sema3A/Fc- or hIgG-treated platelets in Walsh buffer were incubated with 30 μM PAR1-TRAP for the indicated times at 37°C without stirring. Then, cells were lysed with 0.5% Triton-X100 lysis buffer. Guanosine triphosphate (GTP)–form of Rac1 was pull-downed by incubation with glutathione-S-transferase (GST)–p21-activated kinase 1 (PAK1)–p21-binding domain (PBD) and glutathione beads for one hour at 4°C. After washing with lysis buffer, precipitates were eluted with SDS sample buffer with β-ME, followed by electrophoresis on a 12% SDS-PAGE gel. After transfer to a PVDF membrane, Rac1 was detected by a mouse anti-Rac1–specific antibody. Total Rac1 was detected by

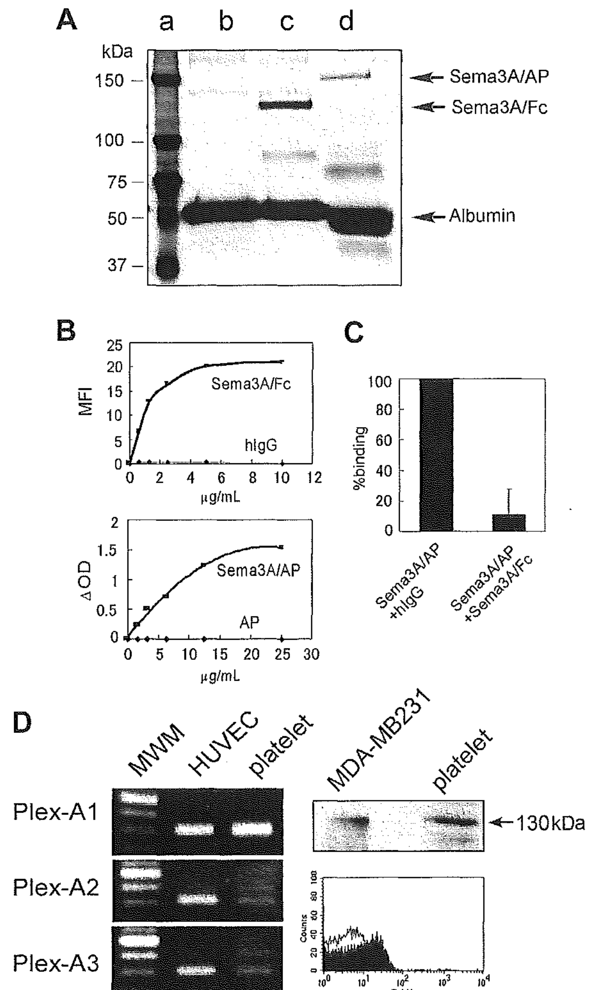

Figure 1. Detection of Sema3A binding to platelets and expression of Sema3A receptors in platelets. (A) Silver stain of purified Sema3A fusion proteins; 0.25 μg of Sema3A/Fc (~ 125 kDa, lane c) and Sema3A/AP (~ 150 kDa, lane d) were loaded on a 7.5% SDS-PAGE gel under reducing conditions and silver staining was performed. Sema3A/Fc and Sema3A/AP samples contain BSA as a carrier protein. In lane c, only BSA was loaded. Molecular weight marker was loaded in lane a. (B) Binding of Sema3A/Fc or Sema3A/AP to platelets. Washed platelets (5×10^5) were incubated with Sema3A/Fc or hIgG, followed by incubation with FITC anti–human Fc. Sema3A/Fc binding was detected by flow cytometry, and mean fluorescence intensity (MFI) was plotted in the top panel. Washed platelets (5×10^6) were incubated with Sema3A/AP or AP, and after washing, AP activity was measured using disodium phenylphosphate as a substrate. Change in optical density (ΔOD) was plotted on the bottom panel. Shown are representative results of 3 independent experiments. (C) Inhibition of Sema3A/AP binding by Sema3A/Fc. Washed platelets were first incubated with 125 μg/mL hIgG or Sema3A/Fc. Then, platelets were incubated with 10 μg/mL Sema3A/AP, and AP activity was measured. Shown is mean and SE of relative binding to hIgG-incubated sample of 3 independent experiments. (D) Expression of plexin-A1, -A2, or -A3 in platelets was detected by RT-PCR assay (left). Human umbilical vein endothelial cell (HUVEC) was used as a positive control. Expression of neuropilin-1 in platelets was detected by Western blotting and flow cytometric analysis (right). In Western blotting, neuropilin-1 expression was detected by anti–neuropilin-1 antibody, followed by incubation with HRP anti–mouse IgG. MDA-MB231 was used as a positive control. In flow cytometry, platelets were incubated with mouse monoclonal anti–neuropilin-1 antibody (filled curve) or control antibody (MOPC21; open curve), followed by incubation with Alexa488-conjugated anti–mouse IgG. MWM indicates molecular weight marker.

electrophoresis of total lysates on an SDS-PAGE gel followed by detection with the Rac1-specific antibody.

Intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} concentrations in fluo-3–loaded platelets were assessed under flow cytometry as described previously.[29] In brief, platelets were labeled with 5 μM fluo-3-AM (Wako Pure Chemical, Osaka, Japan) at 37°C for 15 minutes. After incubation with 20 μg/mL Sema3A/Fc or hIgG, 5×10^5 platelets in 200 μL Walsh buffer were subjected to flow cytometry analysis. After the determination for about 10 seconds of baseline fluo-3 fluorescence from the platelet population, cell aspiration into the flow cytometry was briefly paused, and 1:10 volume of 5 U/mL thrombin was added. The acquisition was then resumed, and changes in log fluorescence versus time were recorded. For each plot, rectangular analysis regions were defined over the time axis, and mean florescence intensity was calculated with CellQuest software (BD Japan).

Quantification of platelet cyclic nucleotide levels

For cAMP quantification, 1.6×10^6 platelets in Walsh buffer were incubated with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature. Iloprost (20 μg/L; Cayman Chemical, Ann Arbor, MI) was used as an agonist for activation of adenylate cyclase. ADP (5 μM) was added to the platelet samples and incubated for 2 minutes at room temperature to study inhibition of adenylate cyclase. After lysing platelets, cAMP contents were measured by an enzyme immunoassay kit according to the manufacturer's directions (Biotrak cAMP EIA System; Amersham, Piscataway, NJ). For cGMP quantification, 3.6×10^6 platelets in Walsh buffer were incubated with 20 μg/mL Sema3A/Fc or hIgG for 10 minutes at room temperature, and cGMP contents were measured by an EIA kit (Biotrak cGMP EIA System; Amersham).

Statistical analysis

Experimental differences over the controls were analyzed by the Student *t* test. Probability values of *P* less than .05 were considered significant.

Results

Binding of Sema3A to platelets and expression of Sema3A receptors in platelets

We used 2 distinct Sema3A chimera proteins in this study: recombinant human Sema3A fused to human Fc fragment (Sema3A/Fc) or to the catalytic domain of human placental alkaline phosphatase (Sema3A/AP) (Figure 1A). We first investigated the binding of Sema3A to platelets. As shown in Figure 1B (upper), Sema3A/Fc bound to platelets in a dose-dependent and saturable manner. Sema3A/AP also bound to the platelets in basically the same manner as Sema3A/Fc, although it needed about 2-fold concentrations, compared with Sema3A/Fc, to saturate the binding to platelets (Figure 1B lower). About 90% of the Sema3A/AP binding was inhibited by preincubation with excess amounts of Sema3A/Fc, confirming the specificity of Sema3A binding to platelets (Figure 1C). We estimated the binding sites of Sema3A were approximately 8000 (7980 ± 500, n = 4) per platelet.

Next, we examined expression of Sema3A receptors in platelets. Western blotting and flow cytometric analysis revealed that neuropilin-1 was expressed in platelets (Figure 1C). Plexin expression was examined by RT-PCR assay, using platelet samples in which the contaminated leukocytes were removed by a leukocyte removal filter. As shown in Figure 1C, plexin-A1 and low levels of plexin-A2 and plexin-A3 were expressed in platelets. These results suggest that platelets express functional Sema3A receptors.

Effects of Sema3A on αIIbβ3 activation by various agonists and platelet aggregation

Since Sema3A inhibits integrin function in endothelial cells,[16] we examined the effects of Sema3A on integrin αIIbβ3 activation using a ligand-mimetic antibody, PAC-1. Sema3A/Fc dose-dependently inhibited PAC-1 binding induced by all agonists examined, including agonists that act via G-protein–coupled receptors (ie, ADP, thrombin, and U46619) and convulxin, which acts via G-protein–uncoupled receptor, GPVI (Figure 2A; Table 1). Sema3A/Fc inhibited A23187- and phorbol 12-myristate 13-acetate (PMA)–induced PAC-1 binding, suggesting that Sema3A inhibits αIIbβ3 activation mainly downstream of intracellular calcium mobilization and protein kinase C activation. Sema3A/AP also inhibited αIIbβ3 activation by thrombin and ADP (Figure 2B), indicating that the inhibitory effects were caused by the Sema3A domain, not by the fused Fc or AP domain. Sema3A/Fc inhibited a physiologic ligand, soluble fibrinogen binding to platelets after

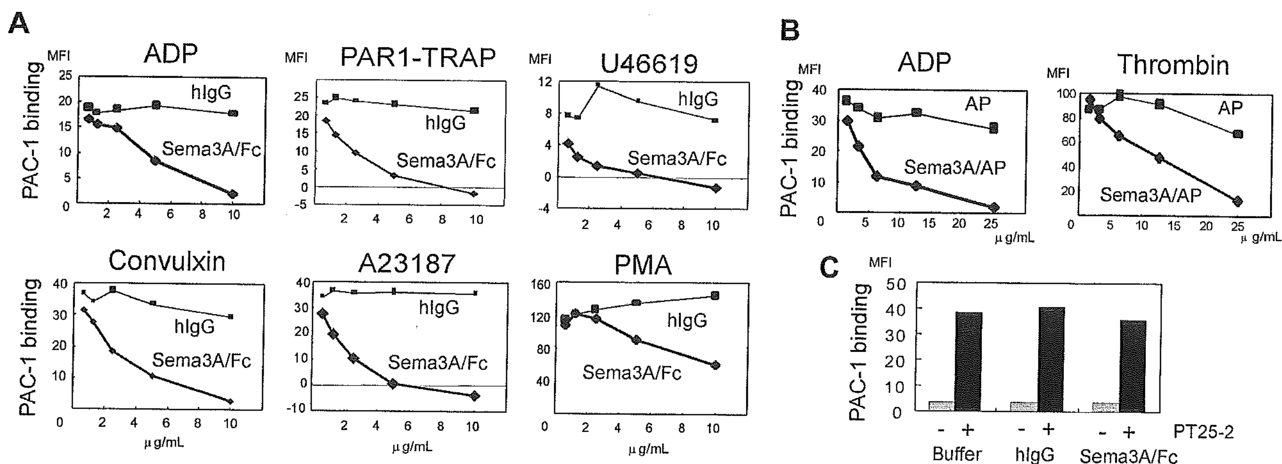

Figure 2. Inhibition of αIIbβ3 activation by Sema3A. (A) Washed platelets preincubated with the indicated concentrations of Sema3A/Fc (◆ and bold lines) or hIgG (■ and thin lines) were activated with ADP (5 μM), PAR1-TRAP (15 μM), U46619 (2 μM), convulxin (5 ng/mL), A23187 (2.5 μM), or PMA (200 nM). Activated αIIbβ3 was detected by binding of FITC–PAC-1. Shown are representative results of 3 to 5 independent experiments. (B) Washed platelets were preincubated with Sema3A/AP (◆ and bold lines) or AP (■ and thin lines) and activated by ADP (5 μM) or thrombin (0.5 U/mL), and then FITC–PAC-1 binding was detected. Shown are representative results of 3 independent experiments. (C) PBS-, hIgG-, or Sema3A/Fc-treated platelets were incubated with or without an αIIbβ3-activating antibody, PT25-2, and PAC1 binding was examined. Shown are representative results of 3 independent experiments.