provided. The synthesized first-strand cDNA was amplified by the polymerase chain reaction (PCR) in a final reaction volume of 50 µl, containing 1 µl of cDNA, 0.5 µM of each oligonucleotide primer, 0.2 mM of each dNTP, 2.5 units of Taq DNA polymerase, and 5 µl of a 10 × PCR buffer. The sequences of primers used were (sense) 5′-TTT GCC TCA AGA TGG AAA CC-3′ and (antisense) 5′-TGT GGG CAA TAG TTG TGC AT-3′ for ET-A (223 bp); and (sense) 5′-GAG TCA ACG GAT TTG GTC GT-3′ and (antisense) 5′-TTG ATT TTG GAG GGA TCT CG-3′ for GAPDH (238 bp). Amplification was carried out in a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA), and the PCR conditions were as follows: 94 °C, 5 min for 1 cycle; 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 30 s (extension) for 25 cycles followed by 7 min final extension at 72 °C. The amplification products were electrophoresed on 1.0% (w/v) agarose gels and visualized by ethidium bromide staining under ultraviolet light.

Cell proliferation assay in vitro

AZ-521 and AZ-H5c cultured in an RPMI 1640 medium were harvested at 80% confluence and seeded at a plating density of 5×10^5 cells/well in six-well plates. They were then cultured for 24 h, and the culture medium was changed to an FBS-free medium supplemented with YM598. We studied seven concentrations of YM598 from 0 to 0.2 mM. Following culture with YM598 for 48 h, the cells were dispersed by trypsinization and counted in a hemocytometer. The experiments were performed in triplicate and repeated twice.

Assay of tumor growth in vivo

Five-week-old female (nu/nu) mice were given injections subcutaneously (s.c.) in the back with 1×10^7 AZ-H5c cells suspended in 100 µl of PBS. The mice were then randomly divided into four groups of eight animals each and received the following treatments: group 1 was injected intraperitoneally (i.p.) with 100 µl of PBS every day for 3 weeks after inoculation; group 2 was injected in the same way with a solution containing 0.01 mg of YM598 dissolved in PBS at a dose of 0.5 mg/kg; group 3 was injected in the same way with a solution containing 0.02 mg of YM598 dissolved in PBS at a dose of 1 mg/kg; and group 4 was injected in the same way with a solution containing 0.2 mg of YM598 dissolved in PBS at a dose of 10 mg/kg. These doses were chosen according to the supplier's recommendations [4]. Treatment started on the same date of the inoculation. Tumor volume was measured with a caliper and calculated as (length × width × height)/2. Mouse body weight was measured once a week.

Assay of liver metastasis in vivo

Five-week-old female (nu/nu) mice were given injections of 5×10^6 AZ-H5c cells suspended in 100 µl of PBS in the spleen. The mice were then randomly divided into four groups of five or six animals each and received the following treatments: group 1 was injected with 100 µl of PBS i.p. every 2 days for 3 weeks after inoculation; group 2 was injected with YM598 every 2 days for 3 weeks after inoculation at a dose of 1 mg/kg i.p.; group 3 was injected with YM598 in the same way at a dose of 10 mg/kg; and group 4 was injected with YM598 at a dose of 1 mg/kg starting 2 days before inoculation. The mice were sacrificed 3 weeks later, and the liver metastasis rate was calculated.

Statistical analysis

The statistical difference was determined by Student's *t*-test. $P < 0.05$ was considered significant.

Results

Microarray assay

A differential gene expression analysis using a DNA microarray demonstrated that many genes were up- and down-regulated in highly metastatic cell lines, in contrast to the parental cell line AZ-521. In these differentially expressed genes, the ET-A gene was the only one of three highly metastatic variants that was generally up-regulated, in contrast to the AZ-521 line. The ratios of the ET-A mRNA expression level in comparison with AZ-521 were 30.1 (AZ-H5c), 57.3 (AZ-P7a), and 47.8 (AZ-L5G) (Table 1).

Detection of mRNAs by RT-PCR

RT-PCR revealed that the mRNA expression of ET-A in AZ-H5c, AZ-p7a, and AZ-L5G was higher than that of the low metastatic AZ-521. The ET-A mRNA up-regulation in AZ-H5c, AZ-p7a, and AZ-L5G was confirmed (Fig. 2).

Effect of YM598 on AZ-521 and AZ-H5c cell proliferation in vitro

After a 48-h incubation with YM598 in the 0.01–0.05 mM concentration, the number of viable cells of AZ-521 and AZ-H5c decreased. In the 0.1–0.2 mM concentrations of YM598, only a few viable cells were seen in the two lines. There was no significant difference between the two lines (Fig. 3). These data suggest that YM598 may have a growth inhibition effect in the 0.01–0.05 mM concentrations on both lines in vitro. In contrast, the cyto-

Table 1. Top three up-regulated genes in highly metastatic cell lines in comparison with the parental low metastatic cell line

Accession no.	Gene name	Intensity in AZ-H5c, AZ-P7a, or AZ-L5G	Intensity in AZ-521	Ratio
AZ-H5c vs. AZ521		AZ-H5c		
D11151	Endothelin-A receptor	987.6	27.3	30.1
L37127	RNA polymerase II	239.3	206.9	7.9
U43142	Human vascular endothelial growth factor-related protein	112.6	–24.0	5.3
AZ-P7a vs. AZ521		AZ-P7a		
D11151	Endothelin-A receptor	1884	27.3	57.3
M35878	Insulin-like growth factor-binding protein-3	739.8	–25.4	20.4
S77154	NGFI-B/nur77 β-type transcription factor homolog	342.3	6.0	11.9
AZ-L5G vs. AZ521		AZ-L5G		
D11151	Endothelin-A receptor	2029	27.3	47.8
S77154	NGFI-B/nur77 β-type transcription factor	220.5	6.0	6.3
D50683	TGFβ-IIRα	229.2	5.5	5.7

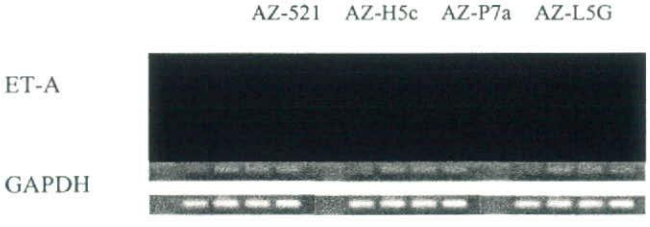

Fig. 2. Reverse transcription polymerase chain reaction (RT-PCR) analysis of ET-A genes in the four cell lines. Electrophoretic images of ET-A mRNA expression by RT-PCR show more up-regulation of the ET-A gene in the highly metastatic cell lines than in the low metastatic AZ-521. Expression of the *GAPDH* gene was used as an internal control for the integrity of the RNA molecules

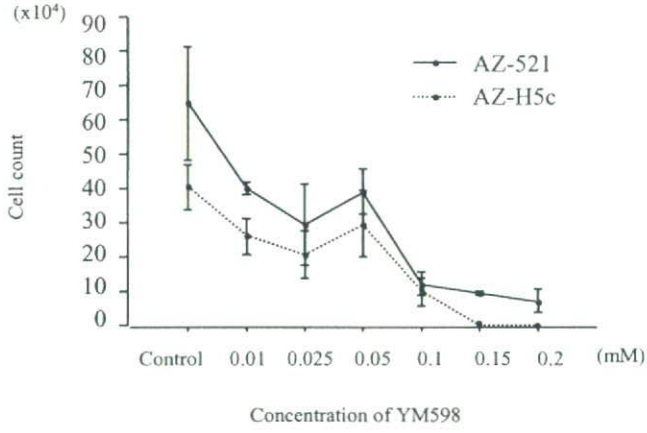

Fig. 3. Effect of YM598 on AZ-521 and AZ-H5c cell proliferation in vitro. After a 48-h incubation with YM598, the number of viable cells of both lines decreased at 0.01–0.05 mM concentrations. At 0.1–0.2 mM concentrations of YM598, only a few viable cells were seen in both lines. There was no significant difference between the two lines. The *vertical bars* show SEs

toxicity of YM598 was shown in the 0.1–0.2 mM concentrations.

Effect of YM598 on the tumor growth of AZ-H5c cells in vivo

As shown in Fig. 4, the administration of YM598 inhibited growth of the AZ-H5c tumors significantly at any dosage. The mean tumor volumes at autopsy were 1453.9 mm^3 (group 1), 502.7 mm^3 (group 2), 448.0 mm^3 (group 3), and 293.7 mm^3 (group 4). The growth inhibition rates, compared with that of group 1, were 34.6% (group 2), 30.8% (group 3), and 20.2% (group 4). There was no evidence of gross toxicity, such as weight loss, resulting from YM598 treatment; and no death occurred during the experiment in any of the groups (Fig. 5).

Effect of YM598 on the liver metastasis of AZ-H5c cells in vivo

In the control group, five of six mice (metastatic rate 83.3%) developed liver metastasis. On the other hand, one of six mice in group 2 (16.7%), one of five mice in group 3 (20.0%), and one of six mice in group 4 (16.7%) developed liver metastasis (Fig. 6). These results demonstrated that YM598 administration significantly reduced the liver metastatic rate of AZ-H5c. No advantage was obtained from dose escalation or pretreatment with YM598.

Discussion

With metastatic progression, gastric cancer is incurable, and existing systemic therapies are largely ineffective. Thus, the development of novel therapeutic strategies is needed. Differential gene expression analysis using a

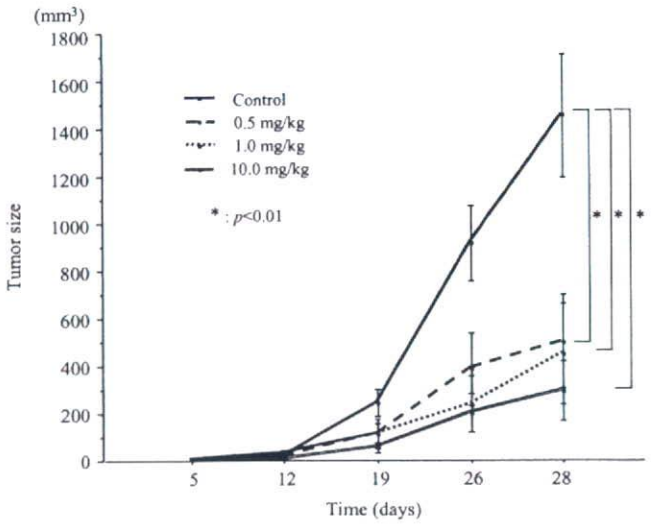

Fig. 4. Effect of YM598 on tumor growth of AZ-H5c in vivo. The administration of YM598 produced significant growth inhibition of the AZ-H5c tumors at any dosage. The *vertical bars* show SEs. $*P < 0.01$ versus control

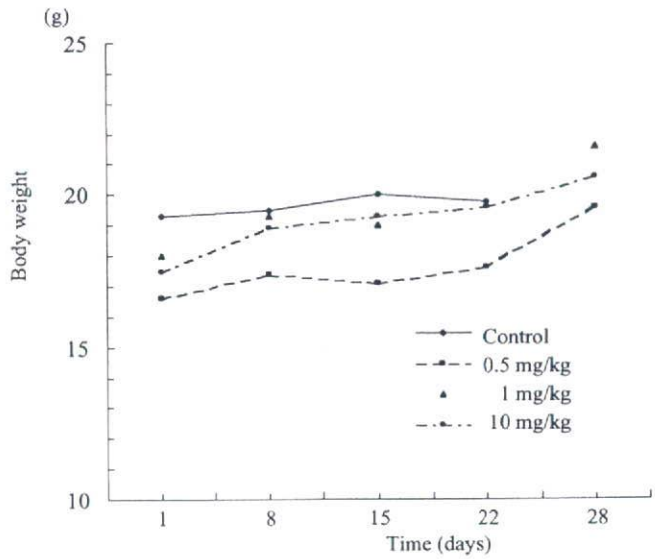

Fig. 5. Changes in the body weight of animals in an in vivo experiment of tumor growth. There was no evidence of weight loss resulting from YM598 treatment during the experiment in any group

DNA microarray is useful for selecting a candidate gene for cancer therapy. To select the metastasis-related genes of gastric cancer, we performed a microarray analysis of three highly metastatic gastric cancer cell lines and compared the results with those from a low metastatic parental cell line. The results suggested that the ET-A gene plays an important role in gastric cancer metastasis.

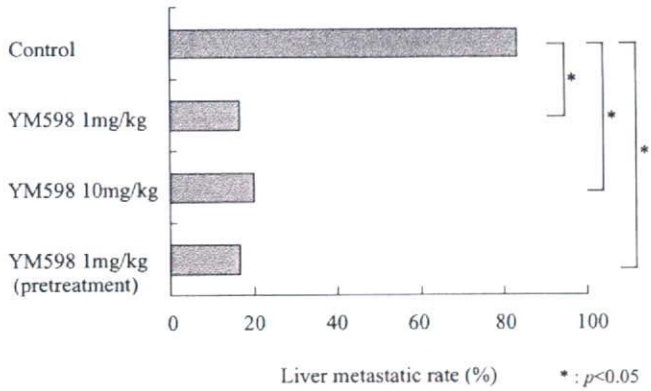

Fig. 6. Effect of YM598 on liver metastasis of AZ-H5c in vivo. YM598 significantly reduced the liver metastatic rate of AZ-H5c. No advantage was obtained from dose escalation or pretreatment

The family of ETs, including ET-1, ET-2, and ET-3, consists of 21 amino acids that mediate a variety of physiological functions. The effects of ETs on mammalian cells are mediated by two distinct subtypes of G protein-coupled receptors: ET-A, which binds ET-1 and ET-2 with high affinity and ET-3 with low affinity; and ET-B, which binds all ET isopeptides with equal affinity [5]. Several human cancer cell lines produce ET-1, which has autocrine/paracrine growth factor functions [6,7]. Elevated plasma levels of ET-1 have also been detected in patients with various solid tumors, including hepatocellular [8] and colorectal [9] cancers. Furthermore, increased ET-A expression in malignant tissue has been demonstrated in several cancer types, including breast [10], ovarian [11], and advanced prostate [12] cancer. The engagement of ET-A by ET-1 is known to trigger activation of tumor growth [13], VEGF-induced angiogenesis [11], cancer cell invasiveness [14], and inhibition of apoptosis [15]. However, little is known regarding how the ET family is involved in cancer metastasis.

In this study, we investigated the effects of ET-A blockade on cell proliferation, tumor growth, and liver metastasis of a highly liver metastatic cell line, AZ-H5c. We used a non-peptide-selective ET-A antagonist, YM598, for the ET-A blockade. It is orally active and has high affinity and selectivity for ET-A with a long duration of action [4]. To date, the effects of the ET-A blockade on gastric cancer have not been investigated. The in vivo study demonstrated that YM598 had a significant growth inhibition effect on AZ-H5c at a dosage of 0.5–10.0 mg/kg. YM598 also decreased the liver metastatic rate of AZ-H5c at 1 mg/kg; however, no advantage was obtained from dose escalation or pretreatment. These results suggested that the liver metastasis-inhibiting effect of YM598 could be largely achieved by

growth inhibition of AZ-H5c. The mechanism through which ET-1 contributes to cell growth is thought to be as follows: Binding of ET-1 to ET-A in the plasma membrane triggers signal-transduction pathways through Gq, a pertussis toxin-intensive G protein that is coupled to the ET-A intracellular domain. Activation of phospholipase C, protein tyrosine kinases, and RAS ultimately results in activation of the RAF/MEK/MAPK pathway. Translocation of intracellular calcium (Ca^{2+}) and activation of protein kinase C and activated MAPK induce nuclear transcription of proto-oncogenes (e.g., c-*FOS*, c-*JUN*, and c-*MYC*), leading to cell growth and mitogenesis [16]. Furthermore, ET-1 not only promotes cell growth, it suppresses apoptosis of cancer cells [15,17]. Therefore, ET-A antagonists could have the effects of both growth inhibition and apoptosis induction on carcinoma xenografts.

Tumor growth is one of multiple steps in a cancer metastatic pathway, and metastasis may possibly be inhibited by blocking one of these steps. In this study, YM598 markedly decreased liver metastasis, which might be due mainly to tumor growth inhibition. However, any other additional effect of the ET-A blockade (e.g., inhibition of cancer cell invasiveness or angiogenesis) could also contribute to reduction of the metastatic rate. Further studies are needed to define the definitive role of the ET-A system in gastric cancer metastasis. Considering its clinical usefulness, it should be determined if YM598 has additional antitumor effects in combination with chemotherapy or radiation therapy.

Conclusion

The present study demonstrated that ET-A blockade by YM598, a non-peptide-selective ET-A antagonist, is effective in inhibiting tumor growth and liver metastasis of a gastric cancer cell line in vivo. Further studies to assess the mechanisms of the effect and the clinical impact of ET-A blockade in gastric cancer are needed. These results suggest that ET-A blockade by YM598 could be a novel therapeutic strategy for inhibiting gastric cancer growth and metastasis.

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Down-Regulation of HLA Class I Antigens in Prostate Cancer Tissues and Up-Regulation by Histone Deacetylase Inhibition

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Purpose: HLA class I down-regulation in cancer cells confers immunological escape from cytotoxic T lymphocytes. We assessed the frequency of down-regulation of HLA class I antigens in a large series of prostate cancer tissues and determined the mechanism of up-regulation by investigating prostate cancer cell lines.

Materials and Methods: Immunohistochemical staining for HLA class I was done in specimens of 419 prostate cancers. We also investigated clinicopathological parameters, and the relationships between HLA class I down-regulation and the parameters. Furthermore, we examined whether HLA down-regulation was caused by epigenetic changes in vitro.

Results: HLA class I was down-regulated in 311 prostate cancers (74.2%) and it significantly correlated with β2-microglobulin down-regulation and a higher clinical stage. Flow cytometric analysis revealed a low level of HLA class I in LNCaP cells, which was up-regulated by the histone deacetylase inhibitor trichostatin A (Sigma®). Trichostatin A up-regulated LNCaP β2-microglobulin at the protein level. Furthermore, chromatin immunoprecipitation assay using an anti-acetylated histone H3 antibody provided direct evidence that trichostatin A up-regulated β2-microglobulin by modulating the acetylation status of the promoter region in LNCaP cells.

Conclusions: The current study shows that the prevalence of HLA class I down-regulation is high in prostate cancer but histone deacetylase inhibitors can up-regulate HLA class I in LNCaP cells by up-regulating β2-microglobulin. We suggest that the combination of an immunotherapeutic approach and histone deacetylase inhibition would accentuate the effects of current immunotherapies for prostate cancer.

Key Words: prostate, prostatic neoplasms, histocompatibility antigens class I, beta 2-microglobulin, trichostatin A

In recent years there have been many studies of specific immunotherapies for various cancers. The rationale for such studies has been supported by strong cellular immune responses, ie introducing cancer specific CTLs from patients. Immune cells must be activated at the tumor site to manifest appropriate effector mechanisms, such as direct lysis or cytokine secretion capable of causing tumor destruction, in addition to the need for a sufficient number of the cells.[1] Most studies showed successful results of in vitro cytotoxicity assays and clinical monitoring, eg by tetramer assay or ELISpot assay (R & D Systems®). However, there have been many cases in which no clinical response was achieved regardless of good immune responses in such assays. Therefore, it is vitally important to evaluate tumor cell factors that influence the clinical responses of specific immunotherapies. Antigen presentation in tumor cells is essential to destroy them, even if an immunotherapy has an excellent effect in eliciting an immune response.

HLA class I has a critical role in the recognition and lysis of tumor cells by CTLs, and defects in antigen presentation could allow the tumor to escape killing by CTLs.[2] The tools for assessing HLA class I expression in surgically removed specimens are limited, although the molecular defects of HLA class I have been studied in detail.[3,4] Recently we developed an excellent mAb for HLA class I, EMR8-5, which is reactive with heavy chains in formalin fixed, paraffin embedded tissue sections. This pan-HLA antibody reacts with all alleles of HLA-A, B and C.

In this study we assessed the frequency of down-regulation of HLA class I antigens using EMR8-5 in a large series of prostate cancer tissues. Furthermore, we determined the mechanism of up-regulation by investigating prostate cancer cell lines.

MATERIALS AND METHODS

Patients and Tissue Samples

We reviewed the clinical pathology archives of 508 consecutive patients who underwent prostate resection or biopsy and were diagnosed with prostatic adenocarcinoma at the Sapporo Medical University Hospital, Sapporo, Japan from 1995 to 2005. Patients whose medical records were incom-

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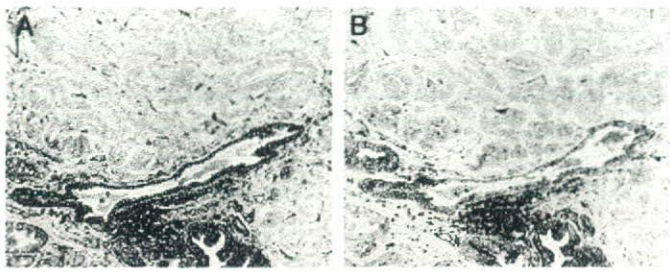

FIG. 1. Representative immunohistochemical staining of HLA class I and β2M down-regulation (score 1) in 68-year-old patient with PSA 5.22 ng/ml and Gleason 4 + 3, clinical T2aN0M0, AJCC stage II prostate cancer shows faint, incomplete membrane staining in cancer cell cytoplasm and/or membranes but complete membrane staining in normal cells, especially lymphocytes and vascular epithelial cells. *A*, staining with mAb for HLA class I (EMR8-5). *B*, staining with mAb for β2M (EMR-B6). Reduced from ×100.

plete were excluded. We selected 419 patients based on the availability of sufficient material for immunohistochemistry. At diagnosis median patient age was 68 years (range 44 to 88) and median serum PSA was 10.9 ng/ml (range 1.1 to 8,670). Informed consent was obtained from patients to use the surgical specimens remaining after pathological diagnosis for the investigational study, which was approved by the Institutional Review Board for Clinical Research at our university. The study included 246 specimens of radical prostatectomy, 136 of prostate biopsy, 28 of cystoprostatectomy for bladder cancer and 9 others, including transurethral prostate resection for benign prostatic hyperplasia, pelvic lymphadenectomy, etc. All hematoxylin and eosin stained slides were reviewed and all of these specimens showed prostatic adenocarcinoma, including Gleason score 3–6, 7–8 and 9–10 in 143, 219 and 57 cases, respectively. Diagnoses were confirmed using the 2002 version of the TNM system. We then classified clinical stage according to the AJCC classification.[5] There were 6 stage I, 320 stage II, 25 stage III and 68 stage IV cases.

Immunohistochemical Staining for HLA Class I

Sections (5 μm) of formalin fixed, paraffin embedded tumors were immunostained after steam heat induced epitope retrieval using mAb EMR8-5, as previously described.[6] We also performed immunohistochemical staining for β2M using our original mAb for β2M, EMR-B6. Human tonsil sections served as positive controls. We also used lymphocytes and vascular epithelium cells in the same specimen as internal positive controls. Negative controls had the primary antibody replaced by buffer. All specimens were reviewed independently using light microscopy by investigators blinded to clinicopathological data (HK and TT).

Membrane immunoreactivity levels for HLA class I and β2M were categorized as undetectable to 2. A score of zero was defined as undetectable staining (fig. 1). A score of 1 was defined as faint, incomplete membrane staining in more than 20% of the tumor cells or as moderate to complete staining in cytoplasm but negative membrane staining in tumor cells. Finally, a score of 2 was defined as complete membrane staining in more than 80% of tumor cells. HLA class I expression was then classified as down-regulated (scores 0 and 1) or positive (score 2).

Statistical Analysis

Spearman's rank correlation was used to test the direction and strength of the relationship between HLA class I expression in cancer cells and other variables, including age, PSA, Gleason score, clinical stage and β2M expression, with $p < 0.05$ considered statistically significant. Calculations were performed using JMP™ software.

Screening of Epigenetic Changes in Prostate Cancer Cell Lines

Treatments with TSA and 5-AC, and flow cytometry. To screen whether HLA class I antigens of prostate cancer cells were down-regulated and whether they could be up-regulated we investigated HLA class I expression on the cell surfaces of prostate cancer cell lines, and the effects of HDAC inhibitor and DNA methyltransferase inhibitor. The human prostate cancer cell lines DU145, PC-3 and LNCaP were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37C with 5% CO_2. Cells with fresh medium were treated with TSA at 300 nM for 24 hours as the HDAC inhibitor and with 5-AC (Sigma-Aldrich) at 5 μM for 72 hours as the DNA methyltransferase inhibitor. After cultivation approximately 2×10^6 cells were incubated with mouse anti-HLA class I mAb W6/32 (American Type Culture Collection, Manassas, Virginia) at 4C for 30 minutes, washed twice with PBS and stained with FITC conjugated goat anti-mouse IgG antibody (BD™ Biosciences) at 4C for 30 minutes. The cells were then washed twice with PBS and resuspended in 1 ml PBS. Flow cytometric detection of FITC positive cells was done using a FACSCalibur™ flow cytometer and CELLQuest™ software. An average of 10,000 events was analyzed.

Western Blot Analysis

Western blotting was performed as previously described[7] using the hybridoma supernatants of EMR8-5 and EMR-B6 for HLA class I heavy chain and β2M, respectively, and mAb to β-actin (Sigma).

ChIP Assay

ChIP assay was performed using an Acetyl-Histone H3 Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, New York) according to the manufacturer protocol. Briefly, cells were plated at a density of 1×10^6 on a 10 cm dish and cultured for 24 hours with 300 nM TSA. Subsequently chromatin was solubilized and subjected to sonication to obtain DNA fragments with an average size of 200 to 1,000 bp. ChIP was performed by incubation with an anti-acetyl histone H3 antibody and a no antibody immunoprecipitation control. Immunoprecipitated DNA served as a template for PCR of β2M promoter. PCR was done in a 50 μl reaction volume using Taq DNA polymerase. Briefly, after heating at 94C for 2 minutes the amplification reaction was performed with primers 5′-GAAAACGGGAAAGTCCC-3′ (forward) and 5′-*AGATCCAGCCCTGGACTAGC*-3′ (reverse) with denaturation at 94C for 30 seconds, annealing at 57C for 30 seconds and extension at 72C for 30 seconds for 30 cycles, followed by incubation at 72C for 7 minutes. For semiquantitative analysis PCR was also performed for

TABLE 1. *Spearman's rank correlation between HLA class 1 expression in cancer cells and other variables*

Variable	Spearman's Rank Correlation Coefficient (ρ)	p Value
Pt age	0.0224	0.6473
PSA	−0.0950	0.0743
Gleason score	−0.0151	0.7575
Clinical stage	−0.1145	0.0191
β2M expression in Ca cells	0.5158	<0.0001

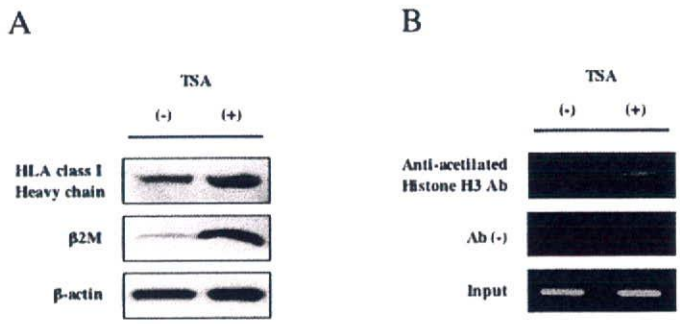

FIG. 2. *A*, Western blotting of LNCaP cells incubated with (+) or without (−) 100 nM TSA for 24 hours. TSA up-regulated β2M protein. *B*, ChIP analysis of histone H3 deacetylation on β2M promoter in LNCaP validated Western blotting result. Input for each reaction served as sample loading internal control. Aliquot precipitated without antibody served as negative control.

genomic DNA obtained from each sample before immunoprecipitation (fig. 2, Input). The PCR product was visualized with ethidium bromide staining under ultraviolet light after electrophoresis on 2.0% agarose gel.

RESULTS

Immunohistochemical study of HLA class I in cancer cells revealed that 91, 220 and 108 of the 419 cases had a score of 0, 1 and 2, respectively. In other words, HLA class I was down-regulated in 311 prostate cancers (74.2%). β2M down-regulation was also found in 24.8% of cases, including 59, 256 and 104 with a score of 0 to 2, respectively. Spearman's rank correlation test revealed that HLA class I down-regulation significantly correlated with β2M down-regulation and higher clinical stage (table 1). Table 2 shows clinical stages in the 419 prostate cancers according to HLA class I and β2M expression.

We next investigated whether HLA down-regulation was caused by epigenetic changes in prostate cancer cell lines. Flow cytometric analysis revealed a low level of HLA class I in LNCaP cells, which was up-regulated by TSA but not by 5-AC (Fig. 3). TSA up-regulated LNCaP β2M at the protein level (fig. 2, *A*). Furthermore, ChIP assay using anti-acetylated histone H3 antibody provided direct evidence that TSA up-regulated β2M by modulating the acetylation status of the promoter region in LNCaP cells (fig. 2, *B*).

DISCUSSION

Although HLA class I down-regulation in prostate cancer has been investigated in several studies,[3,8,9] there were some problems, such as an insufficient number of samples, limited distribution of clinicopathological characteristics and method quality. In this study we included a large number of cases and used the mAb EMR8-5, which reacts with more alleles than other antibodies for HLA class I. Furthermore, we classified the immunostaining patterns of HLA class I heavy chain and β2M, considering cell membrane staining, since these factors are important for specific cancer immunotherapy. Our data suggest that immunotherapy such as an HLA class I associated peptide vaccine may not be suitable for more than 70% of patients with prostate cancer. The down-regulation rate was markedly lower than rates in colon, lung, oral cavity, kidney, bladder and liver cancers with 20% to 42% down-regulation (unpublished data). Although various clinical trials of immunotherapies for prostate cancer have recently been done, none of them achieved satisfactory effects regardless of rationally sufficient immunological responses.[10] This study also demonstrated that prostate cancer in patients with a more advanced clinical stage tended to show HLA class I down-regulation more frequently. We suggest that candidates who wish to participate in clinical trials of cancer immunotherapy should undergo immunohistochemical screening for HLA class I expression in the cancer tissue, especially if clinical stage is advanced.

To our knowledge this study revealed for the first time that HLA class I down-regulation strongly correlates with β2M down-regulation in prostate cancer. Furthermore, it showed that LNCaP cells could present characteristics of prostate cancer cell lines, as previously reported,[11] and histone deacetylation might be involved in down-regulation, as demonstrated in in vitro studies using LNCaP cells. Thus, the current results suggest that HDAC inhibitors can up-regulate the HLA class I of prostate cancer. Huang et al reported that β2M activated phosphorylated cyclic adenosine monophosphate element binding protein with increased expression of its target genes, which could enhance tumor growth and angiogenesis, and facilitate the recruitment of osteoblasts and osteoclasts to the site of tumor colonization in bone.[12] In our study 87.8% of the 41 patients with bone metastasis showed β2M down-regulation with a score of 0 to 2 in 10, 26 and 5, respectively. The rate was higher than that in patients without bone metastasis. Our clinicopathological

TABLE 2. *Expression scores of HLA class 1 and β2M in 419 patients with prostate cancer*

AJCC Stage	Total No.	No. HLA Class 1 (%)			No. β2M (%)		
		Score 0	Score 1	Score 2	Score 0	Score 1	Score 2
I	6	0	3 (50.0)	3 (50.0)	0	4 (66.7)	2 (33.3)
II	320	64 (20.0)	169 (52.8)	87 (27.2)	38 (11.9)	196 (61.2)	86 (26.9)
III	25	9 (36.0)	12 (48.0)	4 (16.0)	8 (32.0)	12 (48.0)	5 (20.0)
IV	68	18 (26.5)	36 (52.9)	14 (20.6)	13 (19.1)	44 (64.7)	11 (16.2)
Totals	419	91 (21.7)	220 (52.5)	108 (25.8)	59 (14.1)	256 (61.1)	104 (24.8)

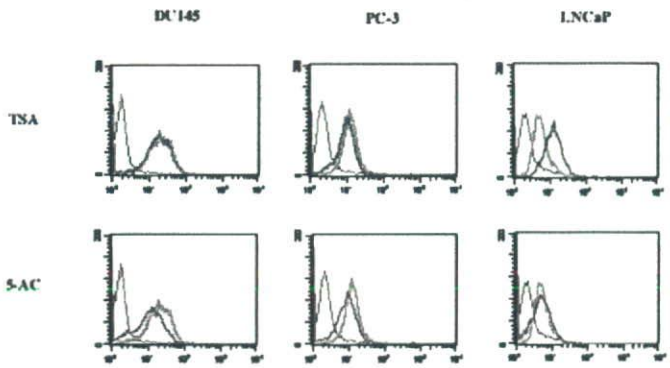

FIG. 3. Prostate cancer cell lines DU145, PC-3 and LNCaP were analyzed for cell surface levels of HLA class I by flow cytometry. Cells were stained with FITC labeled anti-HLA class I antibodies, including neither TSA nor 5-AC (green curves), 100 nM TSA for 24 hours (pink curves) and 5 μM 5-AC for 72 hours (orange curves). Cells were also stained with FITC labeled mouse IgG1 (purple curves). TSA up-regulated HLA class I on LNCaP, whereas 5-AC had no effect on any cell line.

data suggest that β2M expression in prostate cancer cells is not a risk factor for bone metastasis. A reason for the discrepancy between the 2 studies is that Huang et al inoculated human prostate cancer cells into the bone marrow of the host mice, which could be different from the natural history of human prostate cancer. However, we will carefully observe the incidence of bone metastasis, especially in patients in whom cancer tissue shows β2M expression, for a longer time since in vitro study provided evidence that β2M is a growth promoting factor for cancer bone metastasis.[12]

Recently some HDAC inhibitors, eg suberoylanilide hydroxamic acid, were studied in phase I and II trials for hematological and solid tumors.[13–15] Those studies showed that such drugs were well tolerated and could inhibit HDAC activity in tumor tissues with little clinical toxicity.[16] Our data suggest that a combination of an immunotherapeutic approach and HDAC inhibition could accentuate the effects of current immunotherapies for prostate cancer.

CONCLUSIONS

The current study shows that 1) the prevalence of HLA class I down-regulation is high in prostate cancer (especially advanced cancer), 2) HLA class I down-regulation in prostate cancer is related to β2M down-regulation, 3) β2M down-regulation is caused by an epigenetic change in LNCaP cells and 4) TSA can up-regulate HLA class I in LNCaP cells. From these results we suggest that the combination of an immunotherapeutic approach and HDAC inhibition could accentuate the effects of current immunotherapies for prostate cancer.

ACKNOWLEDGMENTS

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Abbreviations and Acronyms

5-AC	=	5-azacytidine
β2M	=	β2-microglobulin
AJCC	=	American Joint Committee on Cancer
ChIP	=	chromatin immunoprecipitation
CTL	=	cytotoxic T lymphocyte
FITC	=	fluorescein isothiocyanate
HDAC	=	histone deacetylase
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
PSA	=	prostate specific antigen
TSA	=	trichostatin A

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European Journal of Immunology Antigen processing

Inhibition of endogenous MHC class II-restricted antigen presentation by tacrolimus (FK506) *via* FKBP51

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The effect of tacrolimus (FK506) on down-regulation of IL-2 production by T cells is considered to be mainly responsible for its strong suppression of immunological events. In this study, we show that FK506 also has an affect on antigen presentation by antigen-presenting cells *in vitro*. FK506 was able to inhibit the presentation of endogenous MHC class II-restricted minor histocompatibility antigens in primary dendritic cells (DC) *in vitro*, but cyclosporine A (CsA) and rapamycin (RAP) were not. RNA interference (RNAi)-mediated reduction of endogenous FK506-binding protein (FKBP)51 expression resulted in a marked decrease in antigen presentation, suggesting that FKBP51 plays a role in endogenous MHC class II-restricted antigen presentation. Since our model used naturally expressed cytosolic antigens in primary DC, these effects might have been due to novel properties of the immunosuppressive drugs and may allow us to elucidate a new paradigm for the immunosuppressive mechanism of FK506.

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Introduction

Immunosuppressive drugs are indispensable for the success of allogeneic transplantation. It is well known that rapamycin (RAP), cyclosporine A (CsA) and tacrolimus (FK506) possess strong immunosuppressive effects in human organ transplantation [1, 2]; they regulate Th1-associated cytokine production by T cells, B cells, dendritic cells (DC) and macrophages following transcriptional signaling blockade by modulating the phosphatase activity of calcineurin in the cytoplasm [3–7]. These therapeutic effects of the drugs, in particular the down-regulation of IL-2 production by T cells, are considered to be responsible for the strong suppression of cellular immunity [8, 9]. On the other hand, evidence has accumulated that these immunosuppressive drugs also affect the development and immunobiologic functions of DC *in vitro* and *in vivo* [10]. Bidirectional influence of CsA and FK506 on DC-T cell interactions was reported: they efficiently blocked intracellular signaling both from DC to T cells, including inhibition of DC-induced T cell activation *via* down-regulation of IL-6 and IL-12 secretion and CD40

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Abbreviations: **BMDC:** bone marrow-derived DC · **CsA:** cyclosporine A · **CyP:** cyclophilin · **FK506:** tacrolimus · **FKBP:** FK506-binding protein · **H:** minor histocompatibility (antigen) · **Ii80:** residues 1–80 of the MHC class II-associated invariant chain · **IL4i1:** IL-4-induced gene 1 · **PPIase:** peptidyl-prolyl isomerase · **RAP:** rapamycin · **siRNA:** small interfering RNA

expression, and from T cells to DC, including T cell-induced DC maturation *via* down-regulation of IL-2 and IFN-γ secretion and CD69 expression [11]. Lee and colleagues reported that FK506 and CsA inhibit MHC class I- and MHC class II-restricted presentation of exogenous antigens without reducing the amount of MHC molecules [12]; FK506 was responsible not only for the induction of T cell anergy but also for inhibition of exogenous antigen presentation by antigen-presenting cells (APC). However, it remained unknown whether FK506 can directly inhibit presentation of endogenous MHC class II-restricted antigens such as minor histocompatibility (H) antigen.

The histocompatibility *H* loci are polymorphic genes that determine the outcome of transplanted tissue grafts as well as graft-*versus*-host disease reactions in both mouse and human [13, 14]. Despite complete matching of MHC molecules between the donor and host strains, natural polymorphisms within the *H* loci result in the presentation of novel peptides by the MHC to T cells [15]. For example, different *H* loci located on the male Y chromosome encode peptides that are presented by both MHC class I and MHC class II molecules and elicit potent CD8 and CD4 T cell responses, respectively [16–18]. We reported that the murine *H46* locus on chromosome 7 encodes the IL-4-induced gene 1 (IL4i1) in an experiment using the specific lacZ-inducible CD4 T cell hybridoma TH3Z, and this antigen was endogenously expressed in bone marrow-derived DC (BMDC) in the context of MHC class II ($I\text{-}A^b$) molecules [19].

Using a model of endogenous minor H antigen-specific T cell recognition of primary BMDC, we found that FK506 could inhibit presentation of endogenous MHC class II-restricted antigens in BMDC *in vitro*, but CsA and RAP could not. From the results showing down-regulated gene expression by small interfering RNA (siRNA), it was suggested that the immunophilin FK506-binding protein (FKBP)51 molecule, but not FKBP12 or FKBP52, played a role in MHC class II-restricted minor H antigen presentation. Since our model used naturally expressed cytosolic antigens in primary BMDC, these effects might have been due to novel properties of the immunosuppressive drugs.

Results

Suppressive effect of FK506 and CsA on exogenous MHC II-restricted antigen presentation

We first investigated the effects of the immunosuppressive drugs FK506, CsA and RAP on exogenous antigen presentation in the context of MHC class II. We have previously shown that recombinant *E. coli* expressing heterologous proteins can serve as an antigen source for generating peptide/MHC class II complexes in BMDC [19]. In this study, recombinant *E. coli* expressing polymorphic IL4i1 protein derived from C57BL/6J (B6) mice was used as an exogenous antigen source, and for detection of generated peptide/MHC class II complexes, we used the B6-derived IL4i1-specific lacZ-inducible CD4 T cell hybridoma TH3Z.

The TH3Z responses to FK506-treated 129/J BMDC that had phagocytosed recombinant *E. coli* expressing IL4i1 were decreased at concentrations from 10 to 1000 nM (Fig. 1A), and responses to 129/J BMDC treated with 1000 nM CsA were also significantly decreased. In contrast, RAP in concentrations ranging from 1 to 1000 nM did not affect the presentation of the exogenous antigen.

These results raised the question of whether FK506 and CsA could affect T cell function by carryover after washing. To clarify this issue, TH3Z cells were cultured in the presence or absence of FK506 or CsA for 48 h and then cocultured with non-treated endogenous IL4i1-expressing B6 BMDC overnight after thorough washing. Responses by TH3Z cells were not influenced by FK506 or CsA in concentrations from 1 to 1000 nM (Fig. 1B). In some experiments, we investigated whether these drugs influence phagocytotic activity by the uptake of fluorescence beads into BMDC; no influence was found (data not shown). Thus, FK506 and CsA, but not RAP, could affect presentation of the exogenous antigen without affecting phagocytotic activity, as previously reported by Lee and colleagues [12].

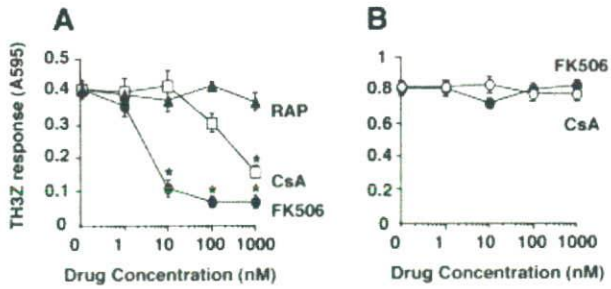

Figure 1. Suppressive effects of FK506 and CsA on MHC class II-restricted presentation of exogenous antigen. (A) 129/J BMDC were treated (or left untreated) with immunosuppressive drugs at the indicated concentrations for 48 h, washed and used as APC. IL4i1 expression-induced transformants were transferred into 96-well plates at 10^6 bacteria/well and cocultured with 10^5 of these APC/well [RAP (▲), CsA (□), FK506 (●)] for 1 h to allow phagocytosis. Then 10^5 TH3Z cells were added to each well; T cell activation was measured as their lacZ response. Results represent one of three independent experiments (*$p<0.01$). (B) TH3Z cells were treated with FK506 (●) or CsA (○) at the indicated concentrations for 48 h, washed and then cocultured with B6 BMDC overnight after thorough washing. T cell activation was measured as their lacZ response. Results represent one of three independent experiments.

Suppressive effect of FK506 on endogenous MHC class II-restricted antigen presentation

We next investigated whether endogenous MHC class II-restricted minor H antigen presentation on primary BMDC is inhibited by the immunosuppressive drugs FK506, CsA and RAP. BMDC from B6 mice naturally present endogenous IL4i1 antigen in the context of I-A^b molecules [19]; they were cultured in the presence or absence of the drugs at the indicated concentrations for 6, 12 and 48 h and then cocultured with the lacZ-inducible CD4 T cell hybridoma TH3Z overnight after thorough washing. As shown in Fig. 2A, TH3Z responded to B6 BMDC that were cultured with FK506 in concentrations from 1 to 1000 nM for 6 h, suggesting that MHC class II-restricted minor antigen presentation was unaffected by FK506 treatment for 6 h. However, TH3Z responses to BMDC that were treated with FK506 for 12 or 48 h were clearly decreased in a dose-dependent manner. In particular, when BMDC were treated with FK506 for 48 h in concentrations from 10 to 1000 nM, T cell responses were clearly decreased. To determine whether FK506 affected the function of antigen-presenting molecules such as MHC class II on the cell surface, 129/J BMDC were treated with or without 100 nM FK506 for 48 h and then incubated for 60 min with the indicated concentrations of the synthetic peptide H46a or H46b and cocultured with TH3Z overnight. The H46a peptide derived from the polymorphic IL4i1 gene of B6 mice is the TH3Z epitope, but the 129/J-derived H46b peptide, which has a single amino acid polymorphism, is not antigenic for TH3Z [19]. In the current study, the H46b peptide was used as a negative control. As shown in Fig. 2B, the TH3Z responses to H46a-pulsed/FK506-treated 129/J BMDC occurred in a dose-dependent manner, but those to H46b did not, indicating that FK506 treatment did not affect presentation of the peptide-MHC class II complex on the cell surface. The TH3Z responses to non-drug-treated BMDC that were cultured with the H46a peptide were higher than those to H46a-pulsed/FK506-treated 129/J BMDC, suggesting that FK506 was not essential but influenced the presentation ability.

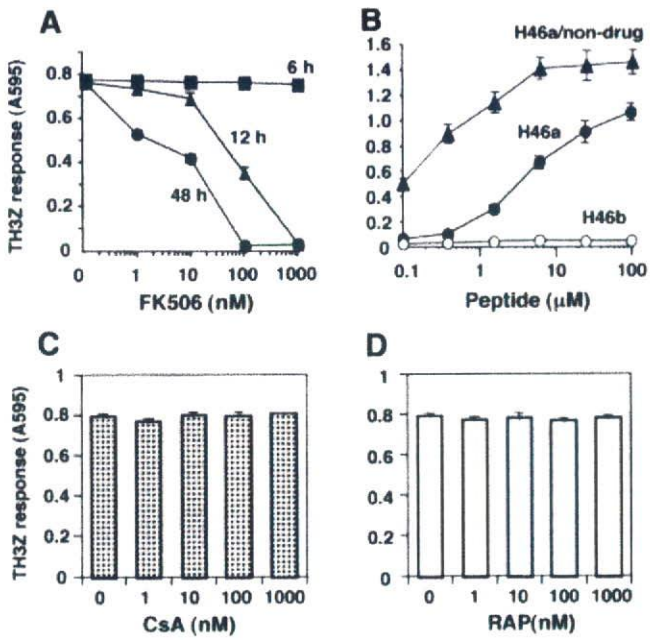

Figure 2. FK506 inhibits endogenous minor H antigen presentation in the context of MHC class II molecules. (A) BMDC of B6 mice were cultured in the presence or absence of FK506 at the indicated concentrations for 6 (■), 12 (▲) or 48 h (●), plated in 96-well plates at 10^5 cells/well and cocultured with 10^5 lacZ-inducible TH3Z cells overnight after thorough washing. (B) 129/J BMDC were treated with or without 100 nM FK506 for 48 h, washed and then plated in 96-well plates at 10^5 cells/well. They were incubated for 60 min with the synthetic peptides H46a (●) and H46b (○) at the indicated concentrations and then cocultured overnight with 10^5 TH3Z. The TH3Z response to H46a-pulsed/non-drug-treated 129/J BMDC was measured as a control (▲). (C, D) BMDC of B6 mice were cultured in the presence or absence of CsA or RAP for 48 h, plated in 96-well plates at 10^5 cells/well and cocultured with 10^5 TH3Z overnight. TH3Z responses (mean ± SD) represent measurement by absorbance at 595 nm, and results from one of three independent experiments are shown.

In contrast, CsA or RAP treatment for 6, 24 or 48 h in concentrations from 1 to 1000 nM did not affect antigen presentation (Fig. 2C, D; the results of treatment for 6 and 24 h are not shown). Unlike the data for presentation of exogenous antigens, these data suggest that FK506 but not CsA or RAP can affect presentation of the endogenous MHC class II-restricted IL4i1 antigen.

FK506 inhibits endogenous MHC class I- and class II-restricted presentation of OVA

Although identical molecules were used as the exogenous and endogenous antigens, presentation of exogenous antigen was affected by both FK506 and CsA, while presentation of endogenous antigen was affected only by FK506. Therefore, we further investigated these effects in another endogenous antigen model, using OVA as a model antigen, the lacZ-inducible CD4 T cell hybridoma KZO for detection of MHC class II (I-A^k)-restricted antigen and the lacZ-inducible CD8 T cell hybridoma B3Z for detection of MHC class I (H-$2K^b$)-restricted antigen [20]. To make OVA antigen cDNA, we used truncated $OVA_{138-386}$, because the OVA_{1-137} amino acid region includes the signal sequence for extracellular secretion and does not include the I-A^k epitope [20]. Moreover, $OVA_{138-386}$ cDNA was fused with residues 1–80 of the MHC class II-associated invariant chain (Ii80), referred to here as Ii80-OVA; it has been reported that when antigens are fused with Ii80, they can be presented on MHC class II molecules as endogenous

antigens [20]. BMDC of B6C3F1 mice (H-$2^{b/k}$) (C57BL/6N Crj × C3H/HeN Crj) were transduced with retroviruses expressing either vector alone, $OVA_{138-386}$ or Ii80-OVA cDNA and then cultured with or without immunosuppressive drugs for 48 h.

As shown in Fig. 3A, KZO responses to OVA antigen-expressing BMDC treated with FK506, even at a concentration of 1 nM, were decreased. These suppressive effects of FK506 on OVA antigen presentation were stronger than the effects on presentation of the endogenous antigen IL4i1. Likewise, we tested the influence of FK506 on the lacZ-inducible CD4 T cell hybridoma KZO; concentrations of FK506 from 1 to 1000 nM did not notably influence KZO responses (data not shown). These data suggest that FK506 can also affect the presentation of the endogenous MHC class II-restricted OVA antigen. In the same setting, CsA and RAP in concentrations from 1 to 1000 nM did not affect antigen presentation (Fig. 3B, C). Responses of the lacZ-inducible CD8 T cell hybridoma B3Z to FK506-treated (but not CsA- or RAP-treated) BMDC were also decreased, indicating that FK506 can affect the presentation of the MHC class I-restricted OVA antigen as well (Fig. 3D–F). Lee and colleagues reported that FK506 specifically inhibits presentation of the SIINFEKL-H-$2K^b$ complex, resulting in B3Z unresponsiveness to FK506-treated DC2.4 cells [12]. Our data indicate that FK506 inhibits B3Z responses to endogenous MHC class I-restricted OVA antigen in primary BMDC.

To further assess whether FK506 can affect the function of antigen-presenting molecules on the cell surface, B6C3F1 BMDC were transduced with an empty retroviral vector and cultured with or without 100 nM FK506. After 48 h, these APC were incubated for 60 min with the indicated concentrations of the synthetic peptide $OVA_{247-265}$ (for I-A^k) or $OVA_{258-265}$ (for H-$2K^b$) and cocultured with KZO or B3Z, respectively, overnight. The KZO and B3Z responses to these APC

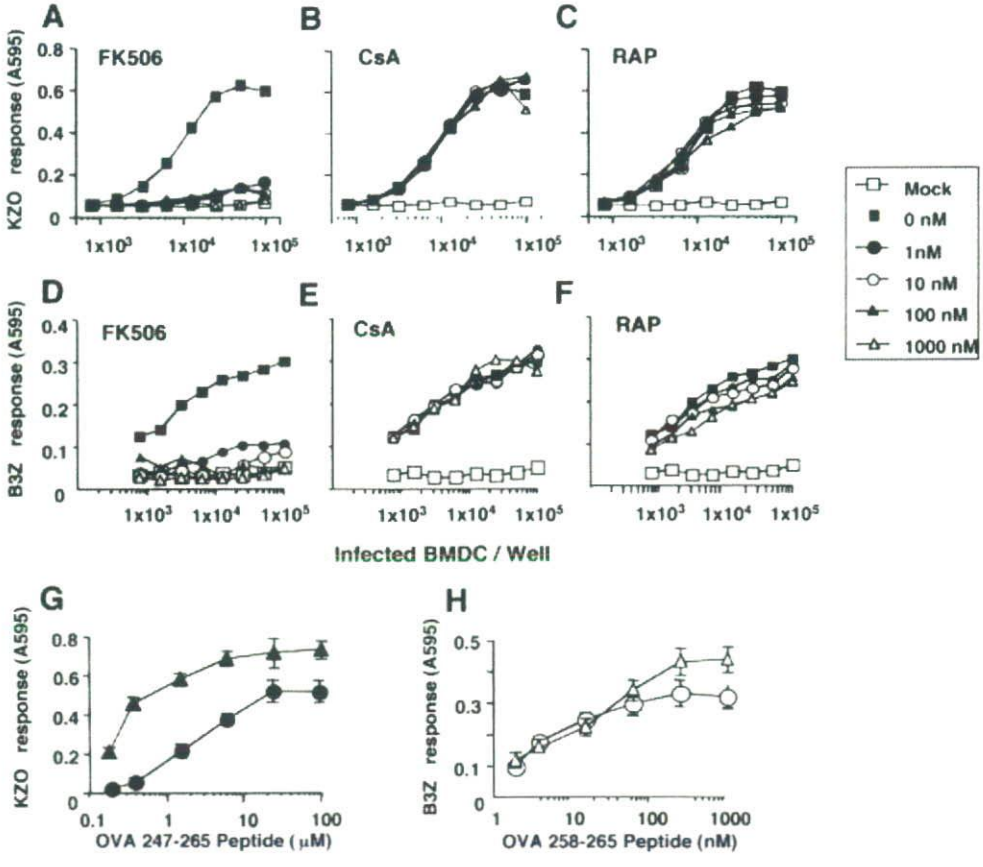

Figure 3. FK506 inhibits presentation of MHC class II- and MHC class I-restricted endogenous OVA by primary BMDC. BMDC of B6C3F1 mice (H-$2^{b/k}$) were transduced with retroviruses expressing OVA cDNA and cultured in the presence or absence of FK506 (A, D), CsA (B, E) or RAP (C, F). After 48 h, the BMDC were titrated in 96-well plates as APC and cocultured with 10^5 KZO or B3Z overnight; the lacZ responses were measured by absorbance at 595 nm. (G, H) B6C3F1 BMDC transduced with empty retroviral vectors were treated with or without 100 nM FK506 for 48 h, washed and then plated in 96-well plates at 10^5 cells/well. They were incubated for 60 min with the OVA synthetic peptides at the indicated concentrations and then cocultured overnight with 10^5 KZO (●) or B3Z (○) cells. The responses of KZO (▲) and B3Z (△) cells to peptide-pulsed, non-drug-treated B6C3F1 BMDC were measured as controls. Results represent one of three independent experiments.

clearly occurred in a dose-dependent manner, indicating that FK506 treatment did not affect presentation of the peptide-MHC class II and -MHC class I complexes on the cell surface (Fig. 3G, H). Similar to TH3Z responses, the KZO and B3Z responses to non-drug-treated BMDC cultured with these peptides were higher than those to peptide-pulsed/FK506-treated B6C3F1 BMDC, suggesting that FK506 was not essential but slightly influenced the presentation ability.

FK506 has no effect on expression of MHC class II and IL4i1 antigens in BMDC

Next we investigated whether the decrease in antigen presentation caused by FK506 was due to a decrease in expression of MHC class II or other relevant molecules. After primary cultivation for 2 days, BMDC were cultured in the presence or absence of 1 μM FK506 for 48 h, and surface expression of selected molecules was analyzed by flow cytometric analysis. The expression levels of MHC class I and class II molecules, CD40, CD80 and CD86 on BMDC were not influenced by FK506 (Fig. 4).

To address the question of whether the decrease in TH3Z responses was due to decreased expression of IL4i1 antigen in BMDC, we examined antigen expression by Western blotting: IL4i1 expression was not affected by FK506 (Fig. 5). These data strongly suggest that FK506 had an effect on MHC class II-restricted processing or presentation of endogenous antigen, resulting in CD4 T cell unresponsiveness.

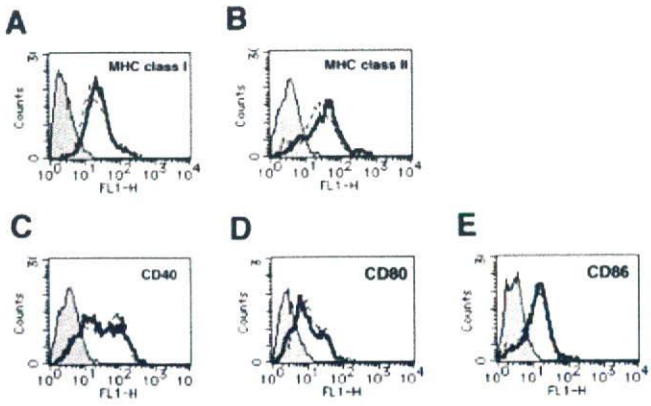

Figure 4. Characterization of FK506-treated BMDC. BMDC of B6 mice were treated with 1 μM FK506 (or left untreated) for 48 h and then stained with FITC-labeled anti-mouse MHC class I (A), MHC class II (B), CD40 (C), CD80 (D) and CD86 (E). After staining, the cells were analyzed on a flow cytometer. The gray peak shows unstained FK506-treated BMDC as a negative control. Thick and dotted lines represent FK506-treated BMDC and non-treated BMDC, respectively.

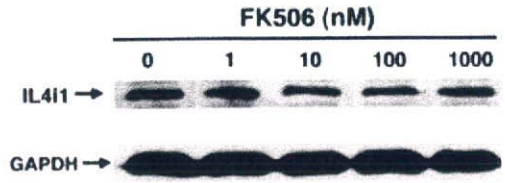

Figure 5. IL4i1 expression profile in the presence of FK506. BMDC of B6 mice were treated with FK506 at the indicated concentrations for 48 h and harvested. Target proteins in the cell lysate were detected by Western blotting using a rabbit anti-IL4i1 antibody and anti-mouse GAPDH as an internal control.

FKBP51 plays a role in MHC class II-restricted antigen presentation

To determine which cellular targets of FK506 are related to antigen presentation, we performed RNA interference (RNAi) experiments. FK506 is known to bind to the immunophilins FKBP12, FKBP51 and FKBP52 [21, 22]. COS cells were transfected with siRNA specific for these proteins, and IL4i1/I-A^b or vector/I-A^b cDNA was then reintroduced for their use as APC 48 h after siRNA transfection. After 24 h (72 h after siRNA transfection), target proteins in the cell lysates were detected using anti-FKBP12, anti-FKBP51, anti-FKBP52, anti-IL4i1 and anti-GAPDH antibodies (Fig. 6A). When siRNA specific for FKBP51 (si-FKBP51) was introduced into COS cells, expression of FKBP51 protein but not FKBP12, FKBP52, IL4i1 or GAPDH protein was specifically down-regulated. Likewise, siRNA specific for FKBP12 (si-FKBP12) and FKBP52 (si-FKBP52) specifically down-regulated the target molecules. IL4i1 protein was detected in all samples, suggesting that siRNA transfection did not influence its expression. To examine whether siRNA/cDNA double-transfected cells could become efficient APC, we performed two studies: 1) flow cytometric analysis of I-A^b expression and 2) examination of antigen presentation on the double-transfected cells.

The expression of I-A^b molecules on siRNA/cDNA (IL4i1/I-A^b) double-transfected COS cells was analyzed by flow cytometry (Fig. 6B). To further confirm whether the siRNA/cDNA (vector/I-A^b) double-transfected COS cells work as efficient APC for activating T cells, these cells were incubated for 60 min with the indicated concentrations of H46a peptide and cocultured with TH3Z overnight. As shown in Fig. 6C, the TH3Z response to double-transfected, H46a-pulsed COS cells occurred in a dose-dependent manner, indicating that siRNA/cDNA double-transfected cells could become efficient APC for TH3Z. Interestingly, TH3Z responses to IL4i1/I-A^b-expressing cells introduced to si-FKBP51 significantly decreased, whereas si-FKBP12- and si-FKBP52-transfected IL4i1/I-A^b-expressing cells stimulated TH3Z

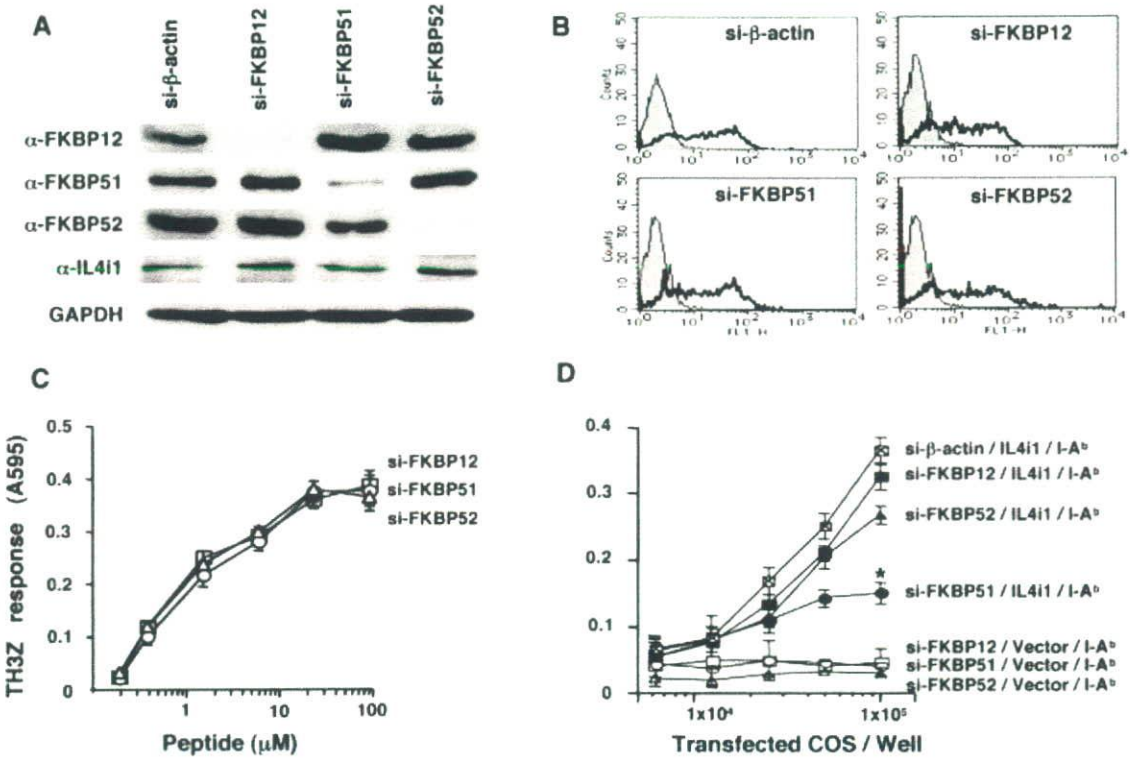

Figure 6. The role of FKBP51 in MHC class II-restricted antigen presentation. COS cells were transfected with siRNA specific for β-actin (si-β-actin), FKBP12 (si-FKBP12), FKBP51 (si-FKBP51) and FKBP52 (si-FKBP52) and then transfected with IL4i1 or a vector and I-A^b cDNA. (A) FKBP12, FKBP 51, FKBP52, IL4i1 and GAPDH as an internal control were detected in total cell lysates by immunoblot analysis. (B) Cells were stained with a FITC-labeled anti-mouse MHC class II (I-A^b) antibody and then analyzed on a flow cytometer. The gray peak represents the negative control, and the thick lines represent the indicated siRNA/cDNA double-transfected cells. (C) The si-FKBP12- (□), si-FKBP51- (○), or si-FKBP52-transfected (△) and vector/I-A^b-transfected COS cells were cultured with the H46a peptide, and then TH3Z cells were added. (D) The si-FKBP-transfected and Ag/I-A^b-expressing [si-β-actin (⊠), si-FKBP12 (■), si-FKBP51 (●) and FKBP52 (▲) /IL4i1/I-A^b] or vector/I-A^b-expressing [si-FKBP12 (□), si-FKBP51 (○) and FKBP52 (△)/vector/I-A^b] cells were cocultured with TH3Z cells. (C, D) TH3Z responses (mean ± SD) represent measurement of absorbance at 595 nm. One of three independent experiments is shown (*$p<0.05$).

to extents similar to the negative control transfected with si-β-actin (Fig. 6D), suggesting that FKBP51 played an important role in endogenous MHC class II-restricted antigen presentation.

Discussion

The biological reaction to FK506 occurs *via* binding to intracellular receptors belonging to the FKBP family of genes. The major cytosolic receptor for FK506 and RAP is reported to be FKBP12 [21, 22]. FKBP12 exhibits peptidyl-prolyl isomerase (PPIase) activity that catalyzes *cis-trans* isomerization of peptidyl-prolyl bonds in peptides and proteins [21, 22]. The immunophilin cyclophilin (CyP), targeted by CsA, also has PPIase activity [23, 24]. However, PPIase activity is unrelated to immunosuppressive effects, indicating that there are other intracellular receptors for FK506 [25, 26]. It was reported that both FK506/FKBP12 and CsA/CyP target and inhibit a Ca^+-dependent serine-threonine phosphatase, calcineurin [27]. Calcineurin dephosphorylates various substrates, including NF-AT family proteins. NF-AT family members play a role in the transcriptional activation of cytokine genes, including IL-2, IL-4 and TNF-α, upon T cell activation initiated by stimulation through T cell receptors [28]. Thus, inhibition of calcineurin has been considered to be the basis of the immunosuppressive effects through direct binding to FK506/FKBP12, RAP/FKBP12 or CsA/CyP.

In the current study, we show that FK506, but not CsA or RAP, has a suppressive effect on endogenous MHC class II-restricted minor H antigen presentation, indicating that the action of FK506 could be related to a pathway other than that of calcineurin; if calcineurin were closely involved, CsA and RAP would also show suppressive effects. Therefore, we focused on the larger FKBP, FKBP51 (the 51-kDa FKBP) and FKBP52 (the 52-kDa FKBP). FKBP51 and FKBP52 are highly homologous, consisting of two consecutive FK506 binding domains and a three-unit repeat of the tetratricopeptide repeat (TPR) domain [29]. The roles of FKBP51 and

FKBP52 are less well known, but they assemble with Hsp90 to form steroid receptor complexes, glucocorticoids (GR) or progesterone (PR) [30, 31]. Recently, it was reported that GR require three functions to efficiently carry out receptor signaling: Hsp90 interaction, association of dynein and PPIase enzyme activity, and differential binding of FKBP52 and FKBP51 to dynein, corresponding to their differential effects on nuclear translocation [32]. Thus, they exhibit only a slight difference in steroid receptor signaling. In this study, we found that endogenous MHC class II-restricted antigen presentation was significantly decreased when FKBP51 protein expression was down-regulated by siRNA, which could not be demonstrated in the case of FKBP12 and FKBP52. These data indicate that FKBP51 played a role in antigen processing or presentation and that there might be a difference between the role of FKBP51 and that of FKBP52.

We observed that exogenous antigen presentation by BMDC was inhibited by FK506 and CsA, but not RAP, as previously reported by Lee and colleagues [12]. The exogenous antigen processing/presentation pathway is well known [33]. In this process, a nascent MHC class II αβ heterodimer is generated into the endoplasmic reticulum (ER) and guided by coassembly with the MHC class II-associated invariant chain (Ii) containing a sorting signal to the late endosome. The internalized exogenous antigen is digested by endosomal/lysosomal proteases in vesicles constructed *via* fusion of the internalized compartment and endosome/lysosomes, which subsequently develop into late endosomal/lysosomal MHC class II compartments (MIIC), and the Ii-derived CLIP peptide in the peptide-binding grove of the MHC II molecule subsequently exchanges fragmented peptide antigens mediated by H-2DM (HLA-DM in humans). However, there has been little or no information on the role of FKBP51 in the presentation of exogenous antigen. In this study, we could not address whether FKBP51 plays a role in exogenous antigen presentation because effective down-regulation of FKBP proteins by FKBP12-, FKBP51- and FKBP52-specific siRNA in primary BMDC was not observed, and COS cells could not become efficient APC with phagocytotic activity for the uptake of exogenous antigens (data not shown).

It has been reported that there are some different points in the pathways for processing of exogenous and endogenous antigens for MHC class II molecules [33]. In particular, the processing pathway of endogenous MHC class II-restricted antigens is poorly understood. The processing pathway relating to FKBP51 molecules examined in the current study provides novel insight into the presentation of endogenous MHC class II-restricted minor antigens on primary BMDC.

Materials and methods

Mice, cells and synthetic peptides

Inbred C57BL/6J (B6) and 129 P3/J (129/J) mice were obtained from The Jackson Laboratory. B6C3F1 mice (C57BL/6N Crj × C3H/HeN Crj) were obtained from Japan Charles River. All procedures were performed in compliance with the guidelines of the Animal Research Center of Sapporo Medical University. Bone marrow cells obtained from the thigh bones of mice were cultured in RPMI 1640 (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum, 10 ng/mL GM-CSF (Biosource International), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 200 U/mL penicillin and 200 μg/mL streptomycin. After 5 days, the characteristic immature DC in $CD11c^+$, $CD86^-$ and $I\text{-}A^b/A^k$-low cultures accounted for more than 65% of the cells in flow cytometric analysis and were designated BMDC and used as APC. The IL4i1-specific lacZ-inducible CD4 T cell hybrid TH3Z (B6 mouse-derived $IL4i1_{567-580}/I\text{-}A^b$-specific) was generated as described [19]. The lacZ-inducible CD4 T cell hybrid KZO ($OVA_{247-265}/A^k$-specific) and CD8 T cell hybrid B3Z ($OVA_{258-265}/K^b$-specific) have been described elsewhere [20]. The H46a peptide (HAFVEAIPELQGHV) derived from B6 $IL4i1_{567-580}$, the H46b peptide (HMFVEAIPELQGHV) derived from 129/J $IL4i1_{567-580}$, the $OVA_{247-265}$ peptide (PDEVSGLEQLESIINFEKL) and the $OVA_{258-265}$ peptide (SIINFEKL) were synthesized, purified by HPLC and confirmed by mass spectrometry.

Treatment of DC and cells with immunosuppressive drugs and T cell activation assays

Tacrolimus (FK506) was kindly provided by Astellas Pharma, Inc. Cyclosporin A (CsA) and rapamycin (RAP) were purchased from Sigma-Aldrich. After 2 or 3 days of primary culture, BMDC from B6, 129/J or B6C3F1 mice were subsequently cultured for 6, 24 or 48 h in the presence or absence FK506, CsA and RAP. In some experiments, TH3Z, KZO and B3Z cells were cultured for 48 h in the presence or absence these drugs. After 48 h, APC and T cell hybrids were thoroughly washed with cultured medium three times and cocultured overnight with lacZ-inducible T cell hybrids and APC, respectively. To examine the physiological APC functions, 129/J BMDC were treated with or without 100 nM FK506 for 48 h, washed and then incubated for 60 min with indicated concentrations of the H46a or H46b synthetic peptide. TH3Z cells were cocultured overnight with these BMDC and their responses examined. The results show lacZ activity measured as the absorbance at 595 nm (with 635 nm as the reference wavelength) of the chromogenic product released after cleavage of the substrate chlorophenol red β-pyranoside [19].

Exogenous antigen presentation by BMDC

The IL4i1 gene from the B6 mouse was inserted unidirectionally into the pLEX prokaryotic expression vector (Invitrogen, CA, USA) between the EcoRI/NotI sites and used to transform GI724-competent bacteria (Invitrogen). Bacterial transformants were grown, and expression of IL4i1 protein was induced according to the manufacturer's instructions (PL

Expression System; Invitrogen). Briefly, 129/J BMDC were treated with immunosuppressive drugs at the indicated concentrations for 48 h (or left untreated), washed and used as APC. Transformants were cultured overnight at 30°C, and 50-fold diluted transformant suspensions with fresh medium were incubated at 30°C to an OD_{550} of 0.5 (approximately 3 h). These transformants were added to 10 μg/mL L-tryptophan for induction of gene expression and cultured at 37°C for 3 h. Antigen expression-induced bacteria were transferred to 96-well plates at 10^6 bacteria/well and cocultured with the 129/J BMDC (10^5 cells/well) in RPMI 1640 medium (antibiotic-free) for 1 h at 37°C in a CO_2 incubator to allow phagocytosis. The plate was centrifuged for 2 min at 1500 rpm, the supernatant removed, and 10^5 TH3Z cells in culture medium (RPMI 1640 culture medium without GM-CSF) with addition of 100 μg/mL gentamicin (to eliminate residual bacteria) were added to each well. T cell activation was measured as their lacZ response.

Retroviral transfection

Ovalbumin (OVA) cDNA was truncated to remove the 1–137 amino acid region, and $OVA_{138-386}$ was used as a model antigen for presentation to MHC class I. Truncated $OVA_{138-386}$ cDNA was fused with Ii80 cDNA (Ii80-OVA) for MHC class II presentation. Each cDNA ($OVA_{138-386}$ and Ii80-OVA) was ligated with the MSCV-IRES GFP retroviral vector. Retroviral transduction into BMDC has been described previously [19]. Briefly, 293T cells, as packaging cells, were transiently transfected with the cDNA vector together with the pGP-KV plasmid encoding Moloney murine leukemia virus structural genes, *gag-pol* and VSV-G plasmids by Lipofectamin 2000 (Invitrogen) transfection as described. After 24 h, the supernatants were filtered through a 0.45-μm filter, and 4 μg/mL polybrene was added before infection. BMDC of B6C3F1 mice ($H\text{-}2^{b/k}$) were cultured for 2 days and then resuspended with retroviral supernatants at 10^6 cells/mL and plated in a 12-well plate at 10^6 cells/well. For infection, the plate was centrifuged at 2500 rpm for 2 h, and the medium was replaced with fresh BMDC culture medium. BMDC were cultured for 48 h in the presence or absence of immunosuppressive drugs, and T cell responses were examined. In some experiments, B6C3F1 BMDC introduced to empty retroviral vectors were treated with or without 100 nM FK506 for 48 h, washed and then incubated for 60 min with the indicated concentrations of OVA synthetic peptides. KZO or B3Z cells were cocultured overnight with these BMDC and their responses examined.

Flow cytometric analysis

FITC-labeled anti-mouse MHC class I, MHC class II ($I\text{-}A^b$), CD40, CD80 and CD86 mAb were purchased from BD Pharmingen (CA, USA). Cells were stained with these primary mAb for 30 min on ice, washed with PBS and resuspended in PBS containing 1% paraformaldehyde. After staining, the cells were analyzed on a flow cytometer (Beckman Coulter, Palo Alto, CA).

Western blotting

Anti-IL4i1 polyclonal antibody serum was generated in rabbits immunized with $IL4i1_{587-600}$, QEKGHAHQNIYPSEC, according to our lab protocol [34]. FKBP12, FKBP51 and FKBP52 antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Human and mouse GAPDH antibodies were purchased from BD Pharmingen. Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% NP40 and protease inhibitor cocktail (Roche Diagnostics, Germany)], and samples were loaded on 12% SDS-PAGE and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Bioscience, NJ, USA). The transferred antigens on the membrane were detected with anti-IL4i1 serum and FKBP antibodies by Western blotting.

FKBP RNA interference and cDNA transfection

Small interfering RNA (siRNA) duplexes (si-FKBP12, 5′-GGUGCUGUUUGUAGACUUA-3′; si-FKBP51, 5′-CGGAAAGGA-GAGGGAUAUU-3′; and si-FKBP52, 5′-GGACAAAUUCUCCUU-UGAC-3′) containing 3′dTdT over the hanging sequence were synthesized by Sigma Genosys (Sigma-Aldrich, Japan). A control nucleotide, si-β-actin control, was purchased from Ambion (predesigned siRNA). IL4i1, $I\text{-}A^b\alpha$ and $I\text{-}A^b\beta$ were cloned as described previously [19] and were reconstructed with pcDNA 3.1 myc-HisA (Invitrogen). COS cells were plated at 10^5 cells/well in a 6-well plate and cultured with Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich) with 10% FCS, 200 U/mL penicillin and 200 μg/mL streptomycin overnight. Then 10 nM siRNA were conjugated with siLentFect Lipid Reagent (Bio-Rad, CA, USA), added to the wells and cultured for 14 h. The culture medium was subsequently replaced with fresh medium, and cells were cultured continually for 34 h. siRNA-transfected COS cells were transiently transfected with IL4i1/$I\text{-}A^b$ ($I\text{-}A^b$ cDNA: a mixture of $I\text{-}A^b\alpha$ and $I\text{-}A^b\beta$ chains at equal concentrations) or vector/$I\text{-}A^b$ cDNA using Lipofectamin 2000. After 24 h, these cells were harvested and examined by Western blotting and flow cytometric analysis. The rest of the cells were used as APC for the TH3Z antigen presentation study. In experiments to examine physiological APC functions, siRNA/cDNA (vector/$I\text{-}A^b$) double-transfected COS cells were incubated for 60 min with the indicated concentrations of H46a peptides and cocultured overnight with TH3Z.

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Suppression of Alloreactivity and Allograft Rejection by SP600125, a Small Molecule Inhibitor of c-Jun N-terminal Kinase

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Background. c-Jun N-terminal kinase (JNK) is reported to play crucial roles in T-cell activation and differentiation, and SP600125 is a small molecule that inhibits JNK. The aim of this study was to examine immunosuppressive action of this compound.
Methods. Rat heterotopic heart transplantation, popliteal lymph node (PLN) hyperplasia bioassay and lymphocyte proliferation assay.
Results. SP600125 treatment reduced histological rejection, and dose-dependently extended median survival time of cardiac allografts from 7 days (vehicle) up to 20 days (40 mg/kg/day). Alloantigen-induced PLN hyperplasia was also inhibited by SP600125 in a similar fashion. SP600125 suppressed mixed lymphocyte reaction and OX52-positive lymphocyte proliferation (IC_{50}: 1.5–5.7 μM). Thus, SP600125 inhibits both T-lymphocyte expansion in vitro and T-cell–mediated alloimmune responses in vivo. In addition, SP600125 interacted with cyclosporine additively to prolong cardiac allograft survival.
Conclusion. Our data provide the first evidence indicating the potential for JNK as a therapeutic target to inhibit the alloimmune response.

Keywords: c-Jun N-terminal kinase, Heart transplantation, Allograft, Rejection, Immunosuppression.

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Acute allograft rejection is primarily a T-cell mediated event and is associated with Th1 phenotype responses (*1*, *2*). Insight into the mechanisms underlying T-cell recognition, activation, and differentiation may provide novel targets for immunotherapy. For example, elucidation of signal transduction pathways downstream from the T-cell receptor has identified the mechanism of action of several immunosuppressive agents and continues to impact the design of new drugs. c-Jun N-terminal kinase (JNK) is a serine threonine protein kinase belonging to the mitogen-activated protein kinase (MAPK) family. After T-cell stimulation, JNK phosphorylates c-Jun and other transcription factors composing activator protein-1 (AP-1) or nuclear factor of activated T-cells (NFAT) family members (*3*). Original studies using Jurkat T-cells implicated roles for JNK in T-cell activation and interleukin (IL)-2 expression (*4*), which has been further supported by studies using genetic approaches (*5*, *6*). However, data reported by other groups has raised questions about the roles for JNK1 or JNK2 in T-cell activation and proliferation (*7–10*). Particularly, in vitro studies using primary CD4 positive T-cells deficient in JNK1 and JNK2 have revealed that JNK is not essential for naïve T-cell activation and proliferation; instead, it is required for differentiation into Th1 cells and cytokine production (*11*).

SP600125, anthra[1,9-cd]pyrazol-6(2H)-one, was identified in a high-throughput biochemical screen by using purified recombinant JNK2 and c-Jun. In vitro cell-based assays further characterized the bioactivity of this compound (*12*). Three major isotypes of JNK have been identified in humans. JNK1 and JNK2 have a broad tissue distribution, whereas JNK3 is primarily localized to neuronal tissues and cardiomyocytes. In the biochemical assays, SP600125 inhibits all three JNK isotypes with similar potency, whereas SP600125 does not inhibit other MAPKs such as extracellular signal-regulated kinase (ERK) and p38 (*12*, *13*). The in vitro assays for the activities in T-cells demonstrated that SP600125 dose-dependently inhibits the phosphorylation of c-Jun, the secretion of cytokines including interleukin (IL)-2, IL-10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and prevents the expression of cell surface activation markers and differentiation of human CD4 positive T-cells.

Although anti-inflammatory effects of this compound on rat adjuvant arthritis were reported (*13*), its in vivo efficacy against the alloimmune response has not been addressed. In the present study, we extended the above-mentioned in vitro observations further to delineate in vivo effects of SP600125 in prolonging graft survival. We postulated that SP600125 might suppress the allograft rejection promoted by T-lymphocytes by suppressing the cell activation and proliferation in vivo.

MATERIALS AND METHODS

Animals

Specific pathogen-free inbred male Lewis rats (LEW) ($RT1^l$) and Brown-Norway rats (BN) ($RT1^n$) weighing 250–

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300 g were obtained from the Charles River Japan Inc., Yokohama, Japan and the Seack Yoshitomi Ltd., Fukuoka, Japan, respectively. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" prepared and formulated by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Reagents

SP600125 was provided by Celgene (San Diego, CA). The drawn structure of anthra[1,9-cd]pyrazol-6-(2H)-one (MW: 220.23), has been shown elsewhere (*12*). Cremophor EL and propylene glycol were purchased from Sigma (St. Louis, MO), while polyethylene glycol 400 and 99.5% ethanol were obtained from Katayama Chemical (Osaka, Japan). Saline was also purchased from Otsuka Pharmaceutical (Tokyo, Japan). For in vivo treatment, SP600125 was prepared daily and dissolved in 30% polyethylene glycol 400, 20% propylene glycol, 15% Cremophor EL, 5% ethanol, 30% saline (PPCES). To obtain a desired dose as indicated, 2.5 ml of the drug solution per kg body weight was administered subcutaneously to a rat. For in vitro study, SP600125 was dissolved in 100% dimethylsulfoxide (DMSO; Sigma) to produce a concentration of 20 mmol/L (mM). Concanavalin A (Con A; Sigma) and lipopolysaccharide (LPS; Sigma) were diluted in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) to 1 mg/mL and 25 mg/mL, respectively, and filtered. These stock solutions were stored at −80 °C before use. Phorbol 12-myristate 13-acetate (PMA) (Sigma) and ionomycin (Sigma) were dissolved in 99.5% ethanol (Katayama Chemical) to produce concentrations of 10 μg/mL and 0.5 mg/mL, respectively, and stored at −20°C before use. Cyclosporine (intravenous formulation) (CsA; Novartis Pharma Stein AG, Stein, Switzerland) was prepared every 3 days and diluted with saline, then stored at 4°C. For in vivo treatments, 1 ml of the drug solution per kg body weight was administered intraperitoneally to obtain a desired dose.

Heterotopic Heart Transplantation

LEW rats served as recipients while BN or LEW rats served as donors. Intraabdominal heterotopic cardiac transplantation was performed using a modified technique described by Ono and Lindsey (*14*) on day 0. Briefly, the venae cavae and pulmonary veins of the donor were ligated with 5-0 silk sutures, and the pulmonary artery and aorta were transected 2–3 mm above their origins in the heart. The donor heart was excised and placed in a saline bath at 4°C. The graft was implanted in the abdominal cavity with end-to-side anastomosis (the donor aorta to the recipient aorta and the donor pulmonary artery to the recipient vena cava) using a continuous running technique with 8-0 monofilament sutures. For treatments, SP600125, PPCES vehicle or CsA was injected to the recipients once a day from day 0 until the day of rejection at doses indicated. The grafts were evaluated daily for viability by abdominal palpation. Operative times ranged from 35 to 45 min with a success rate of approximately 90%. The day of rejection was defined as the posttransplant day on which all contractions had ceased.

Histopathological Examination

Recipient rats were sacrificed on day 3 or day 5. The transplanted hearts were excised, immediately fixed with 10% neutralized buffered formalin and then embedded in paraffin. Transverse sections of the grafts were stained with hematoxylin and eosin, and evaluated by two pathologists blinded to the individual treatment groups. Non-specific postoperative lymphocytic infiltration in the epimyocardium was excluded in the morphologic evaluation because these lesions are known to be related to the heterotopic transplantation model (*15*). The degree of cellular rejection was graded according to the standardized grading system of the International Society for Heart and Lung Transplantation (ISHLT) (*16*), and the histopathologic grade was scored as described previously (*15*) (grade 0=0; grade IA=1; grade IB=1.5; grade II=2; grade IIIA=3; grade IIIB=3.5; grade IV=4).

Popliteal Lymph Node (PLN) Hyperplasia Bioassay

We used a modified method described by Yuh and Morris (*17*). Briefly, splenocytes were isolated from LEW or BN rats. Ficoll density gradient centrifugation was applied to the single cell suspension to sediment erythrocytes and polynuclear leukocytes. Trypan blue exclusion indicated >90% cell viability, and the resultant viable mononuclear cells were resuspended in RPMI 1640 to a final working concentration of 1.25×10^8 cells/ml. For allogeneic stimulation, 2.5×10^6 BN splenic leukocytes (20 μL) were injected subcutaneously into left hind footpads of LEW rats using a repeating syringe fitted with a 27-gauge hypodermic needle on day 0. LEW splenocytes were used for isogeneic control. The rats were untreated, or treated daily with SP600125 or vehicle for 4 days (day 0 to 3) at doses indicated. Each group contained at least five animals, and there was no difference among body weights of all experimental groups at the time when experiments were begun. On day 4, bilateral PLNs were removed and weighed. The enlargement of the left PLN relative to the unstimulated right PLN was expressed as weight ratio of the left to the right node.

Lymphocyte Proliferation Assay

Splenic mononuclear cells were prepared from LEW or BN rats as described above. Single cell suspensions were prepared from mesenteric lymph nodes (MLNs) removed from LEW rats. Viable cells were determined by trypan blue exclusion, and resuspended in RPMI 1640 supplemented with 5% fetal calf serum (Sigma), penicillin G (100 U/mL)-streptomycin (100 μg/mL) (Sigma), 2 mM L-glutamine (Sigma) and 5×10^{-5} M 2-mercaptoethanol (Sigma). Mixed lymphocyte reaction (MLR) was set up in a total volume of 200 μL in 96-well round-bottom microtiter plates (Greiner, Frickenhausen, Germany). LEW lymphocytes (2.5×10^5 cells/well) were stimulated by either x-irradiated (36 Gy) LEW or BN splenocytes (2.5×10^5 cells/well) and incubated at 37°C in 5% humidified CO_2 for 72 hr. For mitogen-induced lymphocyte proliferation, LEW lymphocytes (2.5×10^5 cells/well) were cultured alone or stimulated with Con A (1 μg/mL) or LPS (25 μg/mL), and incubated in 96-well flat-bottom microtiter plates (Greiner) for 24–72 hr. In some experiments, T-cells were positively separated from the lymphocyte suspension. Briefly