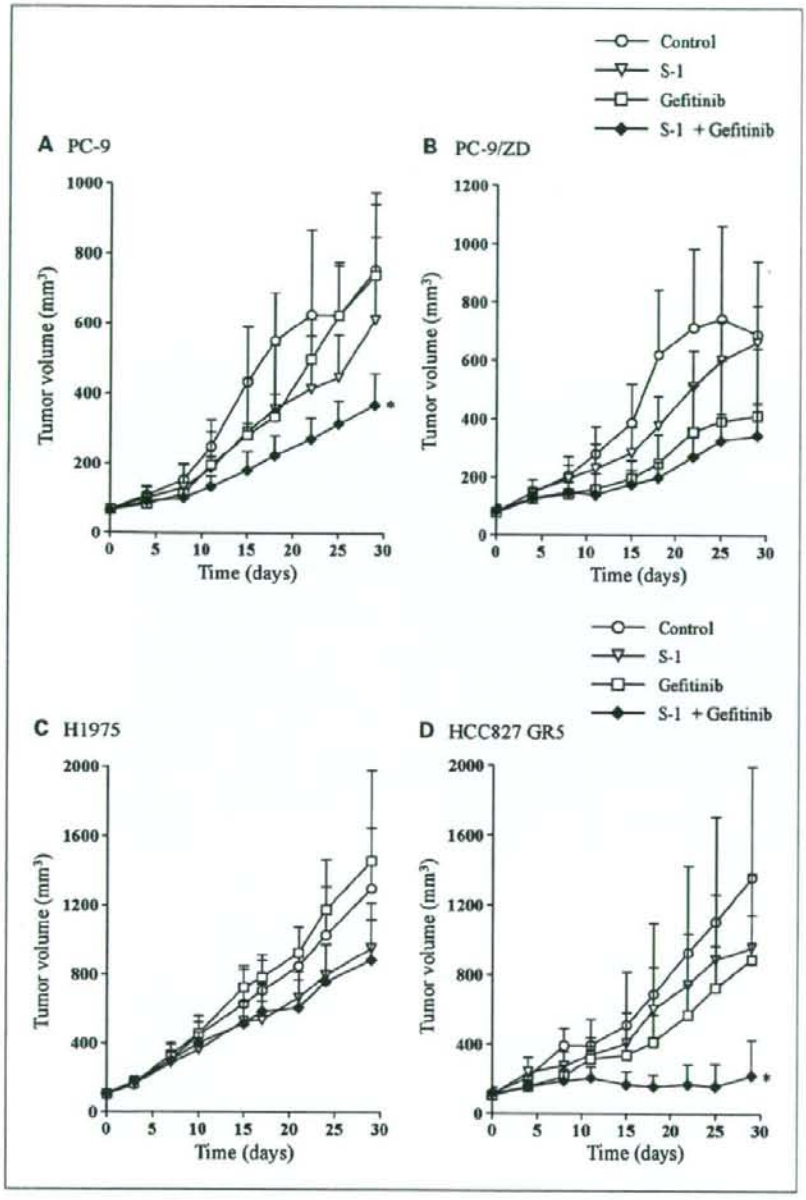

Fig. 4. Effects of the combination of S-1 and gefitinib on the growth of gefitinib-resistant NSCLC cells *in vivo*. *A*, nude mice with tumor xenografts established by s.c. implantation of PC-9 cells were treated daily for 4 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (3 mg/kg), or both drugs by oral gavage. *B* to *D*, nude mice with tumor xenografts of NSCLC cells either harboring the T790M mutation of *EGFR* (PC-9/ZD and H1975) or exhibiting *MET* amplification (HCC827 GR5) were treated daily for 4 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage. Tumor volume was determined at the indicated times after the onset of treatment. *Points*, mean of values from seven mice per group; *bars*, SE. *, $P < 0.05$ for the combination of S-1 plus gefitinib versus control or either S-1 or gefitinib alone (Student's *t* test).

expression that facilitates the antitumor effect of S-1. Pemetrexed, an antifolate drug with multiple targets, has also shown antitumor activity mediated by TS inhibition in a broad range of tumors including NSCLC (49–53). The addition of S-1 or pemetrexed to gefitinib may thus prove effective in NSCLC patients whose gefitinib resistance is attributable to activation of a non-EGFR tyrosine kinase.

In conclusion, we have shown that the combination of S-1 and gefitinib had a synergistic antiproliferative effect in gefitinib-resistant NSCLC cells with *MET* amplification. The inhibition of EGFR phosphorylation and down-regulation of TS by gefitinib were associated with the synergistic interaction between gefitinib and S-1. Our preclinical results suggest that the addition of S-1 to gefitinib is a potential strategy for overcoming EGFR-TKI resistance and warrants clinical evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Circulating Endothelial Cells in Non-small Cell Lung Cancer Patients Treated with Carboplatin and Paclitaxel

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Introduction: Circulating endothelial cells (CECs) increase in cancer patients and play an important role in tumor neovascularization.
Methods: This study was designed to investigate the role of CEC as a marker for predicting the effectiveness of a carboplatin plus paclitaxel based first line chemotherapy in advanced non-small cell lung cancer (NSCLC).
Results: The CEC count in 4 ml of peripheral blood before starting chemotherapy (baseline value) was significantly higher in NSCLC patients, ranging from 32 to 4501/4 ml (n = 31, mean ± SD = 595 ± 832), than in healthy volunteers (n = 53, 46.2 ± 86.3). We did not detect a significant correlation between the CEC count and estimated tumor volume. CECs were significantly decreased by chemotherapy as compared with pretreatment values (175.6 ± 24 and 173.0 ± 24, day +8, +22, respectively). We investigated the correlation between baseline CEC and the clinical effectiveness of chemotherapy. CEC values are significantly higher in patients with clinical benefit (partial response and stable disease, 516 ± 458, 870.8 ± 1215, respectively) than in progressive disease patients (211 ± 150). Furthermore, a statistically significant decrease in CECs, on day 22, was observed only in patients with partial response. Patients who had a baseline CEC count greater than 400/4 ml showed a longer progression-free survival (>400, 271 days [range: 181–361] versus <400, 34 [range: 81–186], p = 0.019).
Conclusion: CEC is suggested to be a promising predictive marker of the clinical efficacy of the CBDCA plus paclitaxel regimen in patients with NSCLC.

Key Words: Circulating endothelial cell, NSCLC, Chemotherapy.

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Angiogenesis plays a critical role in the growth and metastasis of solid tumors.[1] The clinical importance of angiogenesis in human tumors has been demonstrated by several reports indicating a positive relationship between the blood vessel density in the tumor mass and poor prognosis, i.e., survival, in patients with various types of cancers including non-small cell lung cancer (NSCLC).[2–6] Furthermore, Natsume et al.[7] reported the antitumor activities of anticancer agents to be less active against vascular endothelial growth factor-secreting cells (SBC-3/VEGF), in vivo as compared with its mock transfectant (SBC-3/Neo). In recent years, antiangiogenic agents have also been demonstrated to be active against a variety of malignancies, including lung, colorectal, and renal cancer.[8–10] Thus, angiogenesis is a promising target for cancer treatment and is related to the prognosis and efficacy of these drugs, though the tumor vessel biomarkers which predict the effectiveness of antiangiogenic agents and other anticancer agents are not always useful and have not become well-established.

Circulating endothelial cells (CECs) have been recognized as a useful biomarker for vascular damage. CECs are increased in cardiovascular disease, vasculitis, infectious disease, and various cancers.[11–14] Recently, CECs were found to be more numerous and viable in cancer patients than in healthy subjects.[14,15] Furthermore, elevated CECs in cancer patients were found to be nearly normalized when the tumor was removed surgically or with chemotherapy.[15] Therefore, most CECs are considered to be disseminated tissue endothelial cells in the tumors and the CEC number may reflect the extent of tumor angiogenesis. Indeed, the CEC level has been demonstrated to correlate with the plasma level of VEGF, one of the pivotal factors promoting tumor angiogenesis.[15] Mancuso et al. reported that CEC kinetics and viability are promising predictors of the response to chemotherapy with antiangiogenic activity in patients with advanced breast cancer.[16] Thus, CEC is likely to be a useful marker for predicting the effectiveness of chemotherapy as a noninvasive angiogenesis marker.

NSCLC is the leading cause of cancer-related death worldwide. NSCLC accounts for approximately 50% of patients presenting with unresectable advanced stage,[17] and platinum-based chemotherapy offers only a small improve-

ment in survival with advanced NSCLC.[18,19] Over the past decade, several new agents against NSCLC have become available, including the taxanes, gemcitabine, vinorelbine, and irinotecan. The combination of platinum and these new agents has resulted in a high response rate and prolonged survival compared with older chemotherapy regimens (e.g., vindesine, mitomycin, ifosfamide, with cisplatin). Therefore, these regimens are considered standard chemotherapy for advanced NSCLC.[20–26] Although new agents have different mechanisms of action, these combination regimens have not been administered based on the biologic characteristics of each tumor.

Paclitaxel inhibits several endothelial cell functions in vitro such as proliferation, migration, morphogenesis, and metalloprotease production.[27–29] These activities result in antiangiogenic activity in in vivo xenograft models.[27,30] Interestingly, human endothelial cells are more sensitive to paclitaxel than other cellular types.[29] We hypothesized that the CEC value is associated with tumor neovascularization, which is one of the targets of paclitaxel. In the present study, we investigated whether the CEC count at baseline is associated with the effectiveness of the CDDP plus paclitaxel regimen in patients with advanced-stage NSCLC.

MATERIALS AND METHODS

Patients

Patients with histologically or cytologically documented advanced NSCLC were eligible for this study. Each patient was required to meet the following criteria: (1) no prior treatment including chemotherapy, surgery, irradiation, or any fluid drainage; (2) no prior general anesthesia for diagnostic procedures including mediastinoscopy or thoracoscopy; (3) no concomitant diseases including ischemic heart diseases, systemic vasculitis, pulmonary hypertension, or serious complications including infectious disease or diabetes; (4) written informed consent. The trial document was approved by the institutional review board. The clinical characteristics of the patients are shown in Table 1.

TABLE 1. Baseline Characteristics of the Patients

Characteristic	$N = 31$ No. (%)
Gender	
Male	17 (55)
Female	14 (45)
Median age (yr)	60
Range	43–71
ECOG performance status	
0	18 (58)
1	13 (42)
Stage	
IIIA	2 (6)
IIIB	7 (23)
IV	22 (71)
Histology	
Adenocarcinoma	23 (74)
Squamous cell carcinoma	4 (13)
Others	4 (13)

Treatment Schedule and Response Evaluation

All patients were treated according to the following chemotherapeutic regimen: paclitaxel at 200 mg/m^2 over a 3-hour period followed by carboplatin at a dose with an area under the curve of 6 on day 1, repeated every 3 weeks. The treatment was repeated for three or more cycles unless the patients met the criteria for progressive disease (PD) or experienced unacceptable toxicity.

The major axis (a) and minor axis (b) of the tumor mass in each patient were measured with computed tomography. Estimated tumor volume (ETV) was calculated using the following formula; $ETV = 4/3 \times \pi\ (a/2 \times b/2) \times (a/2 + b/2)/2$. Computed tomography examinations were performed before treatment and with every one or two cycles of chemotherapy. Response was evaluated according to the RECIST, and tumor markers were excluded from the criteria.[31]

Assay for CEC

Blood samples from NSCLC patients and healthy volunteers were drawn into a 10-ml Cellsave Preservative Tube (Immunicon Corp. Huntingdon Valley, PA) for CEC enumeration. The CEC protocol used was approved by the Institutional Review Board and written informed consent was obtained from each subject. Samples from NSCLC were obtained before (baseline) and 8 and 22 days after starting chemotherapy. Samples were kept at room temperature and processed within 42 hours after collection. All evaluations were performed without knowledge of the clinical status of the patients. The CellTracks system (Immunicon Corp) which consists of CellTracks AutoPrep system and the CellSpotter Analyzer system was used for endothelial cell enumeration.[32,33] In this system, CD146+/DAPI+/CD105-PE+/CD45APC- cells are defined as CECs. Briefly, cells which express CD146 were immunomagnetically captured using ferrofluids coated with CD146 antibodies. The enriched cells were then labeled with the nuclear dye 4V,6-diamidino-2-phenylindole (DAPI), CD105 antibodies conjugated to phycoerythrin (CD105-PE), and the pan-leukocyte antibody CD45 conjugated to allophycocyanin (CD45-APC). In this system, the CD146-enriched, fluorescently labeled cells were identified as CECs when the cells exhibited the DAPI+/CD105+/CD45- phenotype. We performed CEC enumeration twice, using the same sample, and calculated the mean value.

Statistical Analyses

This study was carried out as exploratory research for detecting CECs from NSCLC patients. The number of enrolled patients was therefore not precalculated. Spearman's correlation analysis was performed to investigate the correlation between CEC count and ETV. Between-group comparisons were made using the t test. The association between CEC count and progression free survival (PFS) was estimated using the Kaplan-Meier method. The log-rank test was used to assess the survival difference between strata. Differences were considered statistically significant at $p < 0.05$.

RESULTS

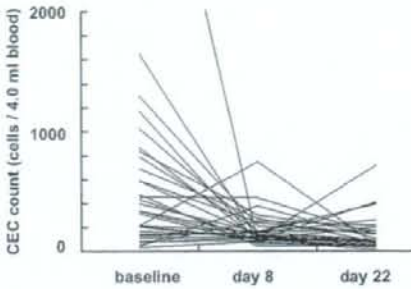
Patient Characteristics

A total of 32 patients were enrolled in the study between August 2005 and March 2006 (Table 1). One patient withdrew consent to participate. Table 1 summarizes the characteristics of the study population. The median age of the patients was 60 years (range, 43–71). The histologic and/or cytologic diagnosis was adenocarcinoma in 23 patients (74.2%), squamous cell carcinoma in 4 (12.9%), and unclassified NSCLC in 4 (12.9%). There were 17 males (54.8%). The clinical stage was IIIA in 2 patients (6.5%), IIIB in 7 (22.6%), and IV in 22 (71.0%).

Ninety-two CEC samples from 31 patients (three samples per patient) were obtained and analyzed. One sample, obtained 22 days after treatment, was not examined because of inadequate collection.

Quantification of CEC

In 31 advanced NSCLC patients, CECs ranged from 32 to 4501 cells/4.0 ml of blood, mean ± SD = 595 ± 832 at baseline. CEC counts were elevated in a large portion of patients with NSCLC as compared with healthy volunteers (n = 53, mean ± SD = 46.2 ± 86.3/4 ml). Case 21 had an exceptionally high CEC count (4501 at baseline). We did not detect a significant correlation between the CEC count and ETV in the 28 assessable patients (p = 0.84, Figure 1). The analysis of CECs during the first course of treatment showed CEC levels to be reduced by CBDCA plus paclitaxel chemotherapy as compared with pretreatment values (176 ± 141 at 8 days and 173 ± 189 at 22 days after treatment) (Figure 2). These reductions were significant (p = 0.011 on day 8 and p = 0.04 on day 22), but there was no significant difference between CEC amounts on day 8 versus day 22 (p = 0.476). There was no difference in the amount of CEC at baseline when patients were subgrouped according to characteristics, such as sex, smoking history, histologic type, and clinical stage. Furthermore, there was no correlation of CEC amounts with the blood examination data (e.g., number of white blood cells, neutrophils, lymphocytes, hemoglobin, platelets, albumin, LDH, CRP, CEA, CYFRA).



	baseline	day 8	day 22
mean ± SD	595 ± 832	176 ± 141 *	173 ± 189 *

FIGURE 2. Circulating endothelial cell (CEC) levels during the first course of CDDP plus paclitaxel chemotherapy. *p < 0.05 versus values at baseline.

CEC Amounts and Objective Tumor Response to Chemotherapy

Thirteen (41.9%) of the 31 patients who received carboplatin and paclitaxel therapy showed a partial response (PR) and 12 (38.7%) showed stable disease (SD). The other 6 patients (19.4%) showed PD. The amounts of CEC at baseline in the patients who showed PR and SD were 516 ± 458/4 ml and 871 ± 1215/4 ml, respectively, and these values were significantly higher than in PD patients (211 ± 150/4 ml, p = 0.023 and p = 0.044, respectively) (Figure 3*A*). Although CEC decrements during chemotherapy were observed in all three subgroups, the extent of the decrements tended to be greater in

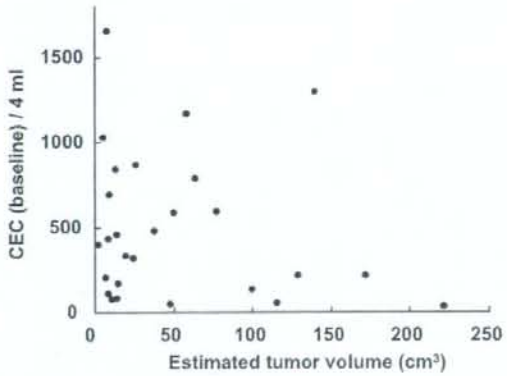


FIGURE 1. Scatter plot analysis to determine the correlation between the number of circulating endothelial cell (CEC) and estimated tumor volume (ETV). ETV is calculated with computed tomography (CT) examination. Case 21 is not included.

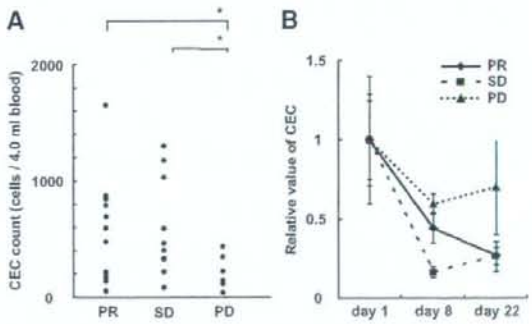


FIGURE 3. *A*, Comparison of circulating endothelial cell (CEC) amount at baseline in non-small cell lung cancer (NSCLC) patients with different clinical responses to CBDCA plus paclitaxel chemotherapy. *p < 0.05 versus values of patients with progressive disease (PD). Case 21 is not included. *B*, Relative change in CEC amount in patients with partial response (PR), stable disease (SD), and PD.

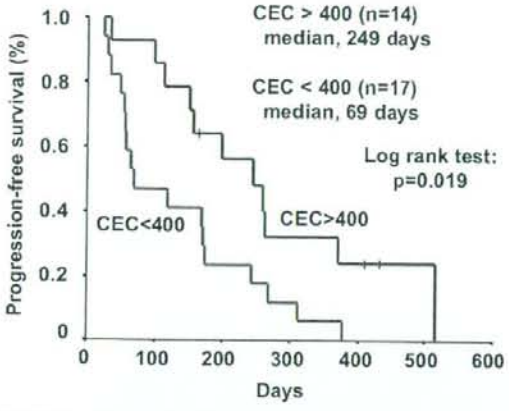


FIGURE 4. Progression-free-survival according to circulating endothelial cell (CEC) count at baseline. The median duration of progression-free survival was greater in patients whose CEC count exceeded 400 (median, 244 days) than in patients whose CEC count was less than 400 (69 days).

patients with PR and SD than in those with PD (Figure 3*B*). In the subgroup analysis, a significant decrease in CECs was observed on day 22 only in PR patients ($p = 0.018$).

CEC Amounts and PFS

For all 31 patients, the median PFS was 154 days (range, 81–361 days). Univariate analysis indicated that patients who had a CEC count of more than 400/4 ml at baseline showed a significantly improved PFS ($n = 14$, median; 244 days) (Log-rank test, $p = 0.019$, Figure 4). A CEC count below 400 at baseline was associated with a poorer PFS ($n = 17$, median; 69 days). The CEC count did not exceed the value of 400/4 ml in any of the healthy volunteers. When we compared the patients whose CEC counts exceeded 200 with those whose counts were less than 200, a consistent difference in PFS was observed between the two groups (>200; $n = 22$, median 227, <200; $n = 9$, median 116, $p < 0.039$).

DISCUSSION

In the present study, we investigated the number of CEC during the first course of CBDCA plus paclitaxel chemotherapy. To our knowledge, this is the first report of CEC in NSCLC patients before treatment. Our findings demonstrated CEC counts in advanced NSCLC at baseline level to be much higher than those in healthy subjects (595 ± 832/4.0 ml versus 32.6 ± 29.5/4.0 ml). Because the NSCLC patients had not yet received anticancer therapy, these increased CECs are likely to be mostly derived from the tumor site. In a previous study, it was found that the amounts of CECs correlate strongly with tumor volume in vivo in an animal model[34]. Nevertheless, we did not find a significant correlation between CECs and ETV. Because the number of CECs could be influenced by many factors related to tumor vasculature, neovascularization, and localization of the tumor, our failure to identify a strong correlation in this study is not surprising. We were also unable to detect a significant direct correlation between CEC amounts and various blood examination data including tumor markers such as CEA and CYFRA. It is unclear at present what biologic characteristics of the tumor or clinical features the CEC number most closely reflects as a biomarker. Mancuso et al. reported that CECs are strongly associated with plasma levels of VCAM-1 and VEGF in breast cancer and lymphoma patients.[15,34] Because VCAM-1 and VEGF are crucial factors for tumor angiogenesis, the variability in CEC values among NSCLC patients might indicate a difference in the neovascularization of each tumor.

We were further able to demonstrate that elevated CECs decreased dramatically after CBDCA plus paclitaxel treatment, but did not reach the level of healthy subjects. Decreased CEC values did not rise again during the first cycle of chemotherapy. Although myelosupression was observed on day 8 and recovered on day 22 in many patients (data not shown), CEC kinetics do not parallel those of WBC, indicating that CEC kinetics might not be influenced by myelopoiesis. Several clinical studies in the field measuring CEC found chemotherapy to be associated with either an increase or a decrease in CECs.[35–39] The different tumor types, stages, prior therapy or not, the anticancer drugs used, measuring points and quantification methods of CEC might have influenced the CEC results after treatment. In the present study, the pretreatment CEC value was much higher than that in lung cancer with metastasis (mean ± SD = 146 ± 270/4 ml), as reported elsewhere.[33] Although the details of the prior therapy in patients with metastatic carcinoma were not provided,[33] chemotherapy can eventually decrease the CEC count.

Schiller et al. compared four standard chemotherapy regimens, cisplatin plus paclitaxel, cisplatin plus gemcitabine, cisplatin plus docetaxel, and carboplatin plus paclitaxel and found no significant difference in survival.[25] Despite the different modes of action of each nonplatinum agent against tumors and different biologic characteristics of each tumor, we could not select the regimen based on these characteristics. In our small study, the patients with PR/SD and longer PFS had higher baseline CEC values. Therefore, it seems that the baseline CEC count is a promising predictor of clinical response to the CBDCA plus paclitaxel regimen and survival in advanced NSCLC. If CEC is a marker for angiogenesis and reflects tumor neovascularization, it is likely that a high CEC is associated with a poor prognosis and lower effectiveness of antiangiogenic therapy. Paclitaxel and docetaxel are categorized as mitotic spindle agents with potent antiangiogenic properties.[27–30] This is why a paclitaxel based regimen might be more effective against tumors with high CEC values. Nevertheless, CEC counts have also been reported to be increased in several clinical syndromes, such as cardiovascular diseases, infectious diseases, and vasculitides.[11–13] The CEC counts in patients with vasculitides have been reported to be dozens of fold higher than those in healthy subjects,[12] therefore, we have to consider the patient condition carefully while interpreting the CEC counts in individual patients, although there were no patients with vasculitis in the present study. Further clinical investigation, with a similar approach, including other nonplatinum anticancer agents, such as

CDDP plus gemcitabine, is essential for the clinical application of CEC for made-to-order chemotherapy in NSCLC.

Antiangiogenic therapy targeting the VEGF pathway such as bevacizumab and VEGFR inhibitors have shown promise in the treatment of solid tumors.[8,39] These agents inhibit endothelial cells through inhibition of the VEGF pathway. It was recently demonstrated that the addition of bevacizumab to CBDCA plus paclitaxel in advanced NSCLC patients produces a significant survival benefit as compared with chemotherapy alone.[40] Considering the outstanding clinical trial and our present study, it would be of great interest to investigate the role of CEC in this regimen.

In conclusion, CECs were measured in NSCLC patients before treatment. Our small clinical study indicates that the CEC count at baseline is a potential biomarker for predicting the response to chemotherapy and PFS, but further clinical evaluation is needed. In the near future, we will start a clinical investigation, using a similar approach, to examine other chemotherapeutic regimens.

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Role of multidrug resistance-associated protein 1 in the pathogenesis of allergic airway inflammation

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[1]*Department of Respiratory Medicine,* [2]*Research Institute for Diseases of Old Ages, and* [3]*Atopy (Allergy) Research Center, Juntendo University School of Medicine, Bunkyo-Ku, Tokyo;* [4]*Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi;* [5]*Department of Genome Biology, Kinki University School of Medicine, Osaka;* [6]*Clinical Research Center for Allergy and Rheumatology, National Sagamihara Hospital, Kanagawa;* [7]*Department of Biostatistics, School of Public Health, The University of Tokyo, Tokyo; and* [8]*Department of Analytical Biochemistry, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan*

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Yoshioka M, Sagara H, Takahashi F, Harada N, Nishio K, Mori A, Ushio H, Okada KS, Ota M, Ito YM, Nagashima O, Atsuta R, Suzuki T, Fukuda T, Fukuchi Y, Takahashi K. Role of multidrug resistance-associated protein 1 in the pathogenesis of allergic airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 296: L30–L36, 2009. First published October 17, 2008; doi:10.1152/ajplung.00026.2008.—Multidrug resistance-associated protein 1 (MRP1) is a cysteinyl leukotriene (CysLT) export pump expressed on mast cells. CysLTs are crucial mediators in allergic airway disease. However, biological significance of MRP1 in allergic airway inflammation has not yet been elucidated. In this study, we sensitized wild-type control mice ($mrp1^{+/+}$) and MRP1-deficient mice ($mrp1^{-/-}$) to ovalbumin (OVA) and challenged them with OVA by aerosol. Airway inflammation and goblet cell hyperplasia after OVA exposure were reduced in $mrp1^{-/-}$ mice compared with $mrp1^{+/+}$ mice. Furthermore, CysLT levels in bronchoalveolar lavage fluid (BALF) from OVA-exposed $mrp1^{-/-}$ mice were significantly lower than those from OVA-exposed $mrp1^{+/+}$ mice. Levels of OVA-specific IgE, IL-4, and IL-13 in BALF were also decreased in OVA-exposed $mrp1^{-/-}$ mice. IgE-mediated release of CysLTs from murine bone marrow-derived mast cells was markedly impaired by MRP1 deficiency. Our results indicate that MRP1 plays an important role in the development of allergic airway inflammation through regulation of IgE-mediated CysLT export from mast cells.

cysteinyl leukotrienes; mast cell

MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 1 (MRP1) is a 190-kDa transmembrane protein belonging to the ATP-binding cassette transporter superfamily (15). The MRP1 gene was isolated from the doxorubicin-resistant human small cell lung cancer cell line H69AR (6), and subsequent in vitro studies established that MRP1 mediates the cellular excretion of many drugs and confers multidrug resistance of cancer cells (3). MRP1 has been shown to be expressed in various human tissues and cells, including mast cells (10). MRP1 transports glutathione *S*-conjugates of endogenous and xenobiotic lipophilic compounds across the cellular membrane into the extracellular space (15). Among these transport substrates, leukotriene C4 (LTC4) is a high-affinity endogenous glutathione *S*-conjugate substrate for the MRP1 (19) and is excreted from mast cells, which play an important role in the pathogenesis of allergy and asthma (2, 20).

Bronchial asthma is a common disorder in adults and children and remains poorly understood and difficult to manage (4). Airway inflammation is a hallmark of this disease (4). Previous studies have indicated that cysteinyl leukotrienes (CysLTs) such as LTC4, LTD4, and LTE4, originally termed slow-reacting substance of anaphylaxis, are crucial mediators in the pathogenesis of allergic asthma (8). LTC4 is synthesized by and excreted from mast cells and is rapidly converted to LTD4 and then to LTE4 (5). CysLTs induce airway smooth muscle contraction, increase vascular permeability and mucus secretion, and may recruit more inflammatory cells to the airway in allergic asthma (13). However, the importance of MRP1, which is the LTC4 export pump on mast cells, for allergic airway inflammation remains poorly defined.

To elucidate the role of MRP1 in the pathogenesis of allergic airway inflammation in vivo, we used an ovalbumin (OVA) sensitization and airway challenge protocol and compared MRP1-deficient mice ($mrp1^{-/-}$) with wild-type control mice ($mrp1^{+/+}$) in a well-established model. We also cultured bone marrow-derived mast cells (BMMCs) from $mrp1^{-/-}$ and $mrp1^{+/+}$ mice and stimulated them with IgE and anti-IgE antibody. The biological significance of MRP1 involvement in allergic airway inflammation and IgE-dependent export of CysLTs from mast cells is discussed.

MATERIALS AND METHODS

Animals. MRP1-deficient $mrp^{-/-}$ mice were generated by gene targeting in embryonic stem cells as described previously (31). $Mrp^{-/-}$ mice originally on the genetic background (129/Ola)/FVB (50:50) were backcrossed 12 times with FVB mice to obtain >99% FVB genetic background. Normal FVB mice were used as wild-type controls ($mrp^{+/+}$). $Mrp^{+/+}$ and $mrp^{-/-}$ mice (male, 6–8 wk of age) were purchased from Taconic Laboratories (Germantown, NY). Mice were maintained in a limited access barrier and housed in a humidity (55 ± 10%)- and temperature (24 ± 2°C)-controlled room under a

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12:12-h light-dark cycle. The study protocol was reviewed and approved by the Juntendo University and Dokkyo University School of Medicine Committee on Animal Care and complies with National Institutes of Health guidelines for animal care.

Sensitization and airway challenge. Mice were sensitized on *days 0* and *14* by an intraperitoneal injection of 50 μg of OVA (Sigma, St. Louis, MO) and 2 mg of aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan) in 200 μl of PBS. Nonsensitized mice received only aluminum hydroxide in PBS. On *days 22*, *24*, *26*, and *28*, the sensitized mice were challenged with aerosolized 1% OVA 30 ml for 30 min. The nonsensitized mice received PBS only. Bronchoalveolar lavage and histological analysis of the lungs were performed 48 h after the last aerosol challenge.

Histological analysis of lung. The murine lungs were infused and fixed with 10% formalin and then embedded in paraffin. Sections of 2.5-μm thicknesses were stained with either hematoxylin and eosin or periodic acid-Schiff (PAS). Semiquantitative scoring systems were used to grade the extent of lung inflammation and goblet cell hyperplasia as previously described (9). Briefly, to determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a five-point scoring system: 0, no cell; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; and 4, a ring of cells >4 cells deep. To determine the extent of mucus production, we quantified goblet cell hyperplasia in the airway epithelium using a five-point grading system: 0, no goblet cells; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Scoring of inflammatory cells and goblet cells was performed in at least 15 different fields for each lung section. Mean scores were obtained from six animals.

Bronchoalveolar lavage fluid and serum analyses. Mice were killed with an overdose of pentobarbital sodium. Blood was drawn, and bronchoalveolar lavage fluid (BALF) was collected with twice repeated washes of excised lungs using 0.7 ml of PBS. Total cell counts and differential cell counts were performed. Cytokine, OVA-specific IgE, and CysLT levels were measured using enzyme-linked immunosorbent assay (ELISA). Mouse IL-4 and IL-13 ELISA were purchased from R&D Systems (Minneapolis, MN). Mouse OVA-specific-IgE ELISA was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). CysLT ELISA was purchased from Cayman Chemicals (Ann Arbor, MI).

Generation of murine BMMCs. BMMCs were generated from the femoral bone marrow cells of $mrp^{+/+}$ and $mrp^{-/-}$ mice and maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated FCS, 100 μM 2-mercaptoethanol, 10 μM MEN-nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% pokeweed mitogen-stimulated spleen-conditioned medium as a source of mast cell growth factors as previously described (11). After 4 wk of culture, >98% of the cells were identifiable as mast cells as determined by toluidine blue staining and fluorescence-activated cell sorting analysis of cell surface expression of c-*kit* and FcεRI.

β-Hexosaminidase release assay. In vitro degranulation of mast cells was determined by β-hexosaminidase release assay as described previously (11, 26). Briefly, BMMCs were incubated with trinitrophenyl (TNP)-specific mouse IgE (BD Pharmingen, San Diego, CA) at the concentration of 1 μg/5 × 10^6 cells for 1 h on ice. BMMCs (1×10^6 cells/ml) were then resuspended in Tyrode's buffer (10 mM HEPES buffer, pH 7.4, 130 mM NaCl, 5 mM KCl, and 5.6 mM glucose) containing 1 mM $CaCl_2$ and 0.6 mM $MgCl_2$ and stimulated with 0.5 μg/ml anti-mouse IgE (BD Pharmingen) for 45 min at 37°C. Cell supernatants and total cell lysate solubilized by sonication were collected, and β-hexosaminidase in the supernatants and cell lysate was quantified by spectrophotometrical measurement of the hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-hexosaminidase (Sigma-Aldrich Japan, Tokyo, Japan) in 0.1 M sodium citrate buffer (pH 4.5). The reaction was terminated by the addition of 0.2 M glycine (pH 10.7). The percentage of β-hexosaminidase release was calculated using the following formula: percent release = (OD of the stimulated supernatant × 100)/(OD of the total cell lysate), where OD is optical density.

IgE-mediated CysLT export from murine BMMCs. BMMCs of $mrp^{+/+}$ and $mrp^{-/-}$ mice were prepared, cultured, and incubated with TNP-IgE and anti-IgE antibody as described above. The cells were separated from the medium by centrifugation. The amount of CysLT secreted into the supernatant was quantitated using the ELISA kit (Cayman Chemicals). The cells were resuspended in lysis buffer, homogenized, centrifuged, and then collected for determination of intracellular CysLT. Each experiment was performed in triplicate.

Statistics. Data are means ± SD and were analyzed using the unpaired *t*-test. Differences between means were considered statistically significant at $P < 0.05$.

RESULTS

Histopathology of the lungs of $mrp1^{-/-}$ and $mrp1^{+/+}$ mice. To investigate the biological significance of MRP1 in vivo in allergic airway inflammation, we sensitized $mrp1^{-/-}$ and $mrp1^{+/+}$ mice to OVA and challenged them with OVA by aerosol. Control mice received PBS. The lungs from $mrp1^{-/-}$ and $mrp1^{+/+}$ mice exposed to PBS aerosol showed normal lung histology in both groups (Fig. 1, *A* and *B*). Sensitization and subsequent exposure to OVA resulted in peribronchial and perivascular inflammation both in the $mrp1^{-/-}$ and $mrp1^{+/+}$ mice, and excessive production of airway mucus glycoproteins by goblet cells in airway epithelium was observed (Fig. 1, *C–F*). However, this inflammation following OVA exposure was reduced in $mrp1^{-/-}$ mice compared with $mrp1^{+/+}$ mice (Fig. 1, *C* and *D*). To evaluate the extent of inflammation, we employed a semiquantitative scoring system as described previously (9). As shown in Fig. 1*G*, blinded semiquantitative grading of the lung sections revealed a statistically significant difference in the degree of airway inflammation between the $mrp1^{-/-}$ and $mrp1^{+/+}$ mice ($P = 0.0143$). In addition, blinded semiquantification of goblet cell staining with PAS also revealed attenuated mucus scores in OVA-exposed $mrp1^{-/-}$ mice compared with OVA-exposed $mrp1^{+/+}$ mice (Fig. 1*H*) ($P = 0.0431$). These data indicate that airway inflammation and goblet cell hyperplasia are reduced in OVA-exposed $mrp1^{-/-}$ mice compared with OVA-exposed $mrp1^{+/+}$ mice.

Inflammatory cell recruitment in BALF. The recovery of cells from the BALF of PBS-exposed $mrp1^{-/-}$ and $mrp1^{+/+}$ mice revealed a predominance of alveolar macrophages in both groups, without any significant differences (data not shown). Aerosol challenge of mice with OVA induced a marked increase in the total cell numbers compared with control groups with PBS (Fig. 2). However, the total cell numbers in BALF were significantly decreased in OVA-exposed $mrp1^{-/-}$ mice compared with OVA-exposed $mrp1^{+/+}$ mice ($P = 0.0243$). Differential cell counts revealed the predominant recruitment of eosinophils into BALF of both OVA-exposed $mrp1^{-/-}$ and $mrp1^{+/+}$ mice. However, OVA-exposed $mrp1^{-/-}$ mice had significantly lower numbers of eosinophils and lymphocytes than the $mrp1^{+/+}$ mice ($P = 0.0243$ and 0.0187, respectively). The numbers of macrophages and neutrophils were not significantly different between groups. These results imply that $mrp1^{-/-}$ mice show reduced recruitment of inflammatory cells, especially eosinophils and lymphocytes, into the airway lumen after OVA challenge compared with $mrp1^{+/+}$ mice.

CysLT levels in BALF. To investigate the role of MRP1 as a CysLT export pump in vivo, we measured total CysLT levels in BALF from $mrp1^{-/-}$ and $mrp1^{+/+}$ mice exposed to PBS or OVA aerosol. As shown in Fig. 3, levels of CysLTs in BALF

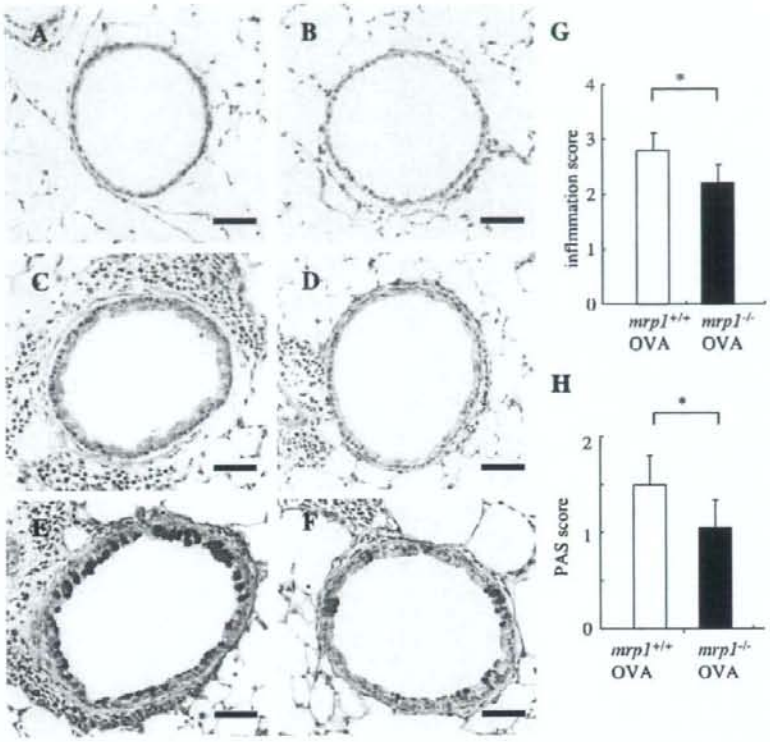

Fig. 1. Histological analysis of lung sections of multidrug resistance-associated protein 1 (MRP1)-deficient ($mrp1^{-/-}$) mice and wild-type $mrp1^{+/+}$ mice. Representative photomicrographs of hematoxylin- and eosin-stained (*A–D*) and periodic acid-Schiff-stained lung sections (*E* and *F*). Scale bar, 50 μm. Lung tissues were obtained 48 h after the last challenge of PBS or ovalbumin (OVA) aerosol. *A*: $mrp1^{+/+}$ mice exposed to PBS aerosol. *B*: $mrp1^{-/-}$ mice exposed to PBS. *C* and *E*: $mrp1^{+/+}$ mice exposed to OVA. *D* and *F*: $mrp1^{-/-}$ mice exposed to OVA. Semiquantitative analyses of inflammatory cell infiltration (*G*) and mucus production (*H*) in lung sections were performed as previously described (14). Scoring of inflammatory cells and goblet cells was performed in at least 15 different fields for each lung section. To prevent observer bias, samples were coded and examined in a blind manner. Mean scores were obtained from 6 animals. $*P < 0.05$.

from OVA-exposed $mrp1^{-/-}$ mice were significantly lower than those from OVA-exposed $mrp1^{+/+}$ mice ($P = 0.0082$).

OVA-specific IgE and cytokine levels. To further assess the mechanism for the reduced airway inflammation in OVA-exposed $mrp1^{-/-}$ mice, we measured OVA-specific IgE levels in BALF and serum samples. As shown in Fig. 4*A*, OVA-specific IgE levels in BALF from OVA-exposed $mrp1^{-/-}$ mice were significantly lower than those from OVA-exposed $mrp1^{+/+}$ mice (P =0.025). OVA-specific IgE levels in serum were also decreased in OVA-exposed $mrp1^{-/-}$ mice (Fig. 4*B*) ($P = 0.0285$). We next measured Th2 cytokines IL-4 and IL-13 in each BALF sample. In PBS-exposed mice of both groups, the levels of IL-4 and IL-13 were below the lower limit of detection (data not shown). As shown in Fig. 4, *C* and *D*, levels of both IL-4 and IL-13 in BALF from OVA-exposed $mrp1^{-/-}$ mice were significantly lower than those from OVA-exposed $mrp1^{+/+}$ mice ($P = 0.0361$ and 0.0101, respectively).

IgE-mediated CysLT export from BMMCs. To examine the role of MRP1 in IgE-mediated CysLT export from mast cells, we cultured BMMCs from $mrp1^{-/-}$ and $mrp1^{+/+}$ mice and stimulated them with TNP-IgE and anti-IgE antibody. Subsequently, the amounts of released CysLTs in the cell-free culture media and the intracellular contents of CysLTs were separately analyzed. MRP1 deficiency did not affect the degranulation as determined by β-hexosaminidase release (Fig. 5*A*) and IgE receptor expression of BMMCs (data not shown). However, CysLT levels in culture media from BMMCs of $mrp1^{-/-}$ mice were significantly lower than those from $mrp1^{+/+}$ mice (Fig. 5*B*) ($P = 0.0017$). In contrast, intracellular CysLT levels in BMMCs of $mrp1^{-/-}$ mice were significantly higher than those of $mrp1^{+/+}$ mice (Fig. 5*C*) ($P = 0.0003$). These results indicate that MRP1 plays a crucial role in IgE-mediated export of CysLTs from activated mast cells.

DISCUSSION

Recent generation of $mrp1^{-/-}$ mice has enabled investigation of the biological function of MRP1 in vivo (30). Wijnholds et al. (31) demonstrated that ear swelling induced by topical application of arachidonic acid was dramatically reduced in the $mrp1^{-/-}$ mice. Importantly, they also revealed that BMMCs from $mrp1^{-/-}$ mice had a reduced capacity to excrete LTC4 after stimulation with calcium ionophore (31). Their report strongly suggests that MRP1 plays an important role in allergic inflammatory response in vivo. However, little is known of the implication of MRP1 in IgE-mediated transport of CysLTs from mast cells and the biological significance of MRP1 in allergic airway disease, including asthma.

In our study, we developed a murine allergic airway inflammation model by intraperitoneal OVA sensitization and airway challenge. We revealed that $mrp1^{-/-}$ mice showed decreased airway inflammation and goblet cell hyperplasia after OVA exposure. CysLT levels in BALF from OVA-exposed $mrp1^{-/-}$ mice were significantly lower than those from $mrp1^{+/+}$ mice.

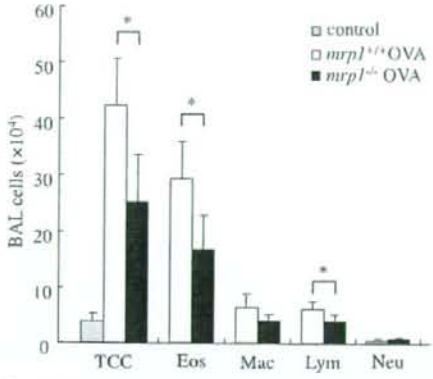

Fig. 2. Bronchoalveolar lavage fluid (BALF) cell counts. BALF were collected from $mrp1^{+/+}$ and $mrp1^{-/-}$ mice 48 h after the last PBS aerosol or OVA aerosol challenge. Total cell counts (TCC) were assessed with a standard hemocytometer. Cell populations were identified on air-dried cytocentrifuged smears (800 rpm for 5 min) after staining with Diff-Quick stain. Differential cell counts were performed on a minimum of 500 cells to identify eosinophils (Eos), macrophages (Mac), lymphocytes (Lym), and neutrophils (Neu). Data are means ± SD of 5 mice per group. $*P < 0.05$.

In addition, OVA-specific IgE, IL-4, and IL-13 levels in BALF were also decreased in OVA-exposed $mrp1^{-/-}$ mice. IgE-dependent release of CysLTs from murine BMMCs was markedly impaired due to MRP1 deficiency. These findings strongly imply that MRP1 plays a key role in the development of allergic airway disease through regulation of IgE-mediated CysLT export from mast cells. To our knowledge, our study is the first report to reveal that $mrp1^{-/-}$ mice are less sensitive to asthmatic response to allergen exposure by using a murine model.

IgE-mediated activation of mast cells in the airway leads to oxygenation of arachidonic acid by 5-lipoxygenase (5-LO) and generation of LTs (12). Among them, secreted CysLTs bind to CysLT receptors and induce bronchoconstriction, mucus hypersecretion, and eosinophil chemotaxis (12, 17). Therefore, inhibition of CysLT biosynthesis or receptor-mediated action is beneficial for patients with bronchial asthma (8). In our murine allergic airway inflammation model, CysLT-synthesizing cells

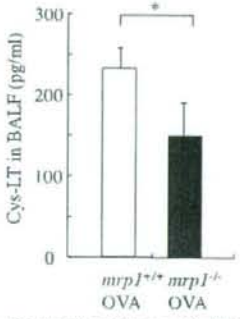

Fig. 3. Measurement of cysteinyl leukotriene (CysLT) levels in BALF. The levels of total CysLTs in the BALF were determined by ELISA. Data are mean ± SD of 5 mice per group. Similar results were obtained in 2 independent experiments. $*P < 0.05$.

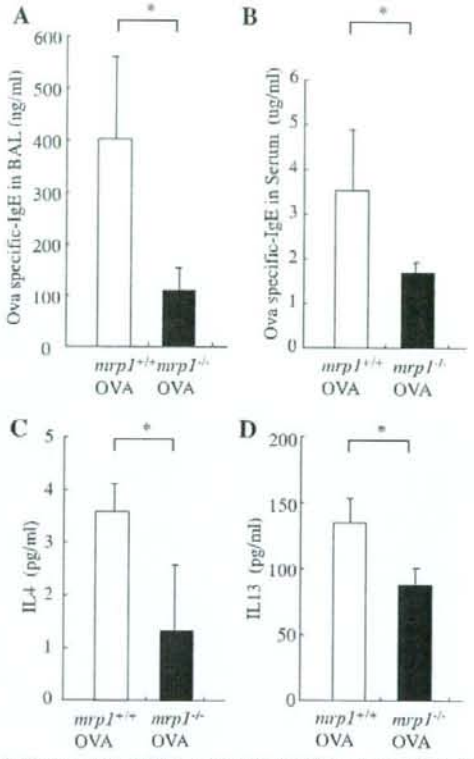

Fig. 4. Measurement of OVA-specific IgE and Th2 cytokine levels. OVA-specific IgE levels in the BALF (*A*) and serum (*B*) were measured by ELISA. The levels of IL-4 (*C*) and IL-13 (*D*) in the BALF were also measured. Data are means ± SD of 4–6 mice per group. Similar results were obtained in 2 independent experiments. $*P < 0.05$.

including mast cells in $mrp1^{-/-}$ mice had a reduced capacity to secrete CysLTs, resulting in decreased CysLT levels in BALF. Suppression of CysLT production due to MRP1 deficiency reduced recruitment of eosinophils and mononuclear cells in the lungs. These findings suggest the possibility that MRP1 inhibitor may be useful as an anti-asthma drug to attenuate airway inflammation to allergen exposure by suppressing IgE-mediated CysLT production.

Th2 inflammatory response is a central component of allergic airway inflammation. In our murine model, Th2 cytokine IL-4 and IL-13 production and lymphocyte recruitment in the lungs were significantly decreased in OVA-exposed $mrp1^{-/-}$ mice, resulting in decreased antigen-specific IgE production. Previous studies have demonstrated that OVA-induced airway eosinophil infiltration and goblet cell hyperplasia were markedly reduced in LTC4 synthase (LTC4S)-deficient mice compared with wild-type control mice (18). Importantly, antigen-specific IgE and Th2 cytokine expression in the lungs were also significantly reduced in OVA-exposed LTC4S-deficient mice, although delayed-type cutaneous hypersensitivity (Th1 cell-dependent response) was intact (18). Others have demonstrated that blockade of $CysLT_1$ receptor reduced elevation of IL-4

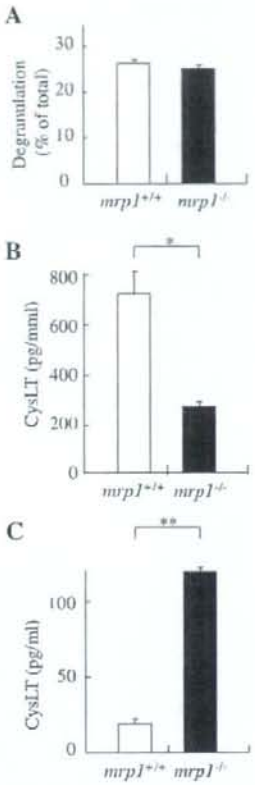

Fig. 5. IgE-mediated CysLT export from bone marrow-derived mast cells (BMMCs). BMMCs from $mrp1^{-/-}$ and $mrp1^{+/+}$ mice were cultured and stimulated with trinitrophenyl (TNP)-IgE and anti-IgE antibody. Degranulation of BMMCs was assessed using the β-hexosaminidase release assay (*A*). The amounts of released CysLTs in the culture supernatant (*B*) and the intracellular contents of CysLTs (*C*) were measured using ELISA. Data are means ± SD of triplicate samples. $*P < 0.05$; $**P < 0.01$. Results are representative of 3 independent experiments that had similar results.

and IL-13 levels in BALF in OVA-exposed mice and attenuated airway inflammation (14). These previous findings provide direct evidence that CysLTs are involved in the regulation of Th2 immune response-dependent pulmonary inflammation. Our current findings in $mrp1^{-/-}$ mice are consistent with these prior reports, because MRP1 is involved in IgE-mediated LTC4 export from mast cells, and a lack of MRP1 resulted in the decrease of CysLT, antigen-specific IgE, IL-4, and IL-13 levels in the lungs of $mrp1^{-/-}$ mice. Impaired Th2 cytokine production due to MRP1 deficiency might be an important mechanism of reducing airway inflammation in our murine model.

Dendritic cells (DCs) are the most potent antigen presenting cells in the airways and initiate immune responses by presenting antigens to T cells (22). Previous studies have demonstrated that DCs express MRP1, and MRP1 regulates the migration of DCs by transporting LTC4, which promotes chemotaxis to the CCL19 (25). In a model of contact hypersensitivity induced by topical application of FITC, DC migration was substantially attenuated in $mrp1^{-/-}$ mice compared with that observed in $mrp1^{+/+}$ mice (25). In addition, MRP1 transporter activity is also crucial for DC differentiation (27). These aforementioned observations on DCs may contribute to the decreased inflammatory response following OVA exposure that we examined in the lungs of $mrp1^{-/-}$ mice.

MRP1 was the first identified ATP-dependent export pump for LTC4. However, the members of the MRP subfamily, including MRP1–6 and MRP10–12, also mediate the ATP-dependent efflux of organic anions, including glutathione conjugates such as LTC4, across the plasma membrane into the extracellular space (7). We questioned whether the lack of MRP1 in mice would be compensated for by induction or altered expression of other ATP-binding cassette transporter subfamily members. However, van der Deen et al. (30) examined immunohistochemical expression of other transporters such as MRP2, -3, -4, -5, -6, and -9 and breast cancer resistance protein (Brcp) in murine lung tissues and observed no differences in expression of all these transporters in MRP1/MDR1a/1b-deficient mice compared with wild-type mice (30). MRP2, also named the canalicular multispecific organic anion transporter (cMOAT), and MRP1 share very similar substrates, including LTC4 (19). However, Wijnholds et al. (31) demonstrated that anti-cMOAT monoclonal antibody does not detect cMOAT protein on the mast cells in $mrp1^{-/-}$ and $mrp1^{+/+}$ mice, whereas cMOAT in the liver and kidney is readily visualized, and the same holds in $mrp1^{-/-}$ and $mrp1^{+/+}$ tissues. These previous findings strongly suggest that MRP1 (and/or MDR1) deficiency does not affect expression of other transporters in lung tissues in mice and supports our conclusion that inhibition of MRP1 might be a major cause of the impaired development of allergic airway inflammation in the lungs of $mrp1^{-/-}$ mice.

Recent studies have demonstrated that MRP4 can transport leukotrienes (LTB4 and LTC4) and contribute to the migration of DCs, like MRP1 (24) (28). Furthermore, MRP4 is expressed in the bronchial epithelial cells in the lungs (29). These previous findings indicate the possibility that other transporters, such as MRP4, also may be important in the lungs of murine allergic airway inflammation model in addition to MRP1, although expression of other ATP-binding cassette transporters was not altered in the lungs of MRP1-deficient mice. This may be the reason why the differences in the degree of airway inflammation between $mrp1^{-/-}$ and $mrp1^{+/+}$ mice were smaller than expected.

In this study, we also investigated the immunohistochemical expression of MRP1 in the lungs of patients with asthma and in a murine allergic airway inflammation model. MRP1 staining was observed in the cytoplasm and on the plasma membrane of the mast cells, and its expression was also found on macrophages, eosinophils, and bronchial epithelial cells (data not shown). These findings were consistent with prior reports (10), and these cells contain the 5-LO/FLAP/LTC4S pathway and generate LTC4 (16, 23). Among them, the mast cell is the most potent IgE-mediated LTC4-synthesizing cell in allergic airway inflammation and expresses MRP1 in human and murine allergic airway disease. However, eosinophils, macrophages, and bronchial epithelial cells are also important sources of CysLTs. We would like to perform in vitro experiments for CysLT export from eosinophils, macrophages, and

bronchial epithelial cells of *mrp1*$^{-/-}$ mice in a future project. In this study, we focused on the involvement of MRP1 in IgE-dependent CysLT export from mast cells and confirmed that MRP1 plays an important role in the IgE-dependent release of CysLTs from mast cells by using murine BMMCs from *mrp1*$^{-/-}$ mice. However, decreases in CysLT levels in BALF from *mrp1*$^{-/-}$ mice in vivo were <50% compared with those in *mrp1*$^{+/+}$ mice, although the difference was statistically significant ($P = 0.0082$). We speculate that residual CysLT production in *mrp1*$^{-/-}$ mice may be due to another export pump and/or derived from eosinophils, macrophages, and bronchial epithelial cells. However, it is thought that MRP1 is at least one of the important transporters on mast cells for LTC4 export in the pathogenesis of allergic airway inflammation, although other transporters may exist, because differences in data between *mrp1*$^{-/-}$ and *mrp1*$^{+/+}$ mice were statistically significant.

There are a few interesting reports of studies that investigated the association between MRP1 and anti-asthma drugs for patients with bronchial asthma. Bandi et al. (1) incubated the human airway epithelial cell line Calu-1 with budesonide, an anti-asthma corticosteroid, and revealed that treatment with budesonide significantly inhibits MRP1 expression and activity in Calu-1 cells. MRP1 has been screened for genetic variations, and several mutations have been identified in the MRP1 gene in the human population (21). Montelukast is a selective $CysLT_1$ receptor antagonist that is clinically used as an anti-asthma drug. Interestingly, genetic variations in MRP1 are associated with variability in montelukast response in patients with asthma (21). These previous studies suggest that MRP1 polymorphism may be useful as a predictive marker for the efficacy of therapy in the management of asthma.

In conclusion, our study revealed that airway inflammation and goblet cell hyperplasia after OVA exposure were reduced in *mrp1*$^{-/-}$ mice compared with *mrp1*$^{+/+}$ mice. Levels of CysLTs, antigen-specific IgE, IL-4, and IL-13 in BALF from OVA-exposed *mrp1*$^{-/-}$ mice were significantly lower than those from OVA-exposed *mrp1*$^{+/+}$ mice. Export of IgE-dependent CysLTs from murine BMMCs was mediated by MRP1. On the basis of these findings, MRP1 expressed on mast cells functions as a CysLT export pump in the development of allergic airway disease. These findings also suggest the possibility that MRP1 may be one of the important therapeutic targets and provide new insights for understanding its role in allergic asthma.

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GRANT

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ORIGINAL ARTICLE

Aberrant expression of Fra-2 promotes CCR4 expression and cell proliferation in adult T-cell leukemia

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Adult T-cell leukemia (ATL) is a mature CD4$^+$ T-cell malignancy etiologically associated with human T-cell leukemia virus type 1 (HTLV-1). Primary ATL cells frequently express CCR4 at high levels. Since HTLV-1 Tax does not induce CCR4 expression, transcription factor(s) constitutively active in ATL may be responsible for its strong expression. We identified an activator protein-1 (AP-1) site in the CCR4 promoter as the major positive regulatory element in ATL cells. Among the AP-1 family members, Fra-2, JunB and JunD are highly expressed in fresh primary ATL cells. Consistently, the Fra-2/JunB and Fra-2/JunD heterodimers strongly activated the CCR4 promoter in Jurkat cells. Furthermore, Fra-2 small interfering RNA (siRNA) or JunD siRNA, but not JunB siRNA, effectively reduced CCR4 expression and cell growth in ATL cells. Conversely, Fra-2 or JunD overexpression promoted cell growth in Jurkat cells. We identified 49 genes, including c-Myb, BCL-6 and MDM2, which were downregulated by Fra-2 siRNA in ATL cells. c-Myb, BCL-6 and MDM2 were also downregulated by JunD siRNA. As Fra-2, these proto-oncogenes were highly expressed in primary ATL cells but not in normal CD4$^+$ T cells. Collectively, aberrantly expressed Fra-2 in association with JunD may play a major role in CCR4 expression and oncogenesis in ATL.

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Keywords: adult T-cell leukemia; CCR4; Fra-2; JunD; c-Myb; MDM2; BCL-6

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Introduction

Adult T-cell leukemia (ATL) is a highly aggressive malignancy of mature CD4$^+$CD25$^+$ T cells etiologically associated with human T-cell leukemia virus type 1 (HTLV-1; Yamamoto and Hinuma, 1985). HTLV-1 encodes a potent viral transactivator Tax that activates the HTLV-1 long terminal repeat (LTR) and also induces the expression of various cellular target genes, including those encoding cytokines, cytokine receptors, chemokines, cell adhesion molecules and nuclear transcriptional factors, collectively leading to the strong promotion of cell proliferation (Yoshida, 2001; Grassmann *et al.*, 2005). However, ATL develops after a long period of latency, usually several decades, during which oncogenic progression is considered to occur through the accumulation of multiple genetic and epigenetic changes (Matsuoka, 2003). Furthermore, circulating ATL cells usually do not express Tax and are considered to be independent of Tax (Matsuoka, 2003). Previously, Mori *et al.* have demonstrated the strong constitutive activation of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) in primary ATL cells (Mori *et al.*, 1999, 2000). However, the molecular mechanisms of ATL oncogenesis still remain largely unknown.

CCR4 is a chemokine receptor known to be selectively expressed by Th2 cells, regulatory T cells (Treg) and skin-homing effector/memory T cells (Imai *et al.*, 1999; Iellem *et al.*, 2001; Yoshie *et al.*, 2001). Previously, we and others showed that ATL cells in the majority of cases are strongly positive for surface CCR4 (Yoshie *et al.*, 2002; Ishida *et al.*, 2003; Nagakubo *et al.*, 2007). Ishida *et al.* have also demonstrated a significant correlation of CCR4 expression with skin involvement and poor prognosis in ATL patients (Ishida *et al.*, 2003). Furthermore, several groups have reported that FOXP3, a forkhead/winged helix transcription factor and a specific marker of Treg (Hori *et al.*, 2003), is frequently expressed in ATL (Karube *et al.*, 2004; Matsubara *et al.*, 2005), supporting the notion that at least a fraction of ATL cases are derived from Treg.

It is also notable that primary ATL cells express CCR4 at levels much higher than normal resting CD4$^+$CD25$^+$ T cells (Nagakubo *et al.*, 2007). Given

that CCR4 is not inducible by Tax (Yoshie *et al.*, 2002), transcription factor(s) constitutively active in ATL cells may be responsible for CCR4 expression. Here, we demonstrate that Fra-2, one of the AP-1 family members (Shaulian and Karin, 2002; Eferl and Wagner, 2003), is aberrantly expressed in primary ATL cells. We further demonstrate that the Fra-2/JunD heterodimer plays a major role in both CCR4 expression and cell proliferation in ATL cells. Furthermore, we demonstrate that the proto-oncogenes c-Myb, BCL-6 and MDM2 (Oh and Reddy, 1999; Pasqualucci *et al.*, 2003; Vargas *et al.*, 2003) are the downstream target genes of the Fra-2/JunD heterodimer and are highly expressed in primary ATL cells. Thus, aberrantly expressed Fra-2 in association with JunD may be involved in ATL oncogenesis.

Results

Analysis of CCR4 promoter activity in ATL-derived cell lines

To examine the transcriptional regulation of CCR4 expression in ATL, we constructed a reporter plasmid carrying the CCR4 promoter region from −983 to +25 bp (the major transcriptional initiation site, +1) fused with the luciferase reporter gene. As shown in Figure 1a, pGL3-CCR4 (−983/+25) showed much stronger promoter activities in ATL cell lines (HUT102 and ST1) than in control human T-cell lines (MOLT-4 and Jurkat). We therefore generated a series of 5′-truncated promoter plasmids and examined their activity in ATL cell lines. As shown in Figure 1b, the promoter region from −151 to −96 bp was the major positive regulatory region in both cell lines. The TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) revealed various potential transcriptional elements in this region (Figure 1c). To identify the actual regulatory elements, we introduced a mutation in each potential element and examined the promoter activity in ATL cell lines. As shown in Figure 1d, a mutation at the AP-1 site or the GATA-3 site significantly reduced the promoter activity. Moreover, double mutations targeting both sites further reduced the promoter activity.

Constitutive expression of Fra-2, JunB and JunD in primary ATL cells

AP-1 is known to be involved in tumorigenesis (Shaulian and Karin, 2002; Eferl and Wagner, 2003), while GATA-3 regulates Th2-type gene expression (Rengarajan *et al.*, 2000). Therefore, we focused on AP-1 in the subsequent study. AP-1 constitutes a heterodimer of a member of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) and a member of the Jun family (c-Jun, JunB and JunD) or a homodimer of the Jun family (Shaulian and Karin, 2002; Eferl and Wagner, 2003). Even though AP-1 was shown to be constitutively active in primary ATL cells (Mori *et al.*, 2000), it has not been clarified which members of AP-1 are actually

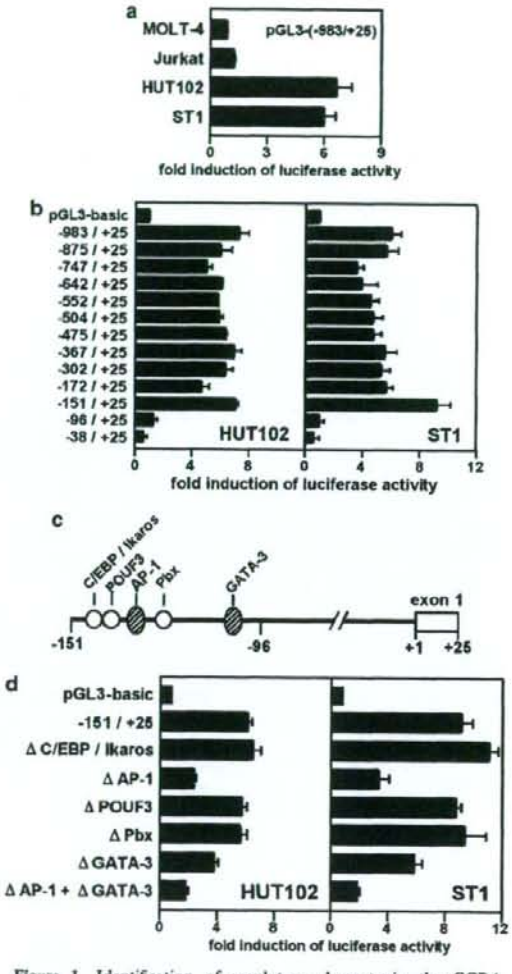

Figure 1 Identification of regulatory elements in the CCR4 promoter. Cells were transfected with pSV-β-galactosidase and pGL3-basic or pGL3-basic inserted with the CCR4 promoter regions as indicated. After 24–27 h, luciferase assays were performed. Promoter activation was expressed by the fold induction of luciferase activity in cells transfected with the CCR4 promoter–luciferase constructs versus cells transfected with the control pGL3-basic. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. (**a**) Selective activation of the CCR4 promoter in adult T-cell leukemia (ATL) cell lines. MOLT-4 and Jurkat: control human T-cell lines; HUT102 and ST1: ATL cell lines. (**b**) Deletion analysis. The promoter region from −151 to −96 bp is necessary and sufficient for reporter gene expression in the two ATL cell lines. (**c**) The schematic depiction of potential regulatory elements in the promoter region from −151 to −96 bp. (**d**) Mutation analysis. ΔC/EBP/Ikaros (from TCTTGGGAAA TGA to TCTTGCAAAATGA), ΔAP-1 (from AATGACTAAGA to AATGTCAAAGA), ΔPOUF3 (from CTTGGGAAATGA to CTTGGGAGGTGA), ΔPbx (from AAGAATCAT to AAGA CCCAT) and ΔGATA-3 (from TTCTATCAA to TTCTGACAA). The potential AP-1 and GATA-3 sites present within the −151 to −96 bp region are the major elements for CCR4 promoter activation in the two ATL cell lines.

expressed in primary ATL cells. We therefore first examined the mRNA expression of the AP-1 family members in primary ATL cells freshly isolated from patients in comparison with normal CD4+ T cells in resting, activated and Th1/Th2-polarized conditions (Figure 2a). As reported previously (Yoshie *et al*., 2002; Nagakubo *et al*., 2007), primary ATL cells consistently expressed CCR4 at levels much higher than various normal CD4+ T-cell populations, including Th2-polarized cultured T cells. Furthermore, primary ATL cells consistently expressed Fra-2 in sharp contrast to various normal CD4+ T-cell populations that were essentially negative for Fra-2 expression. Similar to various normal CD4+ T-cell populations, primary ATL

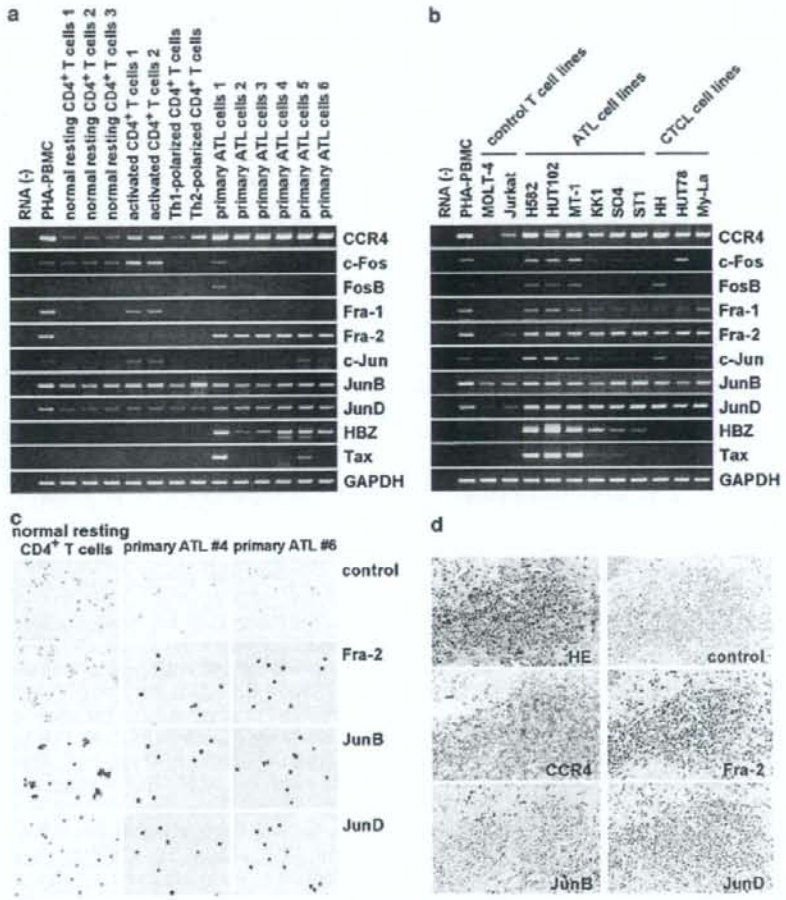

Figure 2 Constitutive expression of Fra-2, JunB and JunD in adult T-cell leukemia (ATL). (**a**) Reverse transcription (RT)–PCR analysis for the expression of the AP-1 family in normal T cells and primary ATL cells. Normal resting CD4+ T cells (purity, >96%) from healthy donors ($n=3$), activated CD4+ T cells from normal donors ($n=2$), Th1-polarized cultured CD4+ T cells, Th2-polarized cultured CD4+ T cells and freshly isolated primary ATL cells (>90% leukemic cells) from patients ($n=6$) were examined as indicated. Normal peripheral blood mononuclear cells treated with phytohemagglutinin (PHA-PBMC) served as a positive control. GAPDH served as a loading control. The representative results from at least two separate experiments are shown. (**b**) RT–PCR analysis for the expression of the AP-1 family in human T-cell lines. Two control human T-cell lines, six ATL cell lines and three CTCL cell lines were examined as indicated. PHA-PBMC served as a positive control. GAPDH served as a loading control. The representative results from two separate experiments are shown. (**c**) Immunocytochemical staining for Fra-2, JunB and JunD in normal CD4+ T cells and primary ATL cells. Normal CD4+ T cells from healthy donors (purity, >96%) and primary ATL cells (leukemic cells, >90%) from two patients were stained with anti-Fra-2, anti-JunB or anti-JunD. Normal rabbit IgG was used as the negative control (control). The representative results from two separate experiments are shown. Original magnification: ×400. (**d**) Immunohistochemical staining of CCR4, Fra-2, JunB and JunD in ATL skin lesions. Tissue sections from ATL skin lesions ($n=6$) were stained with anti-CCR4, anti-Fra-2, anti-JunB or anti-JunD. Mouse IgG_1 and normal rabbit IgG were used as the negative controls (control). Tissue sections were counterstained using Gill's hematoxylin. The representative results from a single donor are shown. Original magnification: ×400.

cells also constitutively expressed JunD and JunB even though JunD expression appeared to be upregulated in primary ATL cells. Other members of the AP-1 family were mostly negative in primary ATL cells, while activated normal CD4+ T cells expressed c-Fos, Fra-1 and c-Jun at high levels. There was no correlation in expression between Fra-2 and the virally encoded HTLV-1 basic leucine zipper factor HBZ or Tax in primary ATL cells. We also confirmed that Fra-2 is not inducible by Tax using JPX-9, a subline of Jurkat carrying the HTLV-1 Tax gene under the control of the metallothionein gene promoter (Nagata *et al.*, 1989; data not shown). Thus, the constitutive expression of Fra-2 is highly unique for primary ATL cells.

We also examined expression of the same set of genes in various human T-cell lines. As shown in Figure 2b, compared to control T-cell lines, ATL cell lines consistently expressed CCR4 and Fra-2 at high levels. ATL cell lines also expressed JunB and JunD at high levels. HTLV-1 Tax has been shown to induce various AP-1 family members (Nagata *et al.*, 1989; Iwai *et al.*, 2001), which may be involved in HTLV-1 gene expression and cell proliferation (Jeang *et al.*, 1991). Consistently, ATL cell lines expressing Tax (H582, HUT102 and MT-1) also expressed other AP-1 family members at low levels. Cutaneous T-cell lymphomas (CTCLs) are a subset of HTLV-1-negative T-cell lymphomas resembling ATL and known to be frequently positive for CCR4 (Kim *et al.*, 2005). CTCL cell lines were also found to strongly express CCR4, Fra-2, JunB and JunD. Thus, the constitutive expressions of Fra-2, JunB and JunD were shared by CCR4-expressing ATL and CTCL cell lines.

We also examined the Fra-2, JunB and JunD protein expression in freshly isolated primary ATL cells and normal resting CD4+ T cells. As shown in Figure 2c, primary ATL cells were indeed stained strongly positive for Fra-2, while normal CD4+ T cells were totally negative for Fra-2. Primary ATL cells were also strongly positive for JunB and JunD, while normal CD4+ T cells were variably positive for JunB and JunD at the single cell level. These results were highly consistent with the results from reverse transcription (RT)–PCR; Figure 2a). We also confirmed the CCR4, Fra-2, JunB and JunD protein expression in skin-infiltrating ATL cells (Figure 2d).

Activation of the CCR4 promoter by Fra-2/JunB and Fra-2/JunD heterodimers

AP-1 is known to function as a heterodimer of a member of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) and a member of the Jun family (c-Jun, JunB and JunD) or a homodimer of the Jun family (Shaulian and Karin, 2002; Eferl and Wagner, 2003). We, therefore, next examined the activation of the CCR4 promoter by individual AP-1 family members singly or in combination. As recipients, we used two T-cell lines, namely, MOLT-4 and Jurkat. The expression levels of AP-1 members, including Fra-2, JunB and JunD, were very low in these cell lines (Figure 2b). As shown in Figure 3a, only Fra-2/JunB or Fra-2/JunD potently activated the CCR4 promoter in both cell lines. We confirmed that other members of the AP-1 family (c-Fos, FosB, Fra-1 and c-Jun) were transcriptionally active by using a synthetic promoter containing two tandem AP-1 consensus-binding sites (pGL3-2xAP-1; Figure 3b). Thus, among the AP-1 family members, only the Fra-2/JunB and Fra-2/JunD heterodimers are uniquely capable of activating the CCR4 promoter. This is highly consistent with their constitutive expression in primary ATL cells (Figure 2a).

Recently, the mRNA of HTLV-1 HBZ has been shown to be expressed in primary ATL cells (Satou *et al.*, 2006). We indeed observed the expression of HBZ in some primary ATL samples (Figure 2a). HBZ has been shown to activate JunB homodimer- or JunD homodimer-dependent transcription (Basbous *et al.*, 2003; Thebault *et al.*, 2004). Therefore, we also examined the effects of HBZ as well as Tax on the CCR4 promoter in MOLT-4 and Jurkat cells. As shown in Figure 3c, HBZ alone or in combination with Fra-2, JunB, JunD, Fra-2/JunB or Fra-2/JunD showed no effect on the activation of the CCR4 promoter. Similarly, Tax had no significant effect on the CCR4 promoter either alone or in combination with Fra-2, JunB, JunD, Fra-2/JunB or Fra-2/JunD. Thus, HTLV-1 encoded HBZ or Tax neither activates the CCR4 promoter nor affects its activation by Fra-2/JunB or Fra-2/JunD.

We have also confirmed that GATA-3 is constitutively expressed in primary ATL cells and activates the CCR4 promoter (data not shown). In normal CD4+ T cells, GATA-3 may be responsible for the selective expression of CCR4 in Th2 cells (Imai *et al.*, 1999; Rengarajan *et al.*, 2000).

Specific binding of Fra-2, JunB and JunD to the AP-1 site in the CCR4 promoter

We next examined the specific binding of AP-1 family members to the AP-1 site in the CCR4 promoter using the NoShift transcription factor assay, an enzyme-linked immunosorbent assay (ELISA)-like colorimetric assay that is an alternative to the electrophoretic mobility shift assay. As shown in Figure 4a, when the nuclear extracts of two control T-cell lines (MOLT-4 and Jurkat) were used, the specific binding of any AP-1 family members to the AP-1 site of the CCR4 promoter was hardly observed. On the other hand, when the nuclear extracts of two ATL cell lines (HUT102 and ST1) were used, we detected a high level of specific binding of Fra-2, JunB and JunD to the AP-1 site. These results are highly consistent with the results from RT–PCR analyses (Figure 2b) and the luciferase reporter assays (Figure 3a).

By using the chromatin immunoprecipitation (ChIP) assay, we further examined the binding of Fra-2, JunB and JunD to the AP-1 site of the CCR4 promoter *in vivo*. As shown in Figure 4b, we detected specific binding of Fra-2, JunB and JunD to the AP-1 site of the endogenous CCR4 promoter in primary ATL cells but not in normal CD4+ T cells. These results further

support the hypothesis that the CCR4 gene is a direct target gene of Fra-2/JunB and Fra-2/JunD heterodimers in primary ATL cells.

Effects of Fra-2, JunB and JunD small interfering RNAs on CCR4 expression and cell proliferation

To examine the role of Fra-2, JunB and JunD in CCR4 expression and cell proliferation in ATL cells, we next employed the small interfering RNA (siRNA) knockdown technique. As shown in Figure 5a, Fra-2 siRNA, JunB siRNA and JunD siRNA specifically reduced Fra-2 mRNA, JunB mRNA and JunD mRNA, respectively, in two ATL cell lines. On the other hand, control siRNA showed no such effect. Under these conditions, we examined the effects of these siRNAs on CCR4 expression and cell growth. As shown in Figure 5b, Fra-2 siRNA and JunD siRNA reduced CCR4 expression by approximately 50% in both cell lines, whereas JunB siRNA had hardly any inhibitory effect and control siRNA showed no inhibitory effect. Furthermore, as shown in Figure 5c, Fra-2 siRNA and JunD siRNA significantly reduced cell proliferation in both cell lines, whereas JunB siRNA or control siRNA did not. None of the siRNAs affected the growth of the control T-cell lines MOLT-4 and Jurkat. We also compared the effects of single and double knockdown of Fra-2 and JunD on cell growth in two ATL cell lines (Figure 5d). Compared to the effect of single knockdown of Fra-2 or JunD, no additive effect was observed by double knockdown of Fra-2 and JunD in both cell lines. These results may be consistent with the notion that Fra-2 and JunD promote growth in ATL cell lines by functioning as a heterodimer.

To further demonstrate the growth-promoting effects of Fra-2 and JunD, we performed stable transfection of Fra-2 and JunD in the control T-cell line Jurkat. As shown in Figure 5e, Jurkat cells overexpressing Fra-2 or JunD (see inset) indeed showed enhanced growth compared to those transfected with the vector alone. We were, however, unable to isolate Fra-2/JunD double transfectants in Jurkat, probably because of some adverse effects on Jurkat cells by the overexpression of both Fra-2 and JunD.

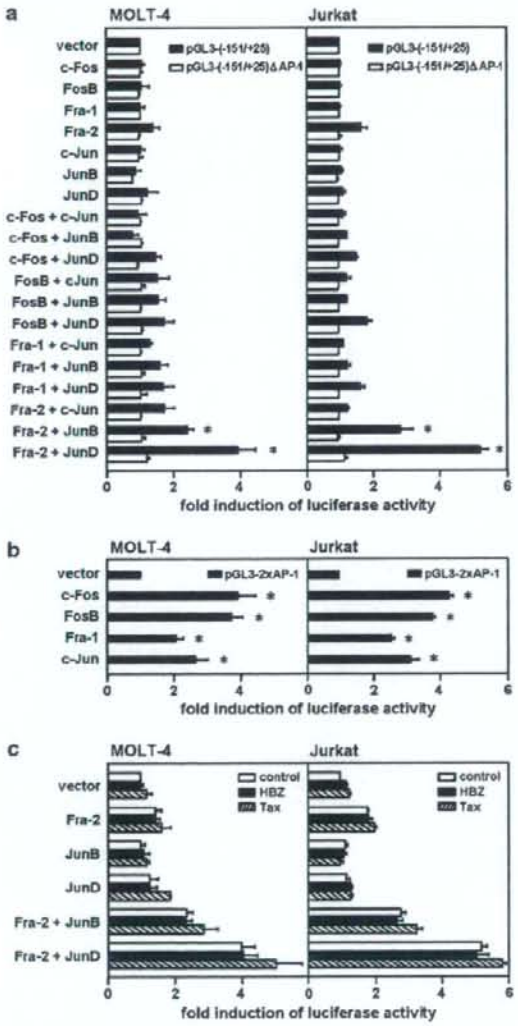

Figure 3 Transactivation of the CCR4 promoter by Fra-2/JunD and Fra-2/JunB. (**a**) Transactivation of the CCR4 promoter with or without the AP-1 site. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and pGL3-CCR4 (−151/+25) or pGL3-CCR4 (−151/+25)ΔAP-1 and an expression vector for c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, JunD or a control vector as indicated. After 24–27 h, luciferase assays were performed in triplicate. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated AP-1 expression vector versus cells transfected with the vector alone. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. *$P<0.05$. (**b**) Transactivation of a synthetic promoter with two copies of the consensus AP-1 site. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and pGL3-2xAP-1 and an expression vector for c-Fos, FosB, Fra-1, c-Jun or the vector alone as indicated. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated expression vector versus cells transfected with a control vector. After 24–27 h, luciferase assays were performed in triplicate. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments. *$P<0.05$. (**c**) Effect of HBZ or Tax on the activation of the CCR4 promoter. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and the pGL3-basic vector or pGL3-CCR4 (−151/+25) and an expression vector for Fra-2, JunB, JunD or a control vector and an expression vector for HBZ, Tax or a control vector as indicated. After 24–27 h, luciferase assays were performed in triplicate. Promoter activation was expressed as the fold induction of luciferase activity in cells transfected with an indicated expression vector versus cells transfected with a control vector. Transfection efficiency was normalized by β-galactosidase activity. Each bar represents the mean ± s.e.m. from three separate experiments.