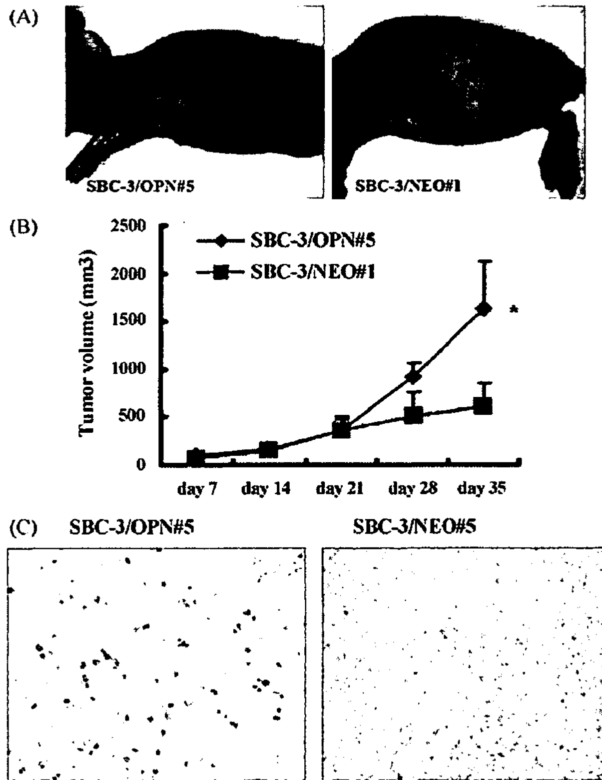

Fig. 3 Effect of OPN gene transfer into SBC-3 cells on tumor growth in mice. The SBC-3/OPN#5 and SBC-3/NEO#1 cells were inoculated s.c. into the left flanks of nude mice. (A) Representative photographs of the tumors at day 35 after inoculation with either the SBC-3/OPN#5 cells or the SBC-3/NEO#1 cells. (B) Tumors were measured with a digital caliper in two perpendicular diameters every week. The tumor volumes were calculated as described in Section 2. Each group consisted of 10 mice. $^{*}P<0.05$ vs. SBC-3/NEO#1. (C) Representative sections of OPN expression in tumors derived from SBC-3/OPN and SBC-3/NEO. Cryostat sections of tumors developing in nude mice were stained with anti-mouse OPN monoclonal antibody (original magnification ×400).

positive vascular endothelial cells was markedly increased in the SBC-3/OPN#5 induced tumor compared to that of the SBC-3/NEO#1 induced tumor. As shown in Fig. 4B, greater than tenfold the number of microvessels was identified in the SBC-3/OPN#5 induced tumor compared with the SBC-3/NEO#1 induced tumor. These results strongly imply that OPN upregulates tumor angiogenesis of SBC-3 cells in mice.

3.8. Effect of OPN transfection on tumor cell apoptosis

We evaluated whether transfection with OPN gene affects tumor cell apoptosis of SBC-3 cells in vivo with immunohistochemical staining for ssDNA. As shown in Fig. 4C, the number of apoptotic cells in the SBC-3/OPN induced tumor was not significantly different from that of the SBC-3/NEO induced tumor. These results suggest the apoptosis of SBC-3 cells in vivo was not affected by transfection with the OPN gene.

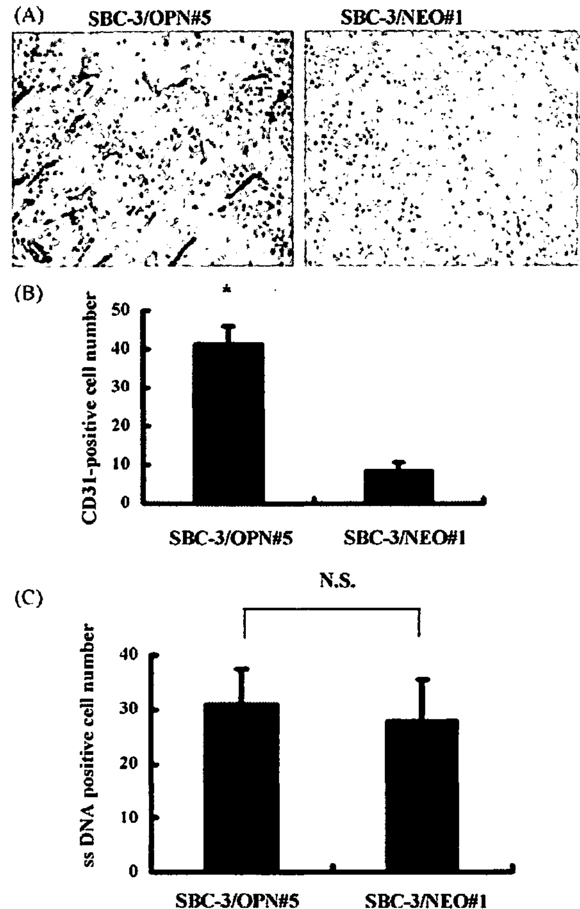

Fig. 4 (A and B) Vascularization of tumors derived from SBC-3/OPN#5 and SBC-3/NEO#1 cells. Cryostat sections of tumors developing in nude mice were stained with anti-CD31 monoclonal antibody. (A) Representative sections were depicted (×200). (B) Quantification of microvessel density in tumors. The number of CD31-positive microvessels in five fields of tumors that demonstrated the highest vascularity was counted at ×200 and presented as mean ± S.D. $^{*}P<0.001$ vs. SBC-3/NEO#1. (C) Quantification of ss DNA staining in SBC-3/OPN and SBC-3/NEO cells developed in nude mice. The number of ss DNA positive cells in SBC-3/OPN#5 tumor was not significantly different from that of SBC-3/NEO#1 tumor.

3.9. Effect of OPN on in vitro HUVEC proliferation

The endothelial cell proliferation is essential for tumor angiogenesis. Therefore, we performed HUVEC proliferation assay using recombinant mouse OPN protein. As shown in Fig. 5, immobilized OPN significantly stimulated HUVEC proliferation compared with immobilized polylysine and BSA. Interestingly, this enhanced HUVEC proliferation mediated by immobilized OPN was significantly inhibited with the addition of anti-human $\alpha v\beta 3$ antibody or GRGDS peptide. These results are consistent with our finding that migration of HUVEC to OPN was mediated by $\alpha v\beta 3$ integrin as shown in Fig. 2. Taken together, these findings imply the interaction between OPN and $\alpha v\beta 3$ integrins on vascular endothelial cells may play an important role in tumor angiogenesis.

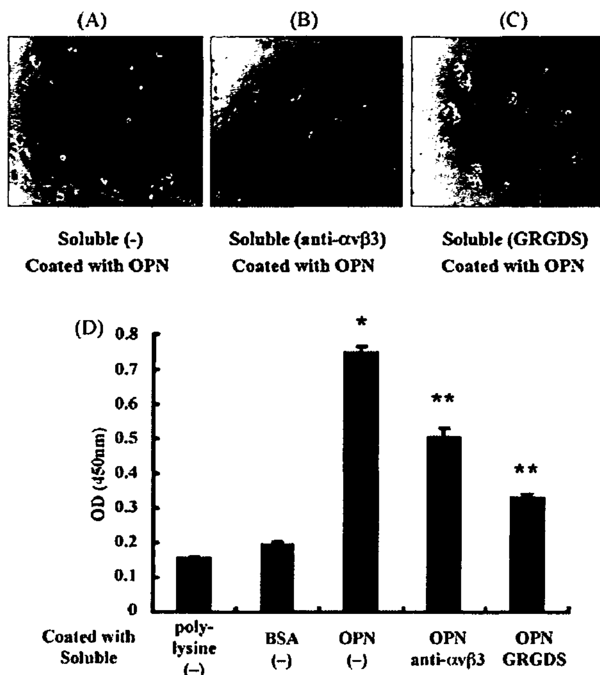

Fig. 5 Inhibitory effect of anti-αvβ3 antibody or RGD peptide on HUVEC proliferation mediated by OPN. (A—C) Representative microphotographs were depicted (×100). (D) Immobilized OPN significantly enhanced HUVEC proliferation and this enhancement was markedly suppressed by treatment with anti-αvβ3 antibody or RGD peptide. Data are presented as mean ± S.D. *$P<0.0001$ vs. coated with BSA, soluble (−); **$P<0.001$ vs. coated with OPN, soluble (−).

3.10. Effect of RMV-7 antibody or TNP-470 on growth of SBC-3/OPN tumor in vivo

Since the SBC-3/OPN#5 induced tumors revealed strong neovascularization and tumor growth, the SBC-3/OPN#5 induced tumors were treated with RMV-7 or anti-angiogenic agent, TNP-470, to investigate whether the accelerated SBC-3/OPN#5 tumor growth *in vivo* was directly associated with neovascularization mediated by the interaction between OPN and its receptor, αvβ3 integrin. As shown in Table 1, TNP-470 and RMV-7 administration significantly reduced *in vivo* tumor growth against SBC-3/OPN#5 cells with growth-inhibitory ratio (GIR) values (%) of 83.8% and 85.6%, respectively. In contrast to strong antitumor activity against SBC-3/OPN tumor, RMV-7 did not reveal any antitumor activity against the SBC-3/NEO tumor. These results suggest that the abrogation of the interaction between OPN and αvβ3 integrin could be an effective therapeutic modality in OPN-overexpressing lung cancer.

4. Discussion

OPN is a secreted multifunctional glycosylated phosphoprotein that is involved in tumor progression and metastasis through interaction with adhesion molecules such as integrins αvβ3, αvβ5, and αvβ1, and CD44 variants in a RGD sequence dependent or independent manner [24,25]. Angiogenesis plays a central role in the growth and metastasis of various cancers. The endothelial cell migration is dependent on their adhesive to extracellular matrix protein such as OPN through a variety of cell adhesion receptor including αvβ3 integrins [26]. It has been reported that overexpression of the αvβ3 integrin on tumor vasculature is associated with an aggressive phenotype of several solid tumor types [27,28]. Recent clinical studies also revealed that OPN, a ligand for αvβ3, overexpression is associated with tumor progression and poor survival of patients with lung cancer [17,18].

Table 1 Antitumor activity of RMV-7 or TNP-470 against SBC-3/OPN and SBC-3/NEO inoculated into nude mice

Cell line	Agent	Tumor volume (mm^3)	GIR (%)
SBC-3/OPN#5	TNP-470 (−)	506.9 ± 246.28	83.8
	TNP-470 (+)[b]	81.79 ± 34.4*	
	RMV-7 (−)	2272.45 ± 1126.73	85.6
	RMV-7 (+)[a]	326.35 ± 157.18**	
SBC-3/NEO#1	TNP-470 (−)	126.7 ± 27.98	27.1
	TNP-470 (+)[b]	92.36 ± 12.64	
	RMV-7 (−)	464.76 ± 167.49	3.6
	RMV-7 (+)[a]	448.17 ± 177.68	

Antitumor activity was evaluated in term of growth-inhibitory ratio (GIR, %), defined as [1 − (mean tumor volume of treated/mean tumor volume of control)] × 100 at day 32[a] after the first administration of RMV-7 or day 28[b] after the first administration of TNP-470. Data are presented as mean ± S.D.
* $P<0.05$ vs. TNP-470 (−).
** $P<0.05$ vs. RMV-7 (−).

In this study, we conducted *in vivo* tumorigenicity experiments using human lung cancer cell line, SBC-3 cells, to reveal whether interaction between OPN and its receptor αvβ3 plays a key role in tumor growth mediated by angiogenesis. The SBC-3 cell line was originally established from bone marrow aspirate of the 24-year-old male patient with small cell lung cancer [29]. Its subcutaneous implantability has been approved by Fukumoto et al. [30]. OPN-overexpressing SBC-3 cells significantly enhanced *in vivo* tumor growth compared to the control cells. Interestingly, *in vitro* cell growth rate and VEGF mRNA expression levels were similar among these cells. In contrast, transfection of SBC-3 cells with OPN gene significantly induced neovascularization *in vivo*. Apoptosis of SBC-3 cells *in vivo* and colony formation of SBC-3 cells *in vitro* were not affected by transfection with the OPN gene. These results imply that promotion of the tumor growth of SBC-3/OPN cells *in vivo* may be attributed to the hypervascularization induced by secreted OPN. In fact, recombinant human OPN protein enhanced HUVEC proliferation *in vitro*, and these effects of OPN were significantly suppressed with the addition of anti-αvβ3 integrin monoclonal antibody or RGD peptide. These results suggest that OPN is implicated in the process of angiogenesis by interacting with the αvβ3 integrin. In addition, we performed *in vivo* experiment to evaluate the metastatic effect of OPN. The cell suspensions of SBC-3/OPN or SBC-3/NEO cells were injected into a lateral tail vein of BALB/c nude mice. Unfortunately, we did not observe metastatic colonies in lungs. Although liver and kidney metastasis were observed, there

was no significant difference in the number of metastatic colonies in livers and kidneys between in SBC-3/OPN and SBC-3/NEO injected mice (data not shown).

The sustained growth of solid tumors is dependent on the vascular network, making tumor blood vessels a potential therapeutic target [3]. Since previous reports confirmed that OPN plays an important role in tumor progression and metastasis, various therapeutical trials targeting the interaction between OPN and its receptors have been proposed. Thalmann et al. reported that anti-OPN antibody inhibits the growth stimulatory effect of endogenous OPN for human prostate carcinoma cells [31]. In addition, a murine anti-human OPN antibody, which recognizes the RGD/thrombin cleavage region, inhibits the adhesion of MDA-MB-435 breast cancer cells to OPN [32]. Recent trials have used the siRNA technique to knock down OPN mRNA expression. Shevde et al. have demonstrated that suppression of OPN mRNA with siRNA reduced tumorigenecity of MDA-MB-435 breast cancer cells [33]. In addition, Wai et al. revealed that inhibition of OPN mRNA reduced metastatic potential in murine colon carcinoma cells [34]. Regarding anti-OPN receptor antibodies, Brooks et al. have reported that monoclonal antibody (LM609) against $\alpha v\beta 3$ integrin induces apoptosis of the proliferative angiogenic blood vessel cells and leads to tumor regression in breast cancer [35]. However, there are no studies with regard to the therapeutic trials targeting OPN and its receptor in lung cancer animal models.

In the present study, we evaluated therapeutic efficacy of anti-$\alpha v\beta 3$ integrin antibody (RMV-7) in OPN-overexpressing human lung cancer cells inoculated mice model. Treatment of mice with RMV-7 completely suppressed the *in vivo* tumor growth of SBC-3/OPN with GIR value of 85.6%, while growth rate of SBC-3/NEO *in vivo* was not attenuated by treatment with RMV-7. In the same way, anti-angiogenic agent, TNP-470, exhibited strong anti-tumor activity against SBC-3/OPN tumor with GIR value of 83.8%. These results suggest that interaction between OPN and $\alpha v\beta 3$ integrin plays a crucial role for tumor growth induced by up-regulated angiogenesis of human lung cancer cells in mice and anti-$\alpha v\beta 3$ antibody could be useful in anti-angiogenic treatment of human lung cancer.

Phase I study using vitaxin (humanized monoclonal anti-$\alpha v\beta 3$ integrin antibody) has demonstrated its safety and potential activity in some human cancers. This study revealed that one patient demonstrated partial response and seven patients exhibited stable disease course among the 14 patients evaluated [36]. Recently, McNeel et al. reported phase I trial of a monoclonal antibody specific for $\alpha v\beta 3$ integrin (MEDI-522) in patient with advanced multiple malignancies including lung cancer [37]. In their study, three patients with renal carcinoma demonstrated a prolonged and stable disease course among the 25 patients investigated. However, none of the patients with lung cancer revealed favorable therapeutic response. According to our previous report, OPN is predominantly expressed in NSCLC, but its expression level is variable [38]. In both phase I trials, they did not mention the issue of OPN expression in NSCLC. The reason why none of the patients with NSCLC revealed any response to treatment with anti-$\alpha v\beta 3$ antibody might have been due to the low expression of OPN in NSCLC cells in these patients. In fact, administration of RMV-7 antibody did not reduce *in vivo* tumor growth in SBC-3/NEO cells inoculated mice in our study. These results suggest that intratumoral OPN expression could be a surrogate marker in the prediction of therapeutic response for treatment with anti-$\alpha v\beta 3$ integrin antibody in lung cancer.

Conclusively, our study revealed that OPN is involved in tumor growth and angiogenesis of lung cancer by up-regulating vascular endothelial cell migration and proliferation via interacting with $\alpha v\beta 3$ integrin. OPN and its receptor could be effective target molecules in the future for anti-angiogenic therapy of patients with lung cancer.

Conflict of interest

None.

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Matuzumab and cetuximab activate the epidermal growth factor receptor but fail to trigger downstream signaling by Akt or Erk

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Molecular inhibition of the epidermal growth factor receptor (EGFR) is a promising anticancer strategy, and monoclonal antibodies (mAbs) to EGFR are undergoing extensive evaluation in preclinical and clinical trials. However, the effects of anti-EGFR mAbs on EGFR signaling have remained unclear. We have now examined the effects of 2 anti-EGFR mAbs, matuzumab (EMD72000) and cetuximab (Erbitux), both of which are currently under assessment for treatment of various cancers, on EGFR signal transduction and cell survival in nonsmall cell lung cancer cell lines. Similar to EGF, matuzumab and cetuximab each induced phosphorylation of EGFR at several tyrosine phosphorylation sites as a result of receptor dimerization and activation of the receptor tyrosine kinase. In contrast to the effects of EGF, however, EGFR activation induced by these antibodies was not accompanied by receptor turnover or by activation of downstream signaling pathways that are mediated by Akt and Erk and are important for regulation of cell proliferation and survival. In addition, clonogenic survival assays revealed that matuzumab and cetuximab reduced the survival rate of H292 cells, in which they also inhibited the EGF-induced activation of Akt and Erk. Although we have examined only a few cell lines, our results indicate that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling mediated by Akt or Erk rather than on inhibition of EGFR itself.
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Key words: EGF receptor; signal transduction; matuzumab; cetuximab; nonsmall cell lung cancer

The epidermal growth factor receptor (EGFR, also known as ErbB1), a member of the ErbB family of receptor tyrosine kinases, is a 170-kDa plasma membrane glycoprotein composed of an extracellular ligand binding domain, a transmembrane region and an intracellular tyrosine kinase domain with a regulatory COOH-terminal segment.[1] Binding of ligand to EGFR induces receptor dimerization, activation of the receptor kinase and autophosphorylation of specific tyrosine residues within the COOH-terminal region of the protein.[1] These events trigger intracellular signaling pathways that promote cell proliferation and survival.[2,3]

EGFR is frequently overexpressed in many types of human malignancy, with the extent of overexpression being negatively correlated with prognosis.[4,5] Recognition of the role of EGFR in carcinogenesis has prompted the development of EGFR-targeted therapies that include both small-molecule tyrosine kinase inhibitors (TKIs) that target the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) that target the extracellular domain.[6–8] Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in nonsmall cell lung cancer (NSCLC), and sensitivity to these drugs has been correlated with the presence of somatic mutations in the EGFR kinase domain or with EGFR gene (*EGFR*) amplification.[9–16] Among anti-EGFR mAbs, cetuximab (Erbitux), a chimeric mouse-human antibody of the immunoglobulin (Ig) G1 subclass, has proved efficacious in the treatment of irinotecan-refractory colon cancer[17] and was recently approved by the U.S. Food and Drug Administration for the treatment of patients with head and neck squamous cell carcinoma.[18] Several clinical studies of anti-EGFR mAbs such as matuzumab (EMD72000, humanized IgG1) and cetuximab are ongoing for other types of cancer including NSCLC.[19–24] Anti-EGFR mAbs bind to the extracellular ligand binding domain of the receptor and are thereby thought to block ligand binding.[18,25] The antitumor effects of these mAbs are thus thought to be attributable to inhibition of EGFR signaling as well as to other mechanisms such as antibody-dependent cellular cytotoxicity.[18,26] However, the detailed effects of anti-EGFR mAbs on EGFR signaling have remained unclear.[27–30]

We have now examined in detail the effects on EGFR signal transduction of 2 anti-EGFR mAbs, matuzumab and cetuximab, both of which are used clinically, to provide insight into the mechanisms of their antitumor effects.

Material and methods

Cell culture and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460) and Ma-1 were obtained as previously described[31] and were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Matuzumab and cetuximab were kindly provided by Merck KGaA (Darmstadt, Germany) and Bristol Myers (New York, NY), respectively; gefitinib was obtained from AstraZeneca (Macclesfield, UK); and trastuzumab (Herceptin; Genentech, South San Francisco, CA) was obtained from Chugai (Tokyo, Japan). Neutralizing antibodies to EGFR (clone LA1) were obtained from Upstate Biotechnology (Lake Placid, NY).

Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated consecutively with primary and secondary antibodies, and immune complexes were detected with the use of enhanced chemiluminescence reagents, as described previously.[31] Primary antibodies to the specific intracellular phosphorylation sites of EGFR (pY845, pY1068 or pY1173), to Erk, to phospho-Akt and to Akt were obtained from Cell Signaling Technology (Beverly, MA); those to the extracellular domain of EGFR (clone 31G7) were from Zymed (South San Francisco, CA); those to the intracellular domain of EGFR (EGFR 1005) and to phospho-Erk were from Santa Cruz Biotechnology (Santa Cruz, CA); and those to β-actin (loading control) were from Sigma. Horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG were obtained from Amersham Biosciences (Little Chalfont, UK).

Chemical cross-linking assay

Cells were incubated first with 1 mM bis(sulfosuccinimidyl) suberate (BS[3]; Pierce, Rockford, IL) for 20 min at 4°C and then with

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mAb, monoclonal antibody; NSCLC, nonsmall cell lung cancer; Ig, immunoglobulin; BS[3], bis(sulfosuccinimidyl) suberate; PE, R-phycoerythrin; PI3K, phosphoinositide 3-kinase.

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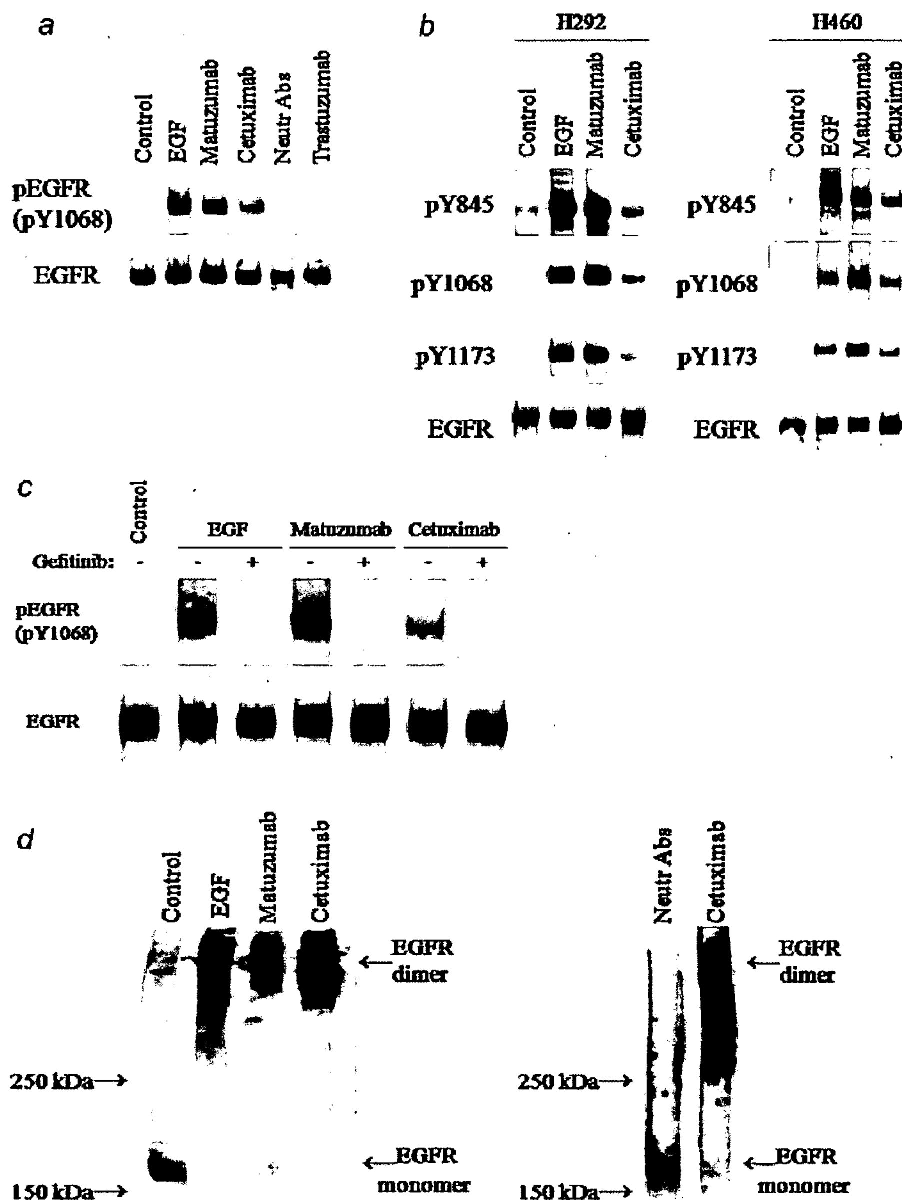

FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM), trastuzumab (50 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR (pY1068) and to total EGFR (the extracellular domain). (*b*) H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y845-, Y1068- or Y1173-phosphorylated forms of EGFR and to total EGFR (the extracellular domain). (*c*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), EGF (100 ng/ml) or gefitinib (10 μM), as indicated. Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR and to total EGFR (the extracellular domain). (*d*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM) or EGF (100 ng/ml). The cells were then washed and exposed to the chemical cross-linker BS^3 after which cell lysates were subjected to immunoblot analysis with antibodies to EGFR (the intracellular domain). The positions of EGFR monomers and dimers as well as of molecular size standards are indicated.

250 mM glycine for 5 min at 4°C to terminate the cross-linking reaction, as described previously.[31] Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis on a 4% gel and subjected to immunoblot analysis with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005).

Immunofluorescence analysis

Cells were grown to 50% confluence in 2-well Lab-Tec Chamber Slides (Nunc, Naperville, IL), deprived of serum overnight, and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were fixed with 4% paraformaldehyde for

30 min at 4°C, permeabilized with 0.1% Triton X-100 for 10 min, and exposed to 5% nonfat dried milk for 1 hr at room temperature. The cells were stained with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005) for 1 hr at room temperature and then incubated for an additional 45 min with Alexa 488-labeled goat antibodies to rabbit IgG (Molecular Probes, Eugene, OR). Cell nuclei were counterstained for 5 min at room temperature with 4′,6-diamidino-2-phenylindole (Sigma) at 2 μg/ml. The chamber slides were mounted in fluorescence mounting medium (DakoCytomation, Hamburg, Germany), and fluorescence signals were visualized with a fluorescence microscope (Eclipse E800; Nikon, Kawasaki, Japan). Negative controls (secondary antibodies alone) did not yield any substantial background staining.

Flow cytometry

Cells were deprived of serum overnight and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were isolated by exposure to trypsin, and aliquots of ~1.0×10^6 cells were incubated for 2 hr at 4°C either with an R-phycoerythrin (PE)-conjugated mouse mAb to EGFR (clone EGFR.1; Becton Dickinson, San Jose, CA), which does not interfere with the binding of EGF to EGFR,[32] or with a PE-conjugated isotype-matched control mAb (Becton Dickinson). The cells were then examined by flow cytometry (FACScalibur, Becton Dickinson) to detect the intensity of EGFR staining at the cell surface.

Clonogenic assay

Cells were plated in triplicate at a density of 200 per 25-cm^2 flask containing 10 ml of medium and were cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated in medium alone for 7 days at 37°C, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted for calculation of the surviving fraction as follows: (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for untreated controls.

Results

Matuzumab and cetuximab induce EGFR phosphorylation in a manner dependent on the receptor tyrosine kinase activity

With the use of immunoblot analysis, we first examined the effects of the anti-EGFR mAbs matuzumab and cetuximab on EGFR phosphorylation in human NSCLC H292 cells, which express wild-type EGFR. Incubation of the serum-deprived cells for 15 min with EGF, matuzumab or cetuximab-induced phosphorylation of EGFR on tyrosine-1068 (Y1068), whereas treatment of the cells with neutralizing antibodies to EGFR or with trastuzumab, a mAb specific for HER2 (ErbB2), had no such effect (Fig. 1*a*). Furthermore, like EGF, matuzumab and cetuximab each induced phosphorylation of EGFR on Y845, Y1068 and Y1173 in H292 and H460 cells (Fig. 1*b*), the latter of which are also human NSCLC cells that express wild-type EGFR.

To determine whether the antibody-induced phosphorylation of EGFR requires the kinase activity of the receptor, we examined the effect of gefitinib, a specific EGFR-TKI. H292 cells were deprived of serum and then exposed to matuzumab, cetuximab or EGF for 15 min in the absence or presence of gefitinib. EGFR phosphorylation on Y1068 induced by EGF, matuzumab or cetuximab was completely blocked by gefitinib (Fig. 1*c*). These findings thus indicated that, like EGF, matuzumab and cetuximab each induce EGFR phosphorylation by activating the tyrosine kinase of the receptor.

Matuzumab and cetuximab induce EGFR dimerization

Ligand-dependent EGFR dimerization is responsible for activation of the receptor tyrosine kinase.[33,34] To examine whether matuzumab or cetuximab induces EGFR dimerization, we incubated serum-deprived H292 cells with the mAbs for 15 min and then exposed the cells to the chemical cross-linker BS^3. Immunoblot analysis of cell lysates with antibodies to the intracellular domain of EGFR revealed that matuzumab and cetuximab each induced EGFR dimerization to an extent similar to that observed with EGF, whereas only the monomeric form of the receptor was detected in control cells or in cells treated with neutralizing antibodies to EGFR (Fig. 1*d*). These data thus suggested that matuzumab and cetuximab activate EGFR through induction of receptor dimerization.

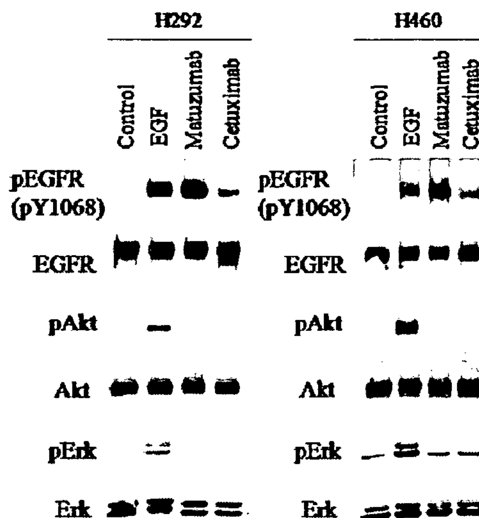

FIGURE 2 – Failure of matuzumab or cetuximab to activate Akt or Erk. H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR, to phosphorylated Akt and to phosphorylated Erk as well as with antibodies to total EGFR (the extracellular domain), Akt or Erk.

Matuzumab and cetuximab fail to induce signaling downstream of EGFR

EGFR signaling is transduced by 2 main pathways mediated by phosphoinositide 3-kinase (PI3K) and Akt and by Ras, Raf and Erk.[35,36] To determine whether EGFR phosphorylation induced by matuzumab or cetuximab is accompanied by activation of these pathways, we examined the levels of phosphorylated (activated) Akt and Erk in H292 and H460 cells treated with these antibodies for 15 min after serum deprivation. In contrast to the effects of EGF, neither matuzumab nor cetuximab induced the phosphorylation of Akt or Erk in H292 or H460 cells (Fig. 2). These results thus indicated that matuzumab and cetuximab induce EGFR activation but fail to activate the downstream Akt and Erk signaling pathways.

Matuzumab and cetuximab do not induce EGFR downregulation

Endocytic trafficking of EGFR is important for full activation of Erk and PI3K.[37] To examine further the defect in signaling downstream of EGFR activation by matuzumab or cetuximab, we determined the effects of these mAbs on receptor turnover. H292 or H460 cells were deprived of serum and then cultured with EGF, matuzumab or cetuximab for various times up to 24 hr, after which the levels of phosphorylated and total EGFR, Akt and Erk were measured. In both H292 and H460 cells treated with EGF, the amount of total EGFR decreased in a time-dependent manner

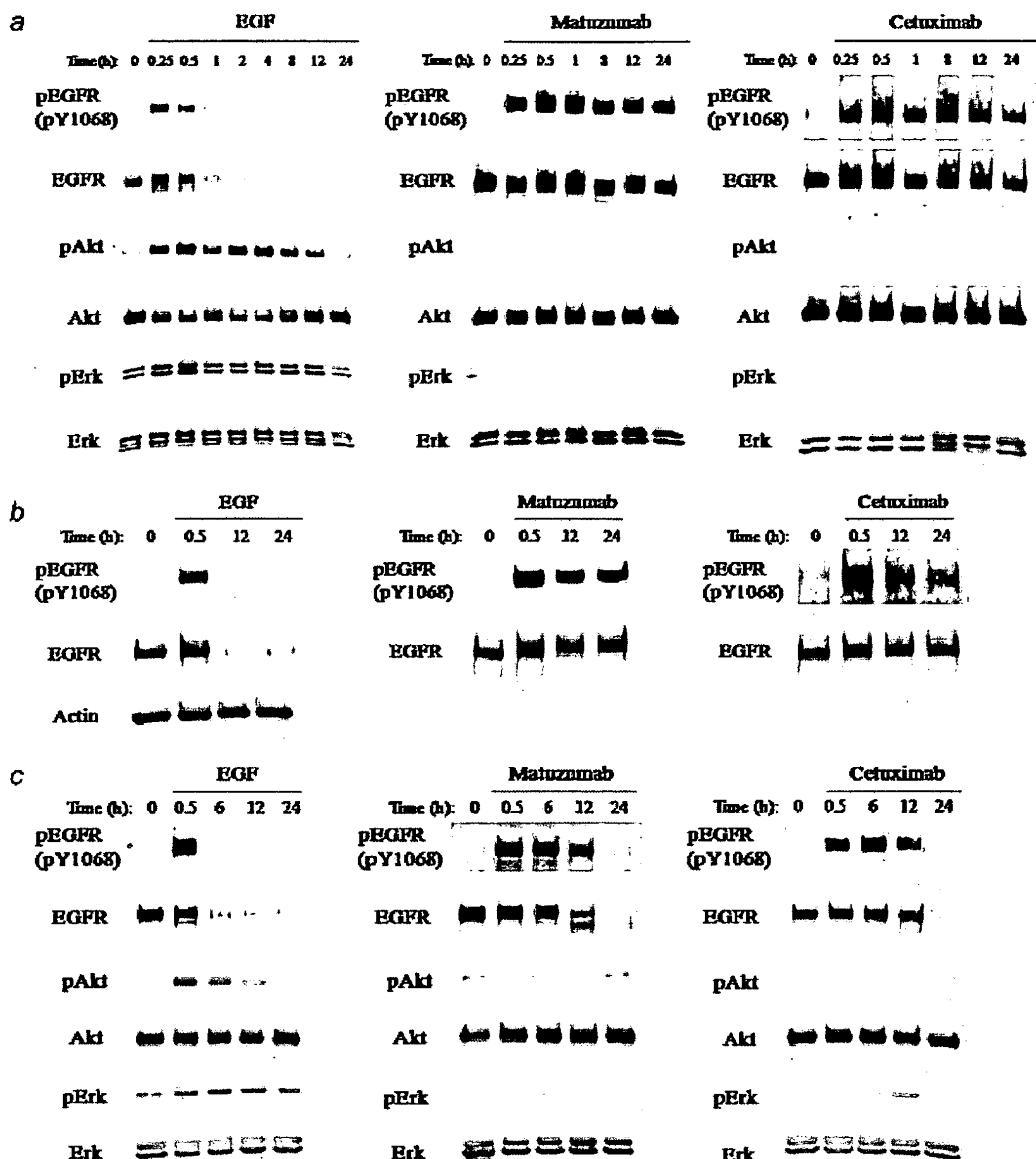

FIGURE 3 – Lack of EGFR turnover in cells treated with matuzumab or cetuximab. (*a*) H292 cells were deprived of serum overnight and then incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), respectively. Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk. (*b*) H292 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM). Cell lysates were subjected to immunoblot analysis with antibodies to theY1068-phosphorylated form of EGFR, to total EGFR (the intracellular domain) or to β-actin (loading control). (*c*) H460 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the intracellular domain), Akt or Erk. (*d*) H292 cells plated on chamber slides were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were fixed, permeabilized, and stained with antibodies to EGFR and Alexa 488-labeled secondary antibodies (green). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Fluorescence signals were visualized with a fluorescence microscope, and the merged images are shown. Scale bar, 20 μm. (*e*) H292 cells were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were stained with either a PE-conjugated mAb to EGFR (right peaks) or a PE-labeled isotype-matched mAb (left peaks) and analyzed by flow cytometry. Representative histograms of relative cell number versus PE fluorescence are shown.

(Figs. 3*a*–3*c*), an effect that has been shown to be the result of receptor internalization and degradation.[30,38] In parallel with this EGFR downregulation, the extent of EGF-induced tyrosine phosphorylation of EGFR also decreased and was virtually undetectable by 4–6 hr (Figs. 3*a*–3*c*). The phosphorylation of Akt and Erk induced by EGF persisted for at least 12 hr but had declined by 24 hr in both cell lines (Figs. 3*a* and 3*c*). In contrast, the levels of phosphorylated and total EGFR in H292 cells treated with

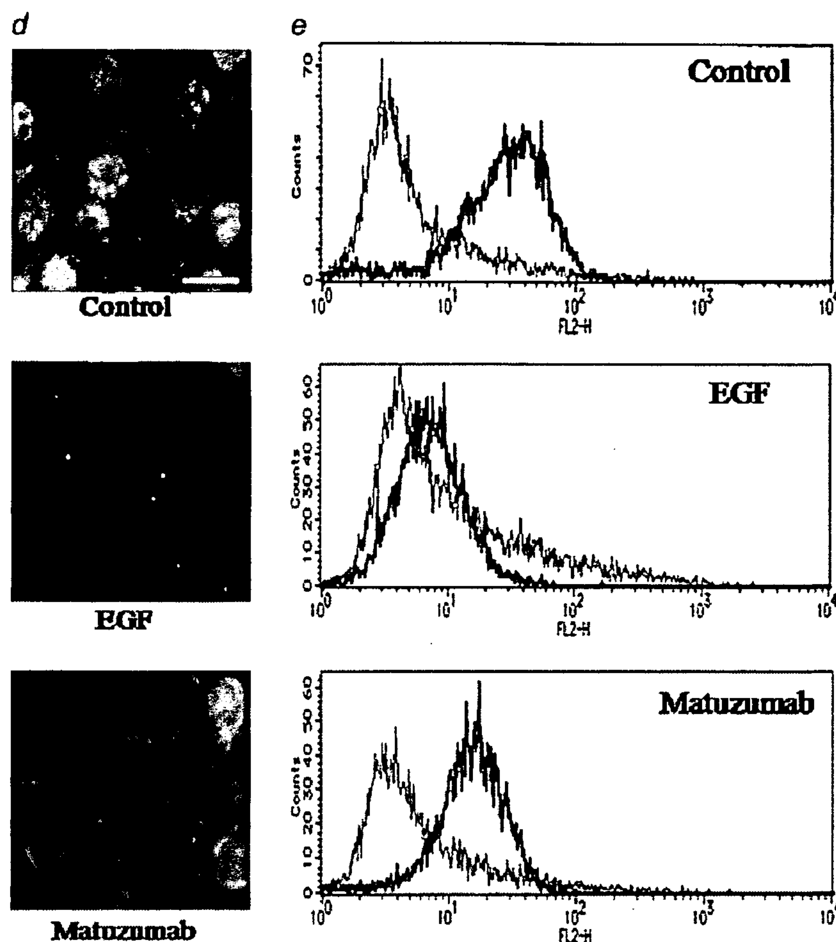

FIGURE 3 – CONTINUED

matuzumab or cetuximab for 24 hr were similar to those apparent after exposure to the antibodies for only 15 or 30 min (Figs. 3*a* and 3*b*). A marked delay in EGFR turnover was also apparent in H460 cells treated with matuzumab or cetuximab (Fig. 3*c*), although EGFR dephosphorylation and downregulation had occurred by 24 hr. Neither matuzumab nor cetuximab induced the activation of Akt or Erk or affected the total amounts of these proteins over a period of 24 hr in either cell line (Figs. 3*a* and 3*c*). We eliminated the possibility that the antibodies to the extracellular domain of EGFR used for the immunoblot analysis shown in Figure 3*a* bind only to the unoccupied form of EGFR (as a result of competition with EGF, matuzumab or cetuximab) by performing the immunoblot analysis shown in Figures 3*b* and 3*c* with antibodies to the intracellular domain of EGFR. These results thus suggested that downregulation of EGFR is impaired in cells treated with matuzumab or cetuximab, likely explaining the failure of these antibodies to activate downstream signaling by Akt and Erk.

To confirm that the inability of the anti-EGFR mAbs to induce EGFR downregulation is attributable to a failure to induce internalization-dependent receptor degradation, we treated serum-deprived H292 cells with matuzumab or EGF for 4 hr and then examined the expression of EGFR by immunofluorescence analysis (Fig. 3*d*) or flow cytometry (Fig. 3*e*). Whereas EGFR was localized at the cell surface in control cells, treatment with EGF resulted in internalization and a decrease in the fluorescence intensity of EGFR. In contrast, EGFR remained at the surface of cells treated with matuzumab. These data suggested that, in contrast to EGF-EGFR complexes, antibody-EGFR complexes remain at the cell surface and do not undergo internalization and degradation.

TABLE I – CHARACTERISTICS OF NSCLC CELL LINES

Cell line	*EGFR* mutation	*EGFR* copy number
H292	Wild type	Polysomy
H460	Wild type	Monosomy
Ma-1	del E746-A750	Gene amplification

Effects of matuzumab and cetuximab on EGF-induced signaling and cell survival

We next determined whether matuzumab or cetuximab inhibits ligand-dependent EGFR signal transduction. To examine also whether the effects of these antibodies are dependent on *EGFR* status, we studied 3 human NSCLC cell lines: 2 cell lines (H292, H460) that possess wild-type *EGFR* alleles and 1 (Ma-1) with an *EGFR* mutation in exon 19 that results in deletion of the residues E746–A750. Our recent fluorescence in situ hybridization analysis[31] revealed that *EGFR* copy number is increased (polysomy) in H292 cells and that H460 cells exhibit monosomy for *EGFR*. Ma-1 cells were also found to manifest *EGFR* amplification (Table I).[31] We treated serum-deprived cells of the 3 NSCLC lines with

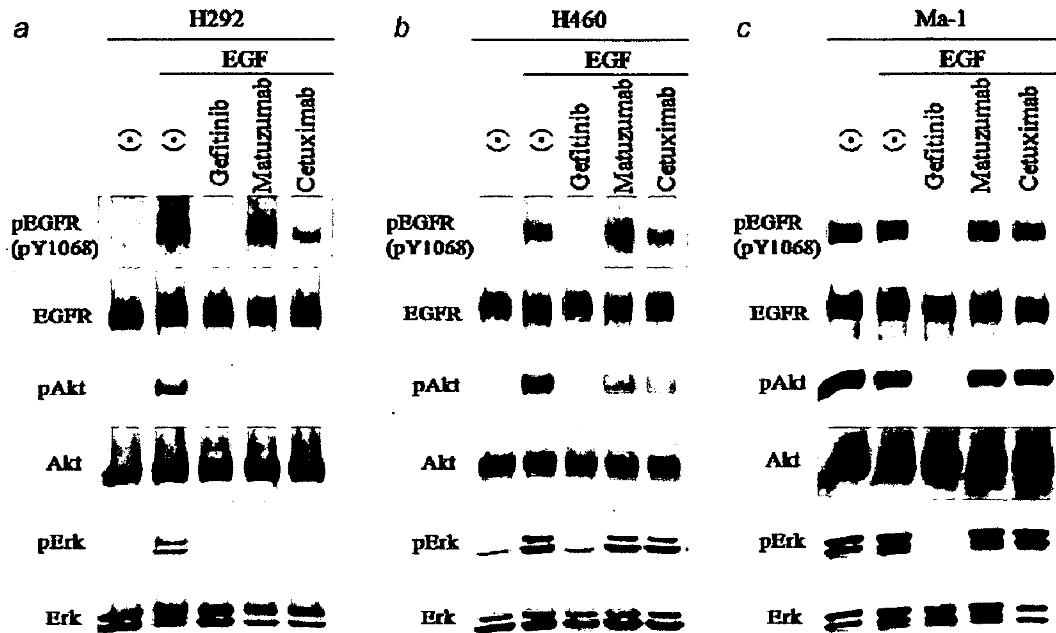

FIGURE 4 – Effects of matuzumab and cetuximab on EGF-induced EGFR signaling. H292 (*a*), H460 (*b*) and Ma-1 (*c*) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk.

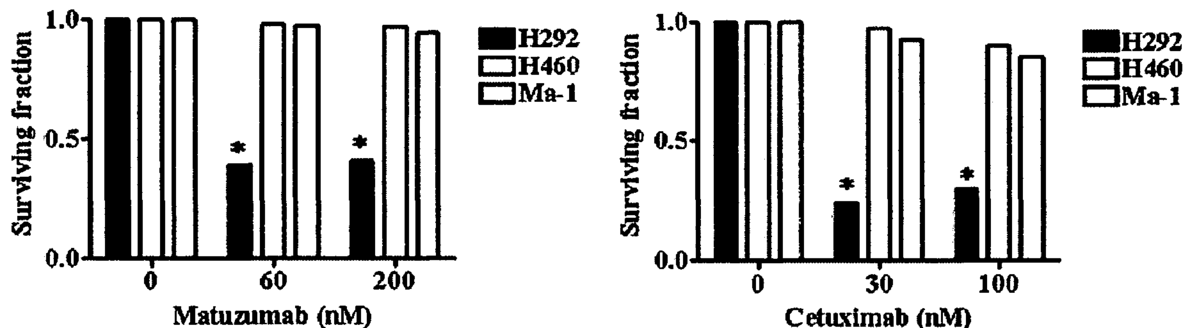

FIGURE 5 – Effects of matuzumab and cetuximab on cell survival. H292, H460 or Ma-1 cells were plated at a density of 200 cells per 25-cm^2 flask in triplicate and cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated with medium alone for 7 days before determination of the number of colonies containing >50 cells for calculation of the surviving fraction. Data are means of triplicates from a representative experiment. $*p < 0.001$ *versus* the corresponding value for cells not exposed to mAb (Student's *t*-test).

matuzumab, cetuximab or gefitinib for 15 min and then stimulated them with EGF for 15 min. Gefitinib prevented the phosphorylation of EGFR, Akt, and Erk induced by EGF in H292 (Fig. 4*a*) and H460 (Fig. 4*b*) cells. The level of EGFR phosphorylation in EGF-treated H292 or H460 cells was not substantially affected by matuzumab or cetuximab, likely because these antibodies also induce EGFR phosphorylation. However, whereas matuzumab and cetuximab did not substantially affect EGF-dependent phosphorylation of Akt or Erk in H460 cells, they markedly inhibited these effects of EGF in H292 cells. As we showed previously,[31] EGFR, Akt, and Erk are constitutively activated in the *EGFR* mutant cell line Ma-1 cell (Fig. 4*c*). Furthermore, whereas gefitinib blocked the phosphorylation of each of these 3 proteins in Ma-1 cells, matuzumab and cetuximab did not.

Finally, we performed a clonogenic assay to determine whether cell survival is affected by the differences in EGF-dependent signaling among H292, H460 and Ma-1 cells after treatment with matuzumab or cetuximab (Fig. 5). Matuzumab and cetuximab each induced a marked reduction in the survival rate of H292 cells, consistent with the inhibition of EGF-dependent EGFR downstream signaling by these antibodies in these cells. In contrast, neither mAb affected the survival of H460 or Ma-1 cells, consistent with the lack of inhibition of EGF-dependent or constitutive EGFR downstream signaling by matuzumab or cetuximab in these cell lines. These results suggested that the effects of matuzumab and cetuximab on EGF-dependent or constitutive EGFR downstream signaling are correlated with their effects on cell survival in NSCLC cell lines.

Discussion

The effectiveness of treatment with anti-EGFR mAbs has been thought to be based on prevention of ligand binding to EGFR and consequent inhibition of EGFR activation.[18,25,26] Matuzumab and cetuximab have recently been developed as EGFR-inhibitory mAbs for clinical use.[17–22,25] A structural study revealed that cetuximab binds to the extracellular ligand binding domain (domain III) of EGFR,[25] and matuzumab is also thought to bind to domain III on the basis of its observed competition with EGFR ligands.[18] We have now shown that matuzumab and cetuximab induced phosphorylation of EGFR at several sites, including Y845, Y1068 and Y1173. These findings are consistent with previous observations that mAb 225, the mouse mAb equivalent to cetuximab, is able to induce EGFR dimerization and activation.[38,39] Cetuximab was also recently shown to induce phosphorylation of EGFR in head and neck squamous cell carcinoma cell lines[29] as well as in NSCLC cell lines including H292.[40] These in vitro results appear to contradict observations that matuzumab and cetuximab inhibit EGFR phosphorylation in vivo.[28,41,42] This apparent discrepancy may be due to the more complex cellular environment in vivo, including the presence of stromal cells that interact with tumor cells. We have also now shown that gefitinib, a specific EGFR-TKI, completely blocked EGFR phosphorylation induced by matuzumab or cetuximab, confirming that this effect of the antibodies is dependent on the intrinsic tyrosine kinase activity of EGFR. Furthermore, our cross-linking analysis showed that matuzumab as well as cetuximab activated EGFR through induction of receptor dimerization. Although recent structural analysis has revealed that cetuximab restricts the range of the extended conformation of EGFR that is required for ligand-induced receptor dimerization,[25] matuzumab and cetuximab likely induce EGFR dimerization in a manner dependent on their immunologically bivalent binding capacities, as was previously shown for mAb 225.[39] We found that neutralizing antibodies to EGFR did not activate EGFR, even though they also recognize the external domain of EGFR and compete with EGFR ligands for receptor binding.[43] The neutralizing antibodies did not induce EGFR dimerization, however, likely accounting for their inability to activate EGFR. This difference in the ability to induce EGFR dimerization between matuzumab and cetuximab on the one hand and the neutralizing antibodies on the other might be due to differences in the corresponding binding sites on EGFR.

To examine the mechanism by which matuzumab and cetuximab exert antitumor effects despite their induction of EGFR activation, we investigated the effects of antibody-induced EGFR activation on EGFR downstream signal transduction. We found that EGFR activation induced by matuzumab or cetuximab was not accompanied by activation of downstream signaling pathways mediated by Akt and Erk, both of which play an important role in regulation of cell proliferation and survival.[35,36] Moreover, we found that the antibody-EGFR complexes were not removed from the plasma membrane, in contrast to the rapid receptor turnover induced by EGF. In response to ligand binding, the ligand-EGFR complex is rapidly internalized and then either recycled back to the cell surface or proteolytically degraded.[44–46] The internalized EGFR interacts with various signaling proteins that are important for sustained activation of the major signaling pathways mediated by PI3K-Akt and Erk.[44,47] The activity of the PI3K-Akt and Erk pathways is thus greatly reduced in cells that are defective in internalization of ligand-EGFR complexes as a result of their expression of a mutant form of dynamin.[37] Furthermore, expression in glioblastoma cells of an EGFR chimeric protein that does not undergo internalization resulted both in a reduction in the extent of EGFR-dependent activation of Akt and Erk as well as in inhibition of tumor growth.[48] These observations thus suggest that inhibition of EGFR turnover by matuzumab or cetuximab is likely responsible for the failure of these mAbs to activate Akt and Erk.

We examined the effects of matuzumab and cetuximab on EGF-dependent EGFR signaling and on cell survival in 3 NSCLC cell lines of differing *EGFR* status. The inhibition of EGF-dependent activation of Akt and Erk by these antibodies appeared related to the inhibition of clonogenic cell survival in the 3 cell lines. With regard to NSCLC cell lines harboring wild-type *EGFR* alleles, matuzumab and cetuximab markedly inhibited EGF-dependent phosphorylation of Akt and Erk in H292 cells but not in H460 cells. Both antibodies inhibited cell survival in H292 cells but not in H460 cells. These results suggest that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling such as that mediated by Akt and Erk rather than on inhibition of EGFR itself. Our present data are consistent with previous observations that cetuximab did not inhibit EGFR phosphorylation completely even in cells sensitive to this antibody.[27,30] It is possible that the difference in sensitivity to matuzumab and cetuximab between the 2 cell lines expressing wild-type EGFR in the present study is due to the difference in gene copy number, given that we found an increase in *EGFR* copy number in H292 cells compared with that in H460 cells.[31] A previous clinical study showed that *EGFR* copy number correlated with the response to cetuximab treatment in individuals with colorectal cancer.[49] *EGFR* copy number was not determined by fluorescence in situ hybridization in previous clinical studies of NSCLC patients treated with matuzumab or cetuximab.[19,22–24] Several clinical studies of the therapeutic efficacy of anti-EGFR antibodies in NSCLC patients are underway, and investigation of the potential of molecular markers including *EGFR* copy number to predict clinical response is warranted. Matuzumab and cetuximab failed to inhibit both activation of Akt and Erk and clonogenic cell survival in Ma-1 cells, which express a mutant form of EGFR that shows an increased sensitivity to EGFR-TKIs such as gefitinib and erlotinib.[9–16] We recently showed that cells expressing EGFR mutants exhibit constitutive, ligand-independent receptor dimerization and activation,[31] likely explaining the lack of effect of matuzumab or cetuximab on EGFR signaling or cell survival in such cells. However, previous studies showed that cetuximab exerted an antitumor effect in a cell line with an *EGFR* mutation, whereas several other cell lines with *EGFR* mutations were resistant to cetuximab.[27,30] Our results are consistent with clinical observations showing that the presence of an *EGFR* mutation is not a major determinant of a positive response to cetuximab in individuals with NSCLC or colorectal cancer.[22,50,51]

In conclusion, we have shown that EGFR turnover is impaired in cells treated with the anti-EGFR mAbs matuzumab or cetuximab, resulting in inhibition of EGFR downstream signaling. Although our study is limited by the small number of cell lines analyzed, our findings provide important insight into the mechanisms by which anti-EGFR mAbs exert their antitumor effects, and they suggest that it may be possible to predict the therapeutic efficacy of such mAbs by assessment of EGFR signal transduction.

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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

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

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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

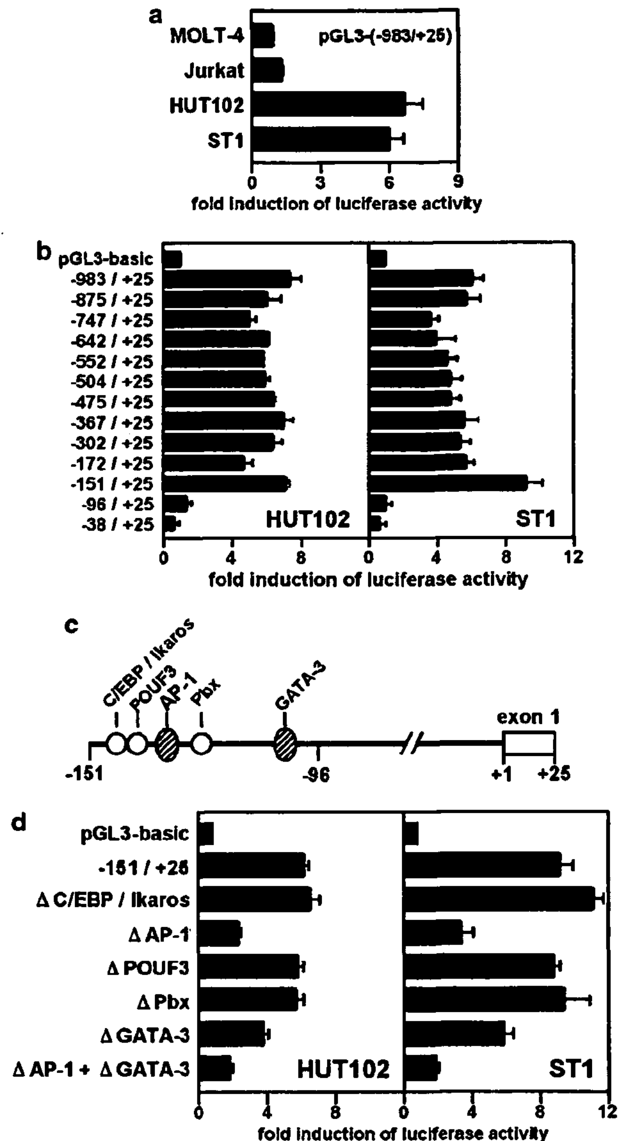

Figure 1 Identification of regulatory elements in the CCR4 promoter. Cells were transfected with pSV-β-galactosidase and pGL3-basic or pGL3-basic inserted with the CCR4 promoter regions as indicated. After 24–27 h, luciferase assays were performed. Promoter activation was expressed by the fold induction 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 TCTTGGGAAATGA to TCTTGCAAAATGA), ΔAP-1 (from AATGACTAAGA to AATGTCAAAGA), ΔPOUF3 (from CTTGGGAAATGA to CTTGGGAGGTGA), ΔPbx (from AAGAATCAT to AAGACCCAT) 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.

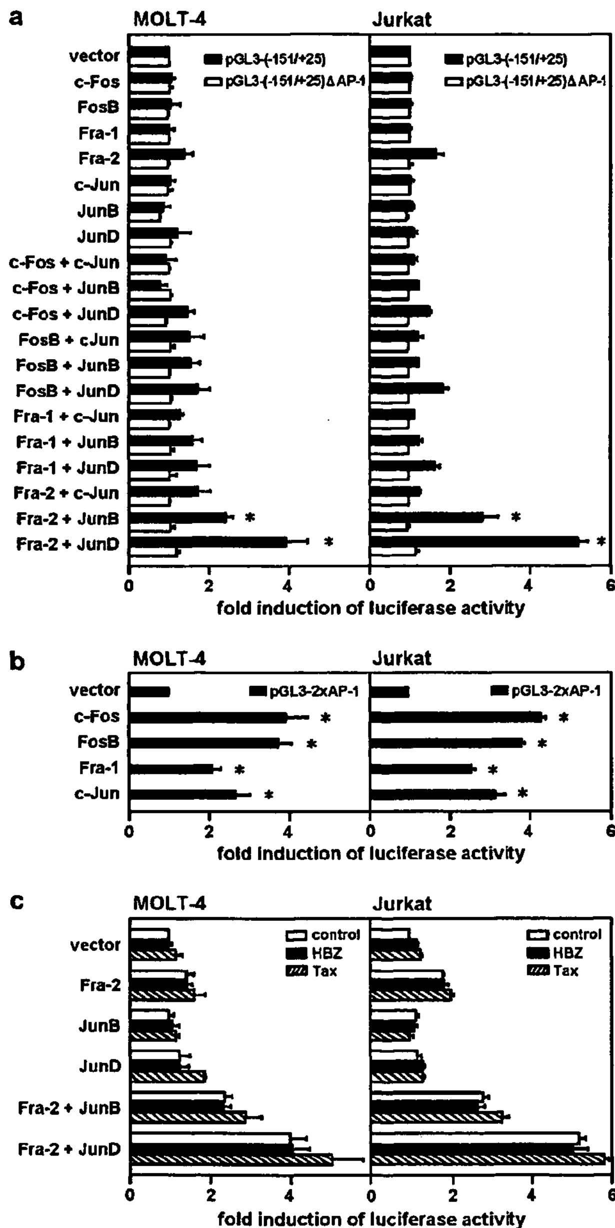

Figure 3 Transactivation of the CCR4 promoter by Fra-2/JunD and Fra-2/JunB. (a) Transactivation of the CCR4 promoter with or without the AP-1 site. MOLT-4 and Jurkat cells were cotransfected with pSV-β-galactosidase and pGL3-CCR4 (–151/+25) or pGL3-CCR4 (–151/+25)ΔAP-1 and an expression vector for c-Fos, 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.

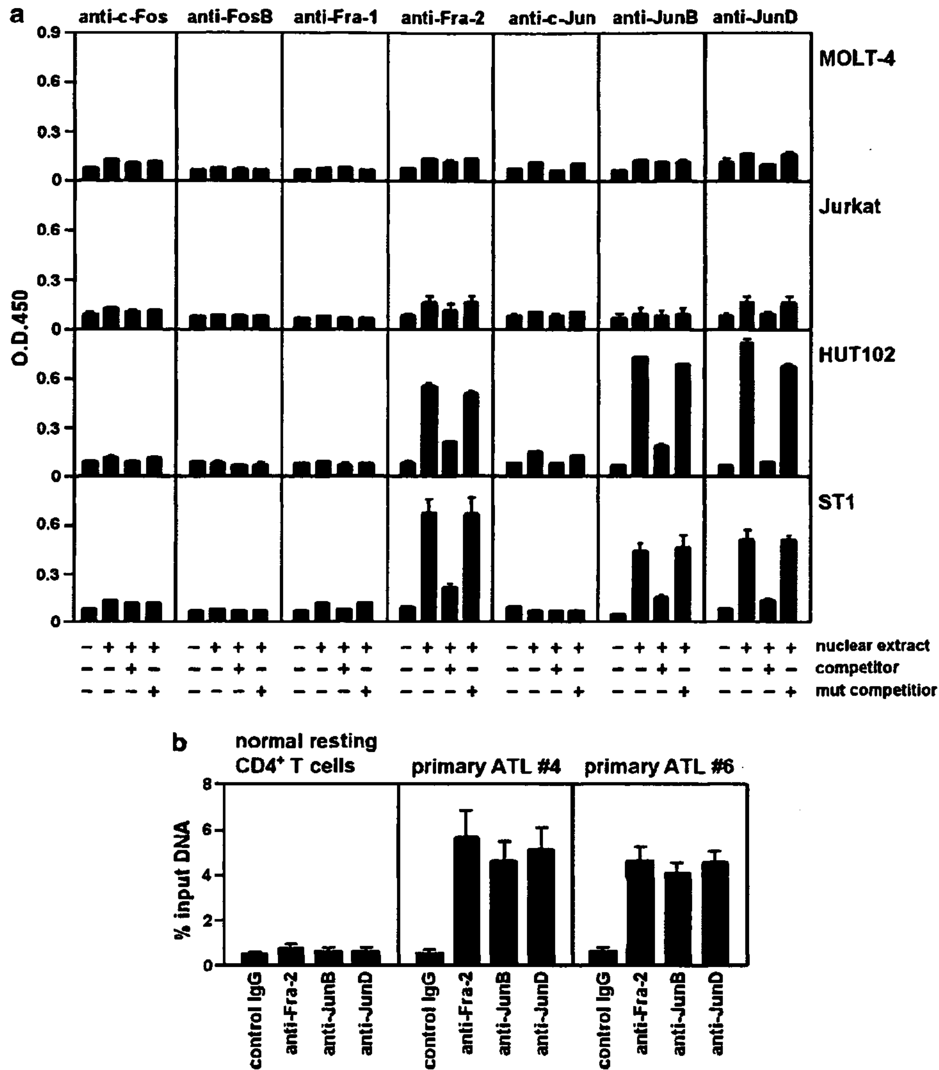

Figure 4 Specific binding of Fra-2, JunB and JunD to the AP-1 site in the CCR4 promoter. (a) NoShift assay. Nuclear extracts were prepared from two control T-cell lines (MOLT-4 and Jurkat) and two adult T-cell leukemia (ATL) cell lines (HUT102 and ST1). Nuclear proteins that bound to the biotinylated AP-1 site oligonucleotide (TGGGAAATGACTAAGAATCAT) were captured on an avidin-coated plate and detected by anti-c-Fos, anti-FosB, anti-Fra-1, anti-Fra-2, anti-c-Jun, anti-JunB or anti-JunD, as indicated. Specificity was determined by adding unlabeled probe (competitor; TGGGAAATGACTAAGAATCAT) or mutant probe (mut competitor; TGGGAAATGTCAAAGAATCAT; differences underlined). Each bar represents the mean ± s.e.m. from three separate experiments. (b) Chromatin immunoprecipitation (ChIP) assay. Chromatins from normal CD4+ T cells from healthy donors (purity, >96%) and primary ATL cells from two patients (leukemic cells, >90%) were immunoprecipitated with anti-Fra-2, anti-JunD or control IgG. The amounts of precipitated DNA relative to total input DNA were quantified by real-time PCR for the CCR4 promoter region containing the AP-1 site. Each bar represents the mean ± s.e.m. from three separate experiments.

Identification of downstream target genes of the Fra-2/JunD heterodimer in ATL cells

To identify the target genes of Fra-2 in ATL cells, we compared the gene expression profiles of ATL-derived ST1 cells transfected with Fra-2 siRNA or control siRNA using the Affymetrix high-density oligonucleotide microarray. As summarized in Figure 6a, at least 49 genes were downregulated more than threefold by Fra-2 siRNA. The classification of these genes according to their biological functions shows that Fra-2 promotes the expression of genes involved in signal transduction (10 genes), protein biosynthesis and modification (8 genes) and transcription (6 genes); it also stimulates the expression of 10 genes of unknown function. Most notably, the list includes the proto-oncogenes c-Myb, BCL-6 and MDM2 (Oh and Reddy, 1999; Pasqualucci *et al.*, 2003; Vargas *et al.*, 2003). As shown in Figure 6b, RT–PCR analysis verified that not only Fra-2 siRNA but also JunD siRNA downregulated these proto-oncogenes in two ATL cell lines. Therefore, c-Myb, BCL-6 and MDM2 are the downstream target genes of the Fra-2/JunD heterodimer in both cell lines. This prompted us to examine the expression of c-Myb, BCL-6 and MDM2 in freshly isolated primary ATL cells by

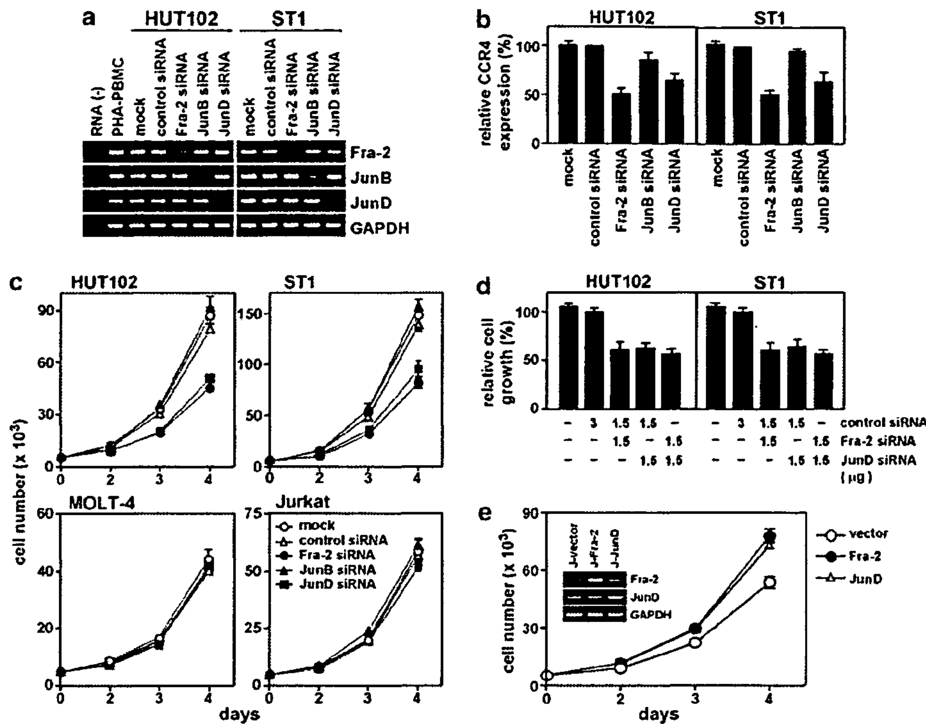

Figure 5 Dominant role of Fra-2/JunD in CCR4 expression and cell proliferation in adult T-cell leukemia (ATL). (**a**) Reverse transcription (RT)–PCR analysis to determine the effect of siRNAs. HUT102 and ST1 were transfected with control siRNA or siRNA for Fra-2, JunB or JunD. After 48 h, total RNA was prepared. The representative results from three separate experiments are shown. (**b**) Real-time RT–PCR analysis for CCR4 expression. HUT102 and ST1 were transfected with control siRNA or siRNA for Fra-2, JunB or JunD. After 48 h, total RNA was prepared and real-time RT–PCR was performed for CCR4 and 18S ribosomal RNA (an internal control). Data are presented as the mean ± s.e.m. of three separate experiments. (**c**) Effect of siRNAs on cell growth. HUT102, ST1, MOLT-4 and Jurkat were transfected with control, Fra-2, JunB and JunD siRNAs and cultured in a 96-well plate at 0.5×10^4 cells per well. At the indicated time points, viable cell numbers were determined using a FACSCalibur by gating out cells stained with propidium iodide. Data are shown as the mean ± s.e.m. of three separate experiments. (**d**) Effect of double knockdown of Fra-2 and JunD on cell growth. HUT102 and ST1 were transfected with control, Fra-2 and JunD siRNAs as indicated and cultured in a 96-well plate at 0.5×10^4 cells per well. At 4 days, viable cell numbers were determined on a FACSCalibur by gating out dead cells stained with propidium iodide. Data are shown as the mean ± s.e.m. of three separate experiments. (**e**) Effect of stable expression of Fra-2 and JunD on cell growth. Jurkat cells were transfected with a control IRES-EGFP expression vector or an IRES-EGFP expression vector for Fra-2 or JunD. Stable transfectants expressing green fluorescence protein were sorted and cultured in a 96-well plate at 0.5×10^4 cells per well. At the indicated time points, viable cell numbers were determined on a FACSCalibur by gating out dead cells stained with propidium iodide. Data are shown as the mean ± s.e.m. of three separate experiments.

RT–PCR. As shown in Figure 6c, we indeed detected the constitutive expression of c-Myb, BCL-6 and MDM2 at high levels in primary ATL cells. In sharp contrast, normal resting CD4$^+$ T cells hardly expressed these proto-oncogenes.

Discussion

The AP-1 transcription factors function as homodimers or heterodimers formed by Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2) and the ATF family proteins (Shaulian and Karin, 2002; Eferl and Wagner, 2003). Most of them are rapidly and transiently induced by extracellular stimuli that trigger the activation of the Janus kinase (JNK), extracellular signal regulated protein kinases 1 and 2 (ERK1/2) or p38 mitogen-activated protein (MAP) kinase pathways (Shaulian and Karin, 2002; Eferl and Wagner, 2003). The AP-1 family is known to be involved in cellular proliferation, oncogenesis and even tumor suppression, depending on the combination of AP-1 proteins and the cellular context (Shaulian and Karin, 2002; Eferl and Wagner, 2003). Previously, by using the AP-1 site of the IL-8 promoter, Mori *et al.* demonstrated a strong Tax-independent expression of JunD in primary ATL cells (Mori *et al.*, 2000). In the present study, we have shown that Fra-2 is constitutively expressed at high levels in primary ATL cells (Figure 2a). Furthermore, except for JunB and JunD, other members of the AP-1 family are mostly negative in primary ATL cells (Figure 2a). Therefore, as demonstrated in the present study, the Fra-2/JunD and Fra-2/JunB heterodimers may be the major AP-1 factors constitutively active in primary ATL cells.

It has been shown that HTLV-1 Tax induces the expression of various AP-1 family members such as c-Fos, Fra-1, c-Jun and JunD (Nagata *et al.*, 1989; Iwai *et al.*, 2001). We indeed observed the expression of