Correspondence

An Algorithm to Tackle Acute Exacerbations in Idiopathic Pulmonary Fibrosis

To the Editor:

The pulmonary perspective by Dr. Collard and colleagues (1) provides an overview of the current understanding about acute exacerbations in idiopathic pulmonary fibrosis (IPF). To tackle this challenging problem, we developed an algorithm (Figure 1). As a first step, we test for the presence of D-dimer and clinical probability of pulmonary embolism. If the test is positive, a specific computed tomography (CT) scan is performed. The CT scan is an accurate modality (2) with which to detect pulmonary embolism; moreover, images can be compared with previous CT scans to evaluate reticular shadowing, honeycombing, and ground-glass appearances. Echocardiography is performed to rule out left heart failure. At the same time, (increasing) pulmonary hypertension can be ruled out. If diffusion capacity is greater than 30%, and if hypoxemia can be corrected to a Po_2 of 75 mm Hg with supplemental oxygen (3), then bronchoalveolar lavage (BAL) is performed to rule out infection (bacterial, viral, opportunistic infections, and fungi).

BAL must be performed soon after admission, and broad-spectrum antibiotics are started immediately after bronchoscopy. We usually start broad-spectrum antibiotics (such as piperacillin-tazobactam or third-generation cephalosporins intravenously). However, atypical bacilli (such as *Legionella* and *Mycoplasma*) must be covered by adding quinolones. As most of these patients with IPF are immunocompromised, *Pneumocystis jiroveci* pneumonia needs to be covered empirically with sulphametoxazole. If infection cannot be proven, corticosteroids are added to the treatment in a dose of 500–1,000 mg for 3 consecutive days. Antiviral agents should be considered when herpesvirus or cytomegalovirus (CMV) is found in BAL, and antifungal agents should be initiated especially when *Aspergillus* is found in an immuno-

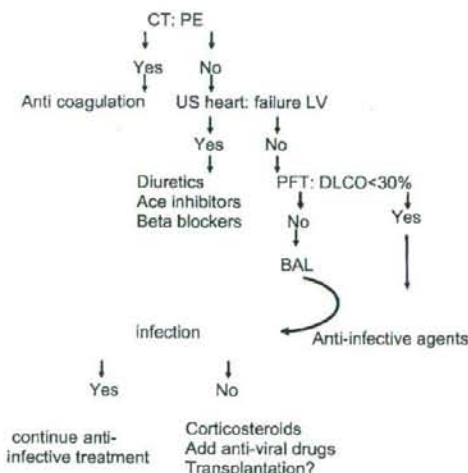

Figure 1. Algorithm of treatment for patients with idiopathic pulmonary fibrosis. BAL = bronchoalveolar lavage; CT = computed tomography; DL_{CO} = diffusion capacity for CO; LV = left ventricle; PE = pulmonary embolism; PFT = pulmonary function testing; US = ultrasound.

compromised patient or if the *Aspergillus* antigen test in BAL is clearly positive (4).

If there is no effect from these therapies, and in the absence of generalized infection, lung transplantation should be considered as a treatment for acute exacerbation in IPF. It is the only treatment that improves survival in patients with interstitial lung disease (5). The patient can only be put on the high-urgency list when a transplant workup has been completed before the acute exacerbation. This is another strong reason to refer patients with IPF in an early stage to a transplant center for transplant evaluation, as an acute exacerbation is not related to pulmonary function tests (1).

In conclusion, Collard and coworkers' article gives an excellent overview of the challenges in acute exacerbations of IPF. We need carefully chosen protocols to guide us in this challenge. We hope that the algorithm we propose will help physicians in dealing with this problem.

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Basis of Acute Exacerbation of Idiopathic Pulmonary Fibrosis in Japanese Patients

To the Editor:

A pulmonary perspective by Dr. Collard and colleagues focused on acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF), trying to establish a worldwide consensus about acutely progressive respiratory failure during the course of IPF (1). Awareness of this serious phenomenon as a distinct pattern of IPF is extremely

important, and helps clinicians worldwide to share the same background information on the diagnosis and management of AE. However, Japanese patients with IPF seem to suffer from AE much more frequently than patients of other ethnic backgrounds. AE is often triggered by infections, drugs, radiotherapy, anticancer agents, and surgery. Epidemiological data suggest that Japanese more frequently suffer from diffuse alveolar damage (DAD) due to various causes such as drugs (2). These observations strongly suggest that genetic factor(s) predispose Japanese to DAD. Reports on AE-IPF are frequent from Japan (3, 4). A large-scale study showed that 5.8% of Japanese patients on gefitinib therapy developed interstitial lung disease, a frequency 10- to 100-fold higher than for patients of other genetic backgrounds (2).

Ethnic differences in the incidence of pulmonary disease have been observed before. For example, most Japanese clinicians have never seen α_1-antitrypsin deficiency or cystic fibrosis, because these diseases are extremely rare in the Japanese population. Population genetics indicates that a founder gene may predominate in a genetically isolated population through the bottleneck effect or random genetic drift (5).

Homo sapiens emerged in Africa around 200,000 years ago and reached Japan about 30,000 years ago, at the end of the Ice Age when the sea level was low and Japan was joined to the Eurasian continent. Subsequent elevation of the sea has genetically isolated Japan, so that the Japanese population may share disease genes that are not found or rarely found in other ethnic groups.

Although there is more movement of people in modern society, the world is still a mosaic of ethnic groups with different diseases. We believe that AE-IPF is actually a collection of diseases with different causes, among which a cause specific to Japanese patients accounts for many cases in Japan. Elucidation of the responsible genetic factor(s) would be important for public health in the world, as well as for understanding pulmonary physiology.

Conflict of Interest Statement: A.A. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.H. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.K. received 220,000 Japanese yen from AstraZeneca for advisory board activities.

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From the Authors:

We thank Dr. Wuyts and colleagues and Dr. Azuma and colleagues for their interest in our pulmonary perspective on acute exacerbations of idiopathic pulmonary fibrosis (IPF) (1). It was our hope in writing this perspective to stimulate thoughtful discussion, debate, and research on this important topic.

Dr. Wuyts and coworkers present an interesting algorithm for the diagnosis and management of acute exacerbations of IPF, and we agree with the authors' emphasis on aggressively looking for alternative etiologies (e.g., infection, pulmonary embolism). While the proposed approach to the diagnosis and management of acute exacerbations of IPF is a reasonable one, it is important to note that data supporting its specific recommendations are lacking. This is particularly true for the management recommendations, including the use of antibiotic, antifungal, and corticosteroid therapies. The authors' comments regarding the appropriateness of early referral for lung transplantation evaluation are strongly endorsed, as many patients with IPF may suffer acute worsening of their disease and death despite previously preserved pulmonary function (2, 3).

Nothing is known about susceptibility to acute exacerbation of IPF, but we agree with Dr. Azuma and coworkers that the Japanese preponderance of published cases may suggest an important genetic predisposition (4, 5). It is interesting to note that genetic and ethnic disparities exist in acute lung injury, a condition clinically and histopathologically similar to acute exacerbation of IPF (6). Better epidemiological data are sorely needed to further investigate Dr. Azuma's hypothesis.

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ORIGINAL ARTICLE

Identification of IGFBP-6 as an effector of the tumor suppressor activity of SEMA3B

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SEMA3B, a member of class 3 semaphorins, is a tumor suppressor. Competition with vascular endothelial growth factor (VEGF)$_{165}$ explains a portion of the activity, whereas the VEGF-independent mechanism was not elucidated. We employed a microarray and screened for the genes whose expression was increased by SEMA3B in NCI-H1299 cells. Insulin-like growth factor-binding protein-6 (IGFBP-6), a tumor suppressor, showed greatest difference in the expression level. Introduction of *IGFBP-6* cDNA reduced colony formation both on the dish surface and in soft agar. Insulin-like growth factor II, which antagonizes IGFBP-6, partly abrogated the effect. Inhibition of IGFBP-6 by small interfering RNA diminished the sub-G0/G1 population that was induced by SEMA3B and abrogated the growth suppressive effect of SEMA3B. We concluded that IGFBP-6 is the effector of tumor suppressor activity of SEMA3B in NCI-H1299 cells. It has been reported that β-catenin suppresses the expression of IGFBP-6. Introduction of β-catenin into the cells partly abrogated the growth suppressive effect of SEMA3B. Our result indicates that semaphorin signaling and β-catenin signaling converge on IGFBP-6 and antithetically affect their functions.

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Keywords: semaphorin 3B; insulin-like growth factor-binding protein-6; β-catenin; microarray; tumor suppression

Introduction

The semaphorins are a family of proteins involved in cell migration, and so are implicated in nerve regeneration (Pasterkamp *et al.*, 1998), tumorigenesis (Christensen *et al.*, 1998), cardiovascular morphogenesis (Behar *et al.*, 1996; Miao *et al.*, 1999) and immune response (Hall *et al.*, 1996). The class 3 semaphorins (SEMA3s) are secreted proteins consisting of seven members, SEMA3A through -3G. Neuropilins (NRP1 and NRP2) and plexins form functional semaphorin receptors that transfer semaphorin signaling into the cells (Kolodkin *et al.*, 1993; He and Tessier-Lavigne, 1997). SEMA3E is the only exception to the above scheme in that it directly binds to a plexin (Gu *et al.*, 2005). An isoform of vascular endothelial growth factor (VEGF), VEGF$_{165}$, is a product of alternative mRNA splicing and also binds to NRPs. The VEGF$_{165}$–NRP complex then binds to VEGFR2 and transmits its signal (Soker *et al.*, 1996, 1998). Therefore, the NRPs are a component of both the SEMA3–NRP–plexin complexes and the VEGF$_{165}$–NRP–VEGFR2 complexes, and are located at the crossroads of SEMA3 and VEGF signaling. SEMA3s and VEGF$_{165}$ inhibit each other by competing for available molecules of NRP (Mac Gabhann and Popel, 2006).

SEMA3B has a tumor suppressor activity. Addition of SEMA3B protein to the medium or introduction of SEMA3B expression plasmid into the cells induces apoptosis and decreases colony-forming activity in NCI-H1299 cells (Sekido *et al.*, 1996; Tomizawa *et al.*, 2001). *SEMA3B* expression is induced by p53 mediated by a p53 responsive element located in the promoter (Ochi *et al.*, 2002). *SEMA3B* expression is frequently suppressed by the methylation of the promoter in lung cancers (Tomizawa *et al.*, 2001). The *SEMA3B* gene is located at 3p21.3 where chromosomal deletions have frequently been observed in lung cancers (Sekido *et al.*, 1996; Tomizawa *et al.*, 2001). These data suggest that SEMA3B may have a tumor suppressor activity that is attributable to the competitive inhibition of VEGF$_{165}$ working as an autocrine survival factor in some cancer cells (Castro-Rivera *et al.*, 2004).

Recent studies on two other members of the SEMA3 family have demonstrated complexity in their signaling pathways. SEMA3A inhibits VEGF activity by mechanisms that are both dependent on and independent of the competition for receptor binding (Guttmann-Raviv *et al.*, 2006). β-catenin/T-cell factor (TCF) signaling has been implicated in the growth inhibitory effects of SEMA3F (Nasarre *et al.*, 2005). These findings suggest that, in addition to the competition with VEGF$_{165}$, SEMA3B may employ an as yet unidentified mechanism for its tumor suppressor activity.

In this study, we have investigated whether SEMA3B activity is mediated by a mechanism other than

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competition with $VEGF_{165}$. Some tumor suppressors function by inducing the expression of their effector genes (Yu *et al.*, 2001; Barz *et al.*, 2006; Chow *et al.*, 2006; Feng *et al.*, 2006). We hypothesized that SEMA3B might be such a tumor suppressor. To test this hypothesis, we employed microarray technology and screened for those genes with increased expression following SEMA3B transfection. We then investigated whether and by what mechanism the identified gene, *insulin-like growth factor-binding protein-6* (*IGFBP-6*), acted as an effector of SEMA3B.

Results

Establishment of stable SEMA3B transfectants
Cell lines (stSEMA3B and stSEMA3B-FLAG) that stably express the native form of SEMA3B or a FLAG-tagged SEMA3B were established by introducing their expression plasmids into NCI-H1299 cells that do not express SEMA3B. This established an experimental system where SEMA3B expressed from the plasmid was secreted and acted on the cells in an autocrine manner. As SEMA3B function may be post-translationally modified, this system was considered more suitable for investigating SEMA3B function than what employs recombinant proteins expressed in prokaryotes. SEMA3B was detected both in the conditioned medium (CM; Figure 1a) and in the cytoplasm (cell lysate, CL; Figure 1b). Expression was not observed in the cell line (stpcDNA) that was established by transfecting an empty plasmid.

Identification of IGFBP-6
We employed a microarray to search for mRNAs that were more highly expressed in stSEMA3B than in stpcDNA in an attempt to find a gene that is an effector of SEMA3B (Figure 2a, also see Supplementary information). Reverse transcription PCR (RT–PCR) was performed on several growth-related genes to confirm the microarray data (Figure 2b). Among the

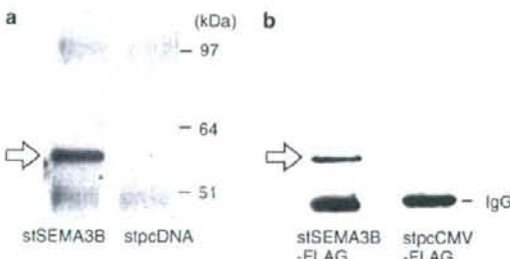

Figure 1 Expression of SEMA3B protein in the stSEMA3B cell line. (**a**) Western blot. SEMA3B protein (60 kDa) in CM of stSEMA3B is indicated by a white arrow. (**b**) Immunoprecipitation. FLAG-tagged SEMA3B protein in CL of stSEMA3B is indicated by a white arrow. IgG that bound nonspecifically to the ANTI-FLAG M2 Affinity Gel serves as a control and confirms the equal loading of the samples. CL, cell lysate; CM, conditioned medium.

cell growth-related genes studied, *IGFBP-6* showed the greatest increase. As expected, IGFBP-6 protein was increased in CM of stSEMA3B, as was in CM of trSEMA3B, stSEMA3B-FLAG and trSEMA3B-FLAG (Figure 2c). IGFBP-6 has been shown to suppress the growth of cancer cells (Sueoka *et al.*, 2000; Leng *et al.*, 2001; Koike *et al.*, 2005) and thus may be an effector of tumor suppressor activity for SEMA3B. Next, we semiquantified the expression levels of *SEMA3B* and *IGFBP-6* in lung cancer cell lines (Figure 2d). The expression of *IGFBP-6* positively correlated with that of *SEMA3B* and was lower than that of normal lung tissue. These observations were consistent with the hypothesis that IGFBP-6 is an effector of SEMA3B and so we decided to further investigate the functions of IGFBP-6.

Suppression of colony formation by IGFBP-6
IGFBP-6 binds to and sequesters insulin-like growth factor II (IGF-II) (Bach *et al.*, 1994). IGF-II often acts as an autocrine growth factor stimulating the growth of some cancer cells, whereas IGFBP-6 suppresses their growth (Kato *et al.*, 1995). Therefore, IGFBP-6 acts as a tumor suppressor for such cancer cells. In addition to this IGF-II-dependent mechanism, IGFBP-6 may act by an IGF-II-independent mechanism to exert its tumor suppressor activity. This mechanism is as yet vaguely understood (Grellier *et al.*, 1998; Sueoka *et al.*, 2000). The effect of IGFBP-6 on the growth of NCI-H1299 cells was studied by measuring colony formation on the dish surface and in soft agar (Figures 3a and b). In both assays, the introduction of an IGFBP-6 expression plasmid significantly reduced the number of colonies, demonstrating that IGFBP-6 exerted a tumor suppressor activity on NCI-H1299 cells.

Inhibition of IGFBP-6 suppressed SEMA3B effects
Results obtained so far suggest that SEMA3B exerts a tumor suppressor activity by inducing the expression of IGFBP-6 as IGFBP-6 is an immediate cause of the activity. To confirm this, we investigated whether the inhibition of IGFBP-6 could abrogate the tumor suppressor activity of SEMA3B.

First, we used small interfering RNA (siRNA) to inhibit IGFBP-6. RT–PCR and western blot analyses showed that 10 nM of *IGFBP-6* siRNA efficiently inhibited the expression of IGFBP-6 (Figure 4a); so this concentration was used. Introduction of *SEMA3B* cDNA into NCI-H1299 cells increased the sub-G0/G1 population, reflecting the tumor suppressor activity of SEMA3B (Figure 4b). Transfection of *IGFBP-6* siRNA canceled this effect (Figure 4b(iii)), whereas that of scrambled siRNA did not (Figure 4b). Transfection of *IGFBP-6* siRNA but not scrambled siRNA also abrogated the growth inhibition by SEMA3B (Figure 4c). Second, we used IGF-II to inhibit IGFBP-6, as the amount of IGF-II higher than that of IGFBP-6 is able to sequester the growth suppressor activity of IGFBP-6 (Bach *et al.*, 1995). In the presence of 50 ng/ml of human IGF-II, the effect of IGFBP-6 was partially abrogated (Figure 4d). These results demonstrate that

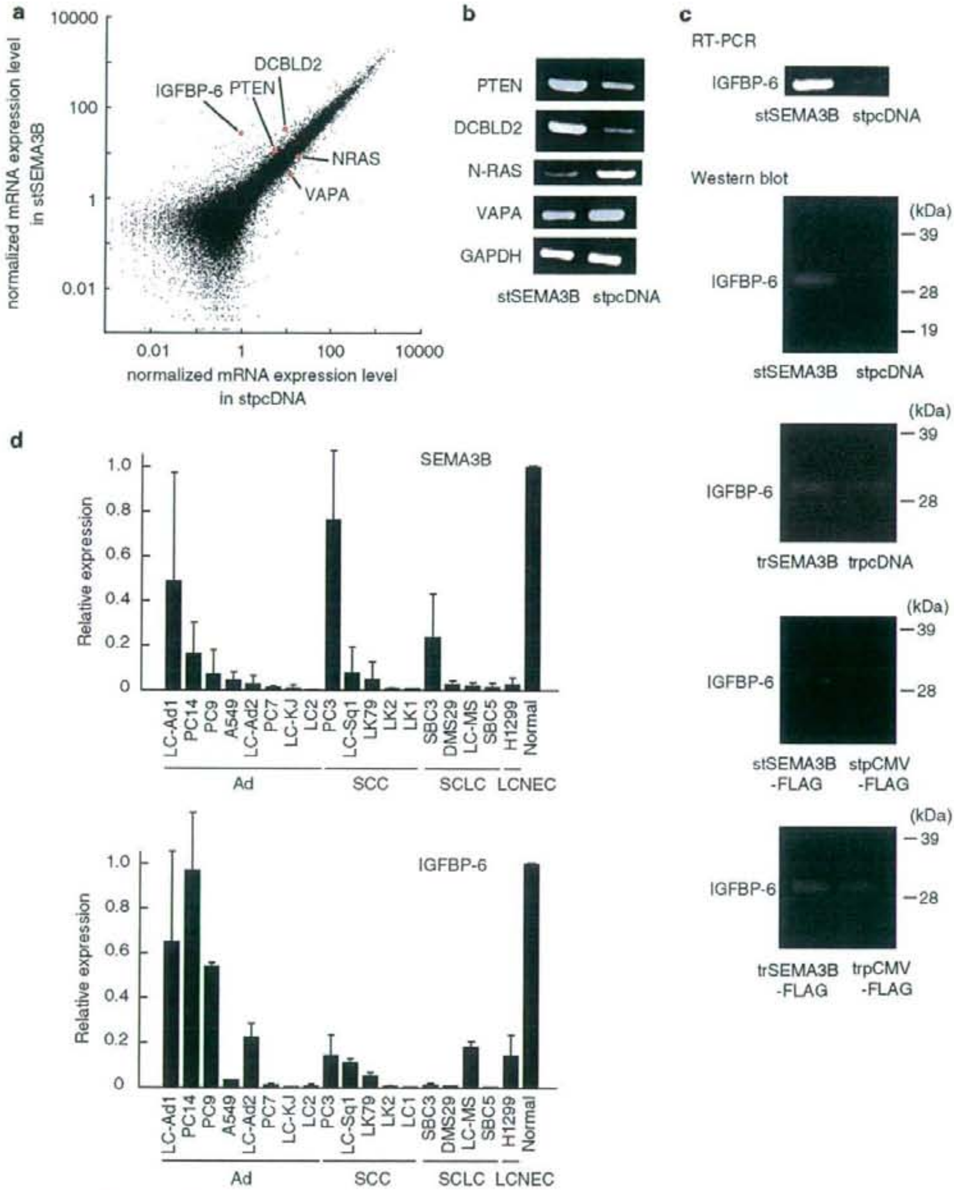

Figure 2 Identification of IGFBP-6. (**a**) A microarray analysis comparing the levels of each mRNA in stSEMA3B and stpcDNA. The spots for *IGFBP-6*, *DCBLD2*, *PTEN*, *NRAS* and *VAPA* are colored red. (**b**) Confirmation of the microarray data by RT–PCR. The results reproduce the microarray data. *GAPDH* mRNA is used as a control. (**c**) *IGFBP-6* expression by RT–PCR and that in CM observed by western blot. (**d**) Expression of *SEMA3B* and *IGFBP-6* in lung cancer cell lines. The expression level was semiquantitatively measured by real-time PCR and normalized to the level of *GAPDH*. The value for the normal lung tissue was set to 1.0. Error bars indicate standard deviations. Ad, adenocarcinoma; AdSq, adenosquamous cell carcinoma; CM, conditioned medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP-6, insulin-like growth factor-binding protein-6; LCNEC, large-cell neuroendocrine carcinoma; RT–PCR, reverse transcription PCR; SCC, squamous cell carcinoma; SCLC, small-cell lung cancer.

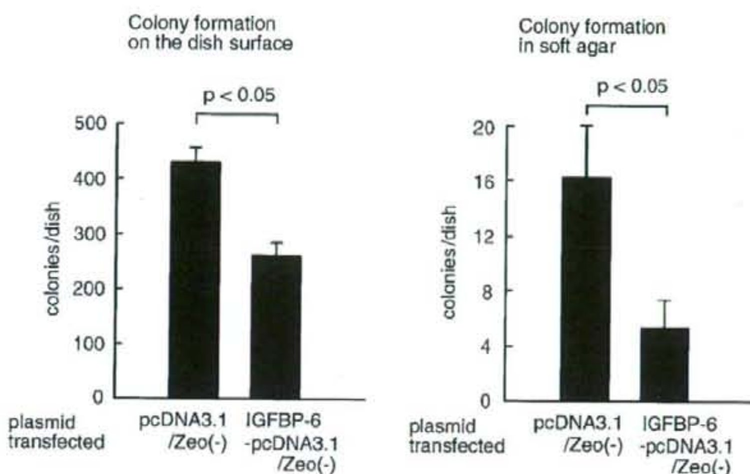

Figure 3 Colony formation on the dish surface and in soft agar. Numbers of colonies formed after the transfection of either the IGFBP-6 expression vector (IGFBP-6-pcDNA3.1/Zeo(−)) or an empty vector (pcDNA3.1/Zeo(−)) are shown. The medium contained 400 μg/ml Zeocin. Experiments were done in triplicates. Error bars indicate standard deviations. IGFBP-6, insulin-like growth factor-binding protein-6.

IGFBP-6 does mediate the tumor suppressor activity of SEMA3B.

Interaction of SEMA3B and β-catenin/TCF signaling
β-catenin is a key component of Wnt/TCF signaling. An accumulation of β-catenin in the cytoplasm activates TCFs and modifies the expression of downstream genes. Excessive activity in the β-catenin/TCF pathway is often observed in cancer cells (Nelson and Nusse, 2004; Clevers, 2006). In NCI-H1299 cells, TCF activity is induced by introducing *β-catenin* cDNA (Usami *et al.*, 2003). The *IGFBP-6* promoter has two copies of TCF-binding motifs, and β-catenin suppresses the expression of IGFBP-6 through these motifs (Denys *et al.*, 2004). These results suggest that, in NCI-H1299 cells, β-catenin may antagonize the tumor suppressor activity of SEMA3B by inhibiting the expression of IGFBP-6. To confirm this, we introduced *β-catenin* cDNA into stSEMA3B to establish stSEMA3B-β-catenin and investigated its effect. The introduction of *β-catenin* cDNA reduced the expression of IGFBP-6, which had been increased by the introduction of *SEMA3B* cDNA (Figure 5a). Moreover, the growth-suppressing activity of SEMA3B was partially abrogated (Figure 5b). These observations are consistent with the inference that β-catenin may antagonize SEMA3B, and indicate that SEMA3B signaling and β-catenin/TCF signaling converge at IGFBP-6 to affect the growth of NCI-H1299 cells.

Discussion

The aim of our study was to find out the as yet unidentified mechanism for the growth inhibition of lung cancer cells by SEMA3B. We have demonstrated that SEMA3B exerted its growth inhibitory effect through the induction of IGFBP-6 expression, and the effect was antagonized by β-catenin.

SEMA3B is an extracellular factor that binds to its receptor, transmits the signal into the cell and affects the cell growth. Signaling that is mediated by a membrane receptor often induces activation or inactivation of a specific transcription factor that changes the transcription of a variety of genes. The transcribed gene in turn exerts its function on cell growth. We speculate that a similar mechanism is at work for SEMA3B because SEMA3B increases the amount of *IGFBP-6* mRNA. Whatever the mechanism, the identification of the effector would have been a tremendous challenge without microarray technology. We focused only on growth-related genes to narrow the number of candidates to identify the effector. Similar approaches are found in the literature where the researchers employed a microarray and showed that peroxisome proliferator-activated receptor γ is a Zac target gene mediating Zac antiproliferation, and that heat shock protein 70 induced by the inhibitor of growth 1 sensitized cells to tumor necrosis factor-α receptor-mediated apoptosis (Barz *et al.*, 2006; Feng *et al.*, 2006). With an enrichment of functional information on individual genes, the strategy taken in this study and others will become much easier and more promising.

Members of the IGFBP family act on cell migration and growth in both IGF-dependent and IGF-independent mechanisms (Firth and Baxter, 2002). IGFBP-6 is unique among the family members as it provides a 20- to 100-fold higher affinity to IGF-II than to IGF-I (Martin *et al.*, 1990; Bach, 1999). The affinity is not so much differentiated in other members of IGFBPs. IGFBP-6 sequesters IGF-II, and thus inhibits growth in lung

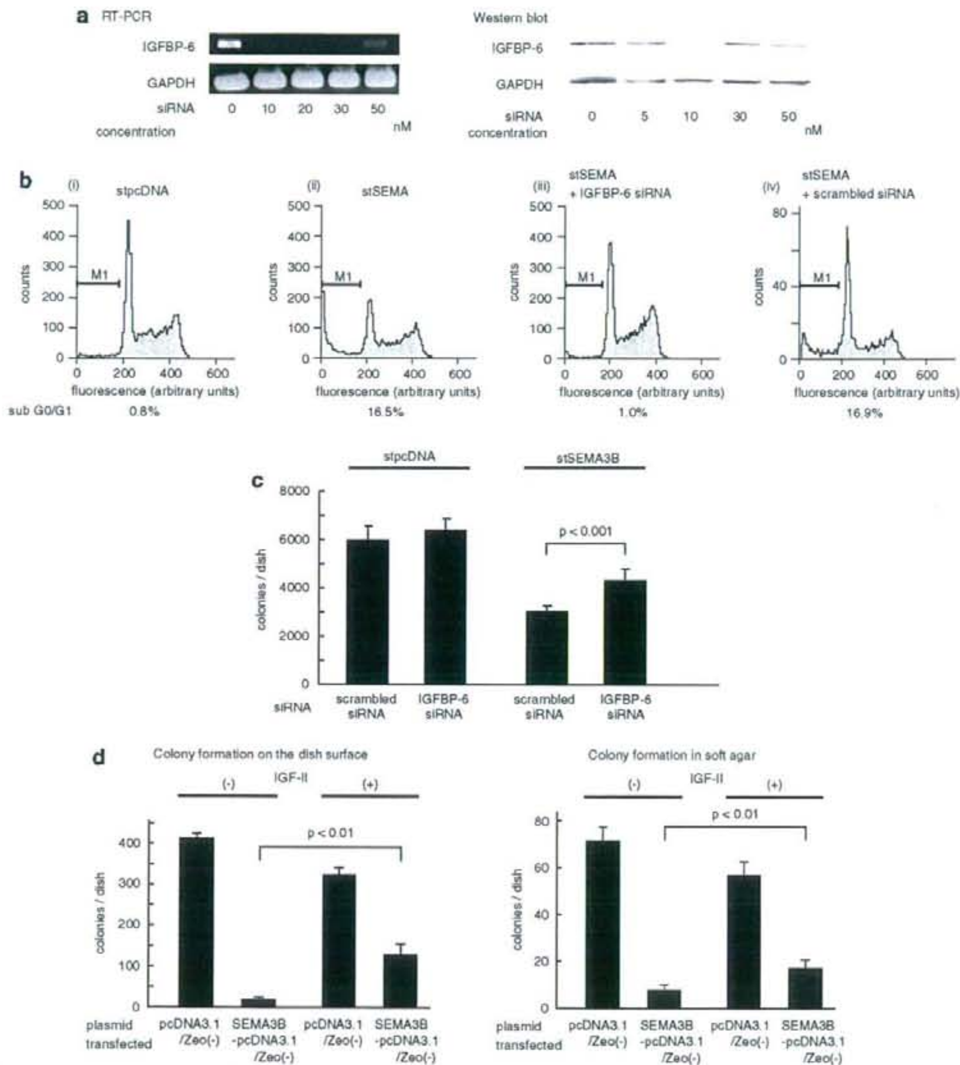

Figure 4 Effects of IGFBP-6 inhibition. (**a**) Optimization of the amount of siRNA monitored either by RT–PCR or by western blot. (**b**) The effect on cell cycle investigated by flow cytometry. The percent of cells that represent sub-G0/G1 fluorescence are indicated. (**c**) The effect on cell proliferation investigated by the cell growth assay. Cell growth suppressed by the introduction of SEMA3B is restored by *IGFBP-6* siRNA. (**d**) The effect on colony formation. Numbers of colonies formed on the dish surface or in soft agar after the transfection of either the SEMA3B expression vector (SEMA3B-pcDNA3.1/Zeo(−)) or an empty vector (pcDNA3.1/Zeo(−)). The medium contained 400 μg/ml of Zeocin together with either 0 or 50 ng/ml of human IGF-II. Experiments were done in triplicates. Error bars indicate standard deviations. IGF-II, insulin-like growth factor II; IGFBP-6, insulin-like growth factor-binding protein-6; RT–PCR, reverse transcription PCR; siRNA, small interfering RNA.

cancer (Sueoka *et al.*, 2000), colorectal cancer (Leng *et al.*, 2001), prostate cancer (Koike *et al.*, 2005), neuroblastoma (Grellier *et al.*, 1998) and rhabdomyosarcoma (Gallicchio *et al.*, 2001), whereas its expression is lower in malignant or metastatic tumors than in benign or non-metastatic tumors (Scholl *et al.*, 2000; Yao *et al.*, 2002). Moreover, the p53 tumor suppressor increases *IGFBP-6* expression (Kannan *et al.*, 2001). These results indicate that IGFBP-6 is a tumor suppressor for a variety of cancers. In our experiment, inhibition of IGFBP-6 by siRNA abolished the effect of SEMA3B on NCI-H1299 cells, indicating that the

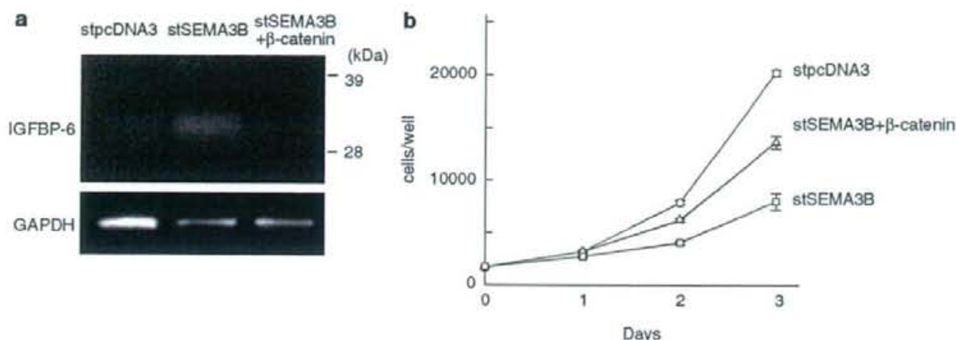

Figure 5 Inhibition of SEMA3B activity by β-catenin. (**a**) Change in the IGFBP-6 expression, western blot. IGFBP-6 expression increases after *SEMA3B* cDNA introduction is reduced by the introduction of *β-catenin* cDNA. (**b**) Change in cell growth. Cell growth suppressed by the introduction of SEMA3B is increased by the introduction of *β-catenin* cDNA. IGFBP-6, insulin-like growth factor-binding protein-6.

growth inhibitory effect of SEMA3B is mediated by IGFBP-6 and links the effects of these two tumor suppressors.

Pathways that transmit growth-related signals often converge on key molecules to antithetically affect their functions. We showed that IGFBP-6 is one of such key molecules where the signaling from SEMA3B and β-catenin converges. Semaphorin increases the expression of phosphatase protein homolog to tensin (PTEN) and activates glycogen synthase kinase-3β, resulting in the inhibition of integrin-mediated adhesion to the extracellular matrix (Tran *et al*., 2007). These results suggest that semaphorin signaling and β-catenin/TCF signaling may also converge at other points, which we have shown in this study.

Carcinogenesis requires both the activation and the inactivation of multiple signaling pathways, leading to the concept of multistep carcinogenesis. Further study into the interrelationship of SEMA3B signaling with other signaling pathways will contribute to our overall understanding of cancers.

Materials and methods

Cell lines

Lung cancer cell lines were obtained as follows. NCI-H1299 (large cell neuroendocrine carcinoma) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). PC-7 and PC-9 (adenocarcinomas) were purchased from IBL (Takasaki, Japan). RERF-LC-Ad1, RERF-LC-Ad2, RERF-LC-MS and PC-3 (adenosquamous carcinomas), as well as RERF-LC Sq-1 (squamous cell carcinomas) were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan). RERF-LC-KJ, LC2/ad and PC-14 (adenocarcinomas), and RERF-LC-A1 (squamous cell carcinomas) were purchased from the Riken Bioresource Center (Tsukuba, Japan). SBC3 and SBC5 (small cell carcinomas) were purchased from the Health Science Research Resources Bank (Osaka, Japan). A549 (adenocarcinoma), LK79 and LK2 (squamous cell carcinomas) were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). Cells were grown in RPMI medium 1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (CELLect GOLD; MP Biomedicals, Eschwege, Germany).

Plasmid construction and transfection

A plasmid, SEMA3B-pcDNA3.1/Zeo(−), that expresses wild-type SEMA3B protein was constructed by inserting human *SEMA3B* full-length cDNA into pcDNA3.1/Zeo(−) (Invitrogen, Carlsbad, CA, USA). A plasmid, SEMA3B-pCMV-Tag2A, that expresses N-terminal FLAG-tagged SEMA3B protein was constructed by inserting human *SEMA3B* full-length cDNA into pCMV-Tag2A (Stratagene, La Jolla, CA, USA). A plasmid, IGFBP-6-pcDNA3.1/Zeo(−), that expresses IGFBP-6 protein was constructed by inserting human *IGFBP-6* full-length cDNA into pcDNA3.1/Zeo(−). A plasmid, β-catenin-pcDNA3.1(+), that expresses β-catenin protein was constructed by inserting human *β-catenin* full-length cDNA into pcDNA3.1(+) (Invitrogen). These plasmids and the three appropriate negative control plasmids, pcDNA3.1/Zeo(−), pCMV-Tag2A and pcDNA3.1(+), were transfected into NCI-H1299 cells using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. In brief, a plasmid (1 μg) and FuGENE 6 (3 μl) were complexed in 100 μl Opti-MEM (Invitrogen) for 15 min. The complex was added to 3×10^5 NCI-H1299 cells seeded in a 35-mm dish and the cells were incubated for 48 h (transient transfectants). To establish the stable transfectants, transient transfectants were incubated in the medium containing 400 μg/ml Zeocin (Invitrogen) or 1 mg/ml Geneticin (Invitrogen) for 14 days. The drug-resistant colonies were cloned, and the clones were expanded. The names of the plasmids transfected and the names of the stable transfectants are paired below and are separated by commas: SEMA3B-pcDNA3.1/Zeo(−), stSEMA3B; SEMA3B-pCMV-Tag2A, stSEMA3B-FLAG; SEMA3B-pcDNA3.1/Zeo(−) and β-catenin-pcDNA3.1(+), stSEMA3B-β-catenin; pcDNA3.1/Zeo(−), stpcDNA; pCMV-Tag2A, stpCMV-FLAG. The names of the plasmids transfected and the names of the transient transfectants are SEMA3B-pcDNA3.1/Zeo(−), trSEMA3B; SEMA3B-pCMV-Tag2A, trSEMA3B-FLAG; SEMA3B-pcDNA3.1/Zeo(−) and β-catenin-pcDNA3.1(+), trSEMA3B-β-catenin; pcDNA3.1/Zeo(−), trpcDNA; pCMV-Tag2A, trpCMV-FLAG.

Western blot analysis
Cells (1×10^7) were plated in a 100-mm dish and incubated in 10 ml RPMI-1640 for 24 h. Cell lysate was prepared using mammalian cell lysis/extraction reagent (Sigma). Both the cell lysate and the cryoconcentrated conditioned medium were subjected to western blot analysis. Samples were dissolved in LDS sample buffer (Invitrogen), heated at 99 °C for 5 min, electrophoresed in NuPAGE 4–12% bis-Tris gel (Invitrogen) and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was immersed in 5% skim milk, 0.1% Tween 20 and dissolved in Tris-buffered saline to block the nonspecific binding. The membranes were incubated overnight at 4 °C with a primary antibody that included goat anti-human SEMA3B polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse anti-human IGFBP-6 monoclonal antibody (R & D Systems, Minneapolis, MN, USA) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Chemicon International Inc., Billerica, CA, USA). The membranes were then incubated for 1 h at 25 °C with a secondary antibody that included horseradish peroxidase-conjugated anti-goat, anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology). The specific signals were visualized by ECL plus (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Immunoprecipitation
Cells were harvested and sonicated in lysis buffer that contained Tris–HCl (50 mM), NaCl (150 mM), EDTA (0.1 mM), 1% Triton-X, 1% NP-40 and Complete Protease Inhibitor Cocktail Tablets (Roche). The FLAG-tagged recombinant protein was captured to the EZview Red ANTI-FLAG M2 Affinity Gel (Sigma) for 24 h at 4 °C. Bound proteins were eluted in an extraction buffer by boiling the gel at 100 °C for 5 min, electrophoresed in an SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The protein was detected as described in the section 'Western blot analysis' except that a mouse monoclonal ANTI-FLAG M2 (Sigma) was used as the primary antibody.

Microarray analysis
stSEMA3B was used as the reference cell line and stpcDNA was used as the control cell line. Total RNA was extracted from the cells by use of the Trizol reagent (Invitrogen). DNA from the RNA sample was completely removed by digesting with RQ1 RNase-free DNase (Promega, Madison, WI, USA), followed by phenol–chloroform extraction. Microarray analysis was performed using the Amersham CodeLink Bioarray Systems (GE Healthcare Bio-Sciences).

RT–PCR
IGFBP-6, *PTEN* and *discoidin, CUB and the LCCL domain containing 2* (*DCBLD2*), *neuroblastoma RAS viral (v-ras) oncogene homolog* (*NRAS*) and *vesicle-associated membrane protein-associated protein A* (*VAPA*) were used as probe genes and *GAPDH* as a control. Total RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). One microgram of cDNA was subjected to PCR (1 cycle of 95 °C for 3 min; 20–35 cycles of 95 °C for 30 s, 60 °C for 15 s and 72 °C for 1 min; 1 cycle of 72 °C for 7 min). The primers used were *IGFBP-6*-F, 5′-GCAGAGGAGAATCCTCCTAAGGAGAG-3′ and *IGFBP-6*-B, 5′-CAGCCAACACCAACACTCTTTC-3′; *PTEN*-F, 5′-CTGACACCACTGACTCTGATCC-3′ and *PTEN*-B, 5′-ACCCTTCGGAAACCTCTCTTAG-3′; *DCBLD2*-F, 5′-GCTGATGTAAACGGTTCGAGTG-3′ and *DCBLD2*-B, 5′-GTGACTACAGTACATGCCGAGGTA-3′; *NRAS*-F, 5′-CAGAGAACCAAACCGCAAAC-3′ and *NRAS*-B, 5′-AAACAGGCCTCTGGAAAACC-3′; *VAPA*-F, 5′-CTGACACCCAGTGTACCTACCTACC-3′ and *VAPA*-B, 5′-CACGGACTGAGATTGTTAGTGG-3′; and *GAPDH*-F, 5′-CCTCAACGACCACTTTGTCA-3′ and *GAPDH*-B, 5′-TTACTCCTTGGAGGCCATGT-3′. The ratios of the amounts of mRNAs were calculated by comparing the intensities of the specific bands measured by a densitometer.

Semiquantification of SEMA3B and IGFBP-6 mRNA by real-time PCR
Total RNAs from 17 human lung cancer cell lines and from normal human lung tissue (negative control; Clontech, Mountainview, CA, USA) were reverse-transcribed, mixed with SYBR Premix Ex Taq (Takara Inc., Shiga, Japan) and amplified by PCR (1 cycle of 95 °C for 120 s; 45cycles of 95 °C for 5 s and 62 °C for 20 s) using the Smart Cycler (Cepheid Innovation, Sunnyvale, CA, USA). The primers used were *SEMA3B*, 5′-GCGTGGAGTGGACTTTCCAG-3′ (forward) and 5′-CAGCCTGCGCAGCAGTAGTC-3′ (reverse); *IGFBP-6*, 5′-CGAGGGGCTCAAACACTCTA-3′ (forward) and 5′-CATCCGATCCACACACCAG-3′ (reverse); and *GAPDH*, 5′-CCTCAACGACCACTTTGTCA-3′ (forward) and 5′-TTACTCCTTGGAGGCCATGT-3′ (reverse). Each mRNA was quantified using its amplification data and then normalized using the amount of *GAPDH* mRNA.

Colony formation on the dish surface
Cells transfected with a plasmid and incubated for 48 h (see 'Plasmid construction and transfection' above) were replated into a 10-cm dish and cultured for 14 days in RPMI-1640 containing 10% fetal bovine serum and 400 μg/ml Zeocin. In the experiment investigating the effects of IGF-II on the growth inhibition through IGFBP-6, the medium also contained 0 or 50 ng/ml of human IGF-II (Strathmann Biotech Gmbh, Hamburg, Germany).

Colony formation in soft agar
NCI-H1299 cells (15 000 cells) transfected with either pcDNA3.1/Zeo(−) or SEMA3B-pcDNA3.1/Zeo(−) and incubated for 48 h (see 'Plasmid construction and transfection' above) were suspended in medium (RPMI-1640, 10% fetal bovine serum, 400 μg/ml Zeocin and 0 or 50 ng/ml of human IGF-II) containing 0.33% melted agar. The suspension was solidified on a presolidified medium containing 0.5% agar. The same medium was placed on top of the double agar layer and was renewed every 2 days. The cultures were incubated for 14 days and then the numbers of colonies were counted.

Transfection of small interfering RNA
The siRNAs for *IGFBP-6* were sense, 5′-CCCGCCCACAGGAUGUGAACC-3′ and antisense, 5′-UUCACAUCCUGUGGGCGGGCA-3′. These sequences were rearranged and used to design negative control siRNAs that were sense, 5′-CGCAUGCGGACUGCCAACACC-3′ and antisense, 5′-UGUUGGCAGUCCGCAUGCGCA-3′. Different concentrations of siRNA were transfected into stSEMA3B by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Total RNA or cellular protein was extracted 48 or 72 h after transfection, respectively.

Flow cytometry analysis
Seventy-two hours after the siRNA transfection, cells were harvested, fixed in 70% ethanol and suspended in phosphate-

buffered saline containing 20 μg/ml Ribonulease A (Sigma), 50 μg/ml propidium iodide, 0.1% sodium citrate and 0.3% NP-40. Flow cytometry was performed using the FACSCalibur equipped with CELLQUEST software (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell growth assay

One thousand cells per well of stpcDNA, stSEMA3B or stSEMA3B-β-catenin were seeded into 96-well dishes containing 100 μl of RPMI-1640 with 2% fetal bovine serum (day 0). On days 1, 2 and 3, 10 μl of Cell Counting Kit-8 reagent (DOJINDO, Kumamoto, Japan) was added to each well. Cells were then incubated for 3 h, and the absorbance at 450 nm/620 nm was measured. Five samples were analysed for each cell group. For the assay treated with siRNA, *IGFBP-6* or scrambled siRNA was transfected into 3000 cells per well of stpcDNA or stSEMA3B on day 1. The medium was changed to remove the siRNAs 6 h after transfection, and the cells were incubated for 72 h. On day 4, the absorbance was measured.

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E-cadherin expression and epidermal growth factor receptor mutation status predict outcome in non-small cell lung cancer patients treated with gefitinib

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Abstract. It is known that an epidermal growth factor receptor (EGFR) gene mutation(s) is present in a percentage of non-small cell lung cancers (NSCLCs). Gefitinib, an inhibitor of the tyrosine kinase activity of EGFR, is effective on most of them. The EGFR mutation status alone cannot fully predict the response to gefitinib and the prognosis for the patients. We hypothesized that information on the expression levels of phosphorylated-EGFR and -Akt, and E-cadherin, alone or in combination with information on the EGFR mutation, may refine our ability of prediction. We investigated 24 NSCLCs that had recurred after surgery and were treated with gefitinib. Specimens resected by surgery were subjected to the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp reaction to determine the EGFR mutation status, and to immunohistochemical staining of phosphorylated-EGFR and -Akt, and E-cadherin to determine their expression levels. The EGFR mutation status was predictive of responsive disease (complete response: CR + partial response: PR) and controlled disease (CR + PR + stable disease: SD). Positive E-cadherin staining was predictive of longer time to progression (12.4 vs. 5.9 months, $p<0.05$) and overall survival (OS) (18.4 vs. 13.0 months, $p<0.05$). Together the patients with an EGFR mutation and the patients with positive E-cadherin staining defined a patient group with a median OS of 18.4 months and excluded the patient group with the median OS of 3.7 months. Neither p-Akt nor p-EGFR staining was associated with the response and survival. In patients with surgically resected NSCLC tumors, the EGFR mutation status and E-cadherin staining can select patients who will benefit from gefitinib therapy.

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Key words: non-small cell lung cancer, gefitinib, E-cadherin, epidermal growth factor receptor mutation

Introduction

Gefitinib (ZD1839, Iressa, AstraZeneca, Wilmington, DE) is a therapeutic reagent for non-small cell lung cancers (NSCLCs). It shows dramatic anti-tumor effects in some patients, but has no effect in others (1). The presence of an epidermal growth factor receptor (EGFR) gene mutation(s) (hereafter EGFR mutation) associates significantly with the gefitinib responsiveness (2,3) and serves as a marker in the choice of therapeutic regimens (4). Some tumors with an EGFR mutation do not respond to gefitinib therapy while those with wild-type gene do (5-7), so additional markers are required to more precisely select tumors that respond to gefitinib.

EGFR transmits signals that direct cell proliferation and survival. The wild-type EGFR preferentially transmits cell proliferation signals through Erk, while the mutant EGFR preferentially transmits cell survival signals through Akt or STAT (8). Gefitinib effectively inhibits the latter (2). This is why gefitinib selectively elicits an apoptotic response in cells with an EGFR mutation, thereby producing its clinical response (8). We hypothesized that molecules that interact with EGFR or are located downstream in the pathway modify the tumor cell response to gefitinib and therefore serve as markers that may help to more precisely predict their responsiveness to gefitinib.

In this study, three molecules were tested for their predictive ability, p-EGFR (phosphorylated at Tyr1173: pTyr1173), p-Akt (phosphorylated at Ser473: pSer473) and E-cadherin, in addition to the EGFR mutation status. p-EGFR(pTyr1173) transmits a signal that directs cell proliferation (9), p-Akt(pSer473) mediates signals that direct cell survival (10) and E-cadherin has been shown to interact with EGFR by modifying its activity (11). We investigated the expressions of these three molecules by immunohistochemistry

in 24 NSCLCs that had been resected by surgery, recurred afterward and were treated with gefitinib. The results enabled us to test their staining intensity, alone, or in combination with the EGFR mutation status. It also improved our ability to predict the responsiveness to gefitinib and patient outcome.

Materials and methods

Patients. This study was approved by the Tsuboi Cancer Center Hospital ethics board. After the written informed consent was obtained, we enrolled 24 Japanese patients who had suffered from lung cancers which were resected between 1996 and 2004 (Tsuboi Cancer Center Hospital, Fukushima, Japan) and then had recurred. The patient characteristics are summarized in Table I. Gefitinib, 250 mg per day, was initiated between July 2002 and October 2006 to treat the recurrent disease. The median time between the surgery and the start of the gefitinib treatment was 740 days (range: 113-2.012). Treatment was continued until the disease progressed, intolerable toxicity developed or a patient refused treatment for other reasons.

Evaluation of the response to gefitinib and patient outcome. Every 4 weeks chest X-rays or computed tomography (CT) scans were done to evaluate tumor response and lung toxicity, and blood tests were done to monitor systemic toxicity. Tumor response that remained stable for at least 30 days was graded according to the Response Evaluation Criteria in Solid Tumors (12). Time to progression (TTP) in these patients was defined as the interval from the start of gefitinib administration to disease progression or death. The outcomes were evaluated up to May 31, 2007, with an average follow-up time of 20.6 months (range: 1.1-50.0). Both mutation and immunohistochemical analyses were performed after completion of the response evaluation.

DNA extraction and mutation analysis. DNA was extracted from the paraffin-embedded tumor tissue (13-15). EGFR mutations were detected using the peptide nucleic acid-locked nucleic acid (PNA-LNA) polymerase chain reaction (PCR) clamp. This method, which has been described in detail elsewhere, is a rapid and sensitive detection system for EGFR gene mutations and can detect point mutations G719C, G719S, L858R and L861Q and deletions in exon 19 in the presence of a 100- to 1.000-fold background of wild-type EGFR (4,6,16).

Immunohistochemistry and scoring. Formalin-fixed, paraffin-embedded tumor tissue was tested for immunoreactivity to p-EGFR, p-Akt and E-cadherin. The primary antibodies used were: anti-p-EGFR that detects EGFR protein phosphorylated at Tyr1173 (Cell Signaling Technology Beverly, MA), anti-p-Akt that detects Akt protein phosphorylated at Ser473 (Cell Signaling Technology) and anti-E-cadherin (BD Biosciences, Beverly, MA). Tissue sections cut at a thickness of 5 μm were placed on glass slides, deparaffinized and then rehydrated. Antigen was quantified using the following procedure. The slides were incubated in citrate buffer in a steamer for 15 min. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide for 5 min and non-specific background staining was blocked by incubation in a protein block for 5 min. Sections were then reacted with primary antibody dilutions (p-EGFR a 1/400 dilution at 37˚C for 15 min, p-Akt a 1/50 dilution at 4˚C for 16 h and E-cadherin a 1/100 dilution at 37˚C for 32 min). The bound antibody was detected by biotinylated secondary antibody and visualized using diaminobenzidine (DAB) chromogen. Sections were then counterstained with Mayer's hematoxylin and mounted using the resinous mounting medium.

The p-EGFR and p-Akt stainings were scored by their cytoplasmic and nuclear staining, while E-cadherin staining was scored by its membrane staining (17-19), all without the knowledge of clinical or laboratory information. The cytoplasmic and nuclear staining of the entire tumor was scored as follows: First, 500 randomly selected tumor cells (50 cells per randomly chosen microscopic field at x40 magnification) were scored as 0 (no staining), 1 (mild), 2 (moderate) or 3 (strong staining). Second, for p-EGFR, the most intense staining observed in >1% of the cells was the staining score for the tumor. For p-Akt, the mode of the cytoplasmic or nuclear staining score, whichever was greater, was the staining score for the tumor. Tumors with staining scores of 0 or 1 were ranked negative and scores of 2 or 3 were ranked positive. The membrane staining of the entire tumor was scored as 0 when no tumor cells were stained, 1 when <10%

Table I. Patient characteristics.

Characteristic	No. of patients (n=24)	%
Gender		
Male	13	54.2
Female	11	45.8
Median age, years (range)	63.2	(44-84)
ECOG performance status		
0	6	25.0
1	18	75.0
Histology		
Adenocarcinoma	21	87.5
Squamous cell carcinoma	1	4.2
Adenosquamous cell carcinoma	2	8.4
Prior chemotherapy		
0-1 regimens	18	75.0
>2 regimens	6	25.0
Smoking history		
Never smoked	19	79.2
Smoker (current/former)	5	20.8
Stage		
I-II	19	79.2
III-IV	5	20.8

ECOG, Eastern Cooperative Oncology Group.

Table II. Response to gefitinib therapy.

	CR	PR	SD	PD	NE
No. of patients	0	8	6	5	5
Median TTP (months)		16.1	9.3	1.0	
Median OS (months)		25.9	20.8	6.5	

TTP, time to progression; OS, overall survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated.

showed weak membrane staining, 2 (weakly positive) when >10% showed complete membrane staining although with weak to moderate intensity and 3 (strongly positive) when >10% had complete and strong membrane staining. Entire tumors with staining scores of 0 or 1 were considered negative while those scored as 2 or 3 were considered positive.

Statistical analyses. All statistical analyses were performed by StatView version 5 software (SAS institute Inc, Cary, NC). Comparisons of the proportions between two populations utilized the χ^2 test. Comparisons of patient outcome (TTP and overall survival, OS) between patient groups utilized the Kaplan-Meier method and the log-rank test. All statistical tests were two-sided and $P<0.05$ was considered significant.

Results

Response to the gefitinib and patient outcome. The responses to gefitinib are summarized in Table II. The responders [complete response: CR + partial response: PR, (8/24) 33%] had significantly longer TTP and OS than non-responders ($p<0.005$ and $p<0.05$, respectively). In addition, the patients with controlled disease [CR + PR + stable disease: SD (13/24) 54%] had significantly longer TTP and OS ($p<0.001$ and $p<0.001$, respectively). We found no significant differences in the OS between patients with PR and SD nor could we prove that CR + PR better defined patients who benefited from the therapy than CR + PR + SD. We therefore performed the analyses based on the two groupings.

Analyses of the EGFR mutation status and staining of p-EGFR, p-Akt and E-cadherin. We investigated the EGFR mutation status and the staining of p-EGFR, p-Akt and E-cadherin. We chose these proteins because they are intimately connected with the activity of EGFR and thus may predict responsiveness to gefitinib and/or patient outcome. In the mutation analysis, 10 patients were found to have an EGFR mutation: one had a point mutation L858R(T2573G), two had a deletion E746-A750del(2235-2249del), six had a deletion E746-A750del (2236-2250del), and one had a deletion L747-S752del, P753S(2240-2257del). All these mutations have been observed in gefitinib responders in the literature (2,3). Representative immunohistochemical staining is shown in Fig. 1 with the results summarized in Table III. The results of the EGFR mutation status are also shown. Positive p-Akt staining was associated with EGFR mutation, which is plausible because mutant EGFR stimulates the cell survival signal that is mediated by p-Akt. The staining intensity of p-EGFR and E-cadherin failed to show an association with the EGFR mutation and thus may be an independent parameter.

Predictors of the responsiveness to gefitinib. We then investigated the association between the expression of these proteins and the responsiveness to gefitinib (Table IV). The presence of an EGFR mutation significantly associates with responsive diseases (CR + PR) or controlled diseases (CR + PR + SD). This is consistent with the results presented in previous reports (20-22). We found no significant associations in the staining result for p-EGFR, p-Akt and E-cadherin.

Predictors of patient outcome. We compared the Kaplan-Meier curves to identify predictors of longer TTP and/or OS. As shown in Fig. 2A the positive staining of E-cadherin predicts a longer TTP (12.4 vs. 5.9 months, $p<0.05$) and longer OS (18.4 vs. 13.0 months, $p<0.05$). The presence of EGFR mutation(s) ($p=0.13$ and $p=0.11$, respectively, Fig. 2B), as well as p-EGFR and p-Akt staining intensity failed to predict outcome. We then looked at the EGFR mutation status in conjunction with the E-cadherin staining intensity as predictors of these same parameters. As shown in Fig. 2C in the right panel, the patients with EGFR mutation-positive tumors and those with E-cadherin-positive tumors defined a patient group with a median OS of 18.4 months and excluded the patient group with the median OS of 3.7 months, although we failed to show a significant difference in TTP (Fig. 2C, left panel). Therefore, we consider that the patients with EGFR mutation-positive or E-cadherin-positive tumors are the most likely to benefit from gefitinib therapy.

Discussion

It was shown that NSCLC tumors with an EGFR mutation(s) respond to gefitinib at a rate of 65 to 100% (5-7,20-24). Several prospective phase II studies have shown that gefitinib therapy significantly lengthened TTP in NSCLC patients with EGFR mutation-positive tumors (5-7). Thus far, no prospective studies have reported on OS. Several retrospective studies have suggested that gefitinib therapy may result in a longer OS in patients with EGFR mutation-positive tumors (20,21,23), however, we did not observe any significant differences in either TTP or OS. This is likely due to the size of the current study, as is discussed later.

We showed that positive E-cadherin staining is significantly associated with TTP and OS. Possible mechanisms that may explain this observation include that i) tumors with a lower E-cadherin expression progress faster than those with a higher expression and ii) E-cadherin modifies EGFR function and thus contributes to the effect of gefitinib treatment. The former mechanism is supported in reports that show that tumors with a positive E-cadherin staining are more frequent in early stage than in locally advanced or metastasizing NSCLCs (25-28). Similar results have been obtained in other malignancies such as the esophagus (29,30), stomach (31,32), colon (33),

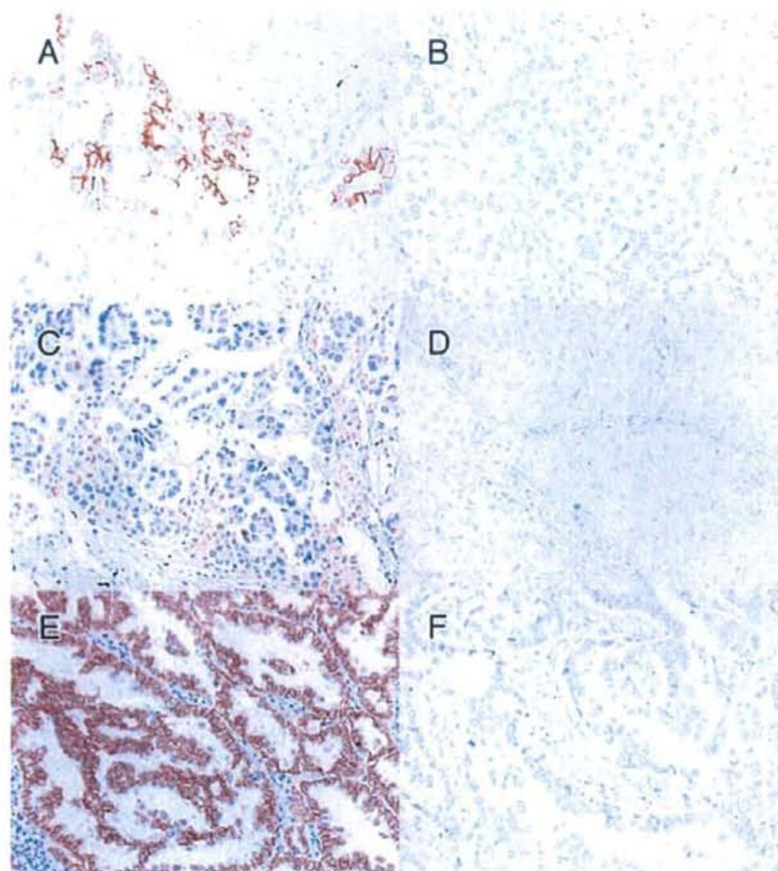

Figure 1. Immunohistochemistry. Positive (A) and negative (B) staining for p-EGFR; positive (C) and negative (D) staining for p-Akt; positive (E) and negative (F) staining for E-cadherin; magnification, x200.

Table III. EGFR mutation and staining of p-EGFR, p-Akt and E-cadherin.

	p-EGFR		p-Akt		E-cadherin	
	Positive	Negative	Positive	Negative	Positive	Negative
All patients	3	21	3	21	19	5
EGFR mutation						
Positive	2	8	3	7	9	1
Negative	1	13	0	14	10	4
P	0.35		<0.05		0.27	

EGFR, epidermal growth factor receptor; p-EGFR, phosphorylated-EGFR; p-Akt, phosphorylated-Akt.

liver (34), pancreas (35) and urinary bladder (36,37). Moreover, in NSCLCs, a positive E-cadherin expression associates with a more differentiated histology (26,28) and a better prognosis (25,27,28). The latter mechanism is supported by reports showing that E-cadherin interacts with EGFR, thereby decreasing ligand-affinity (38,39) and inhibiting activation (40) in several human tumor types including the esophageal, breast and lung (41-43). Mechanisms i) and ii) stated above are not mutually exclusive and both may contribute to a better prognosis.

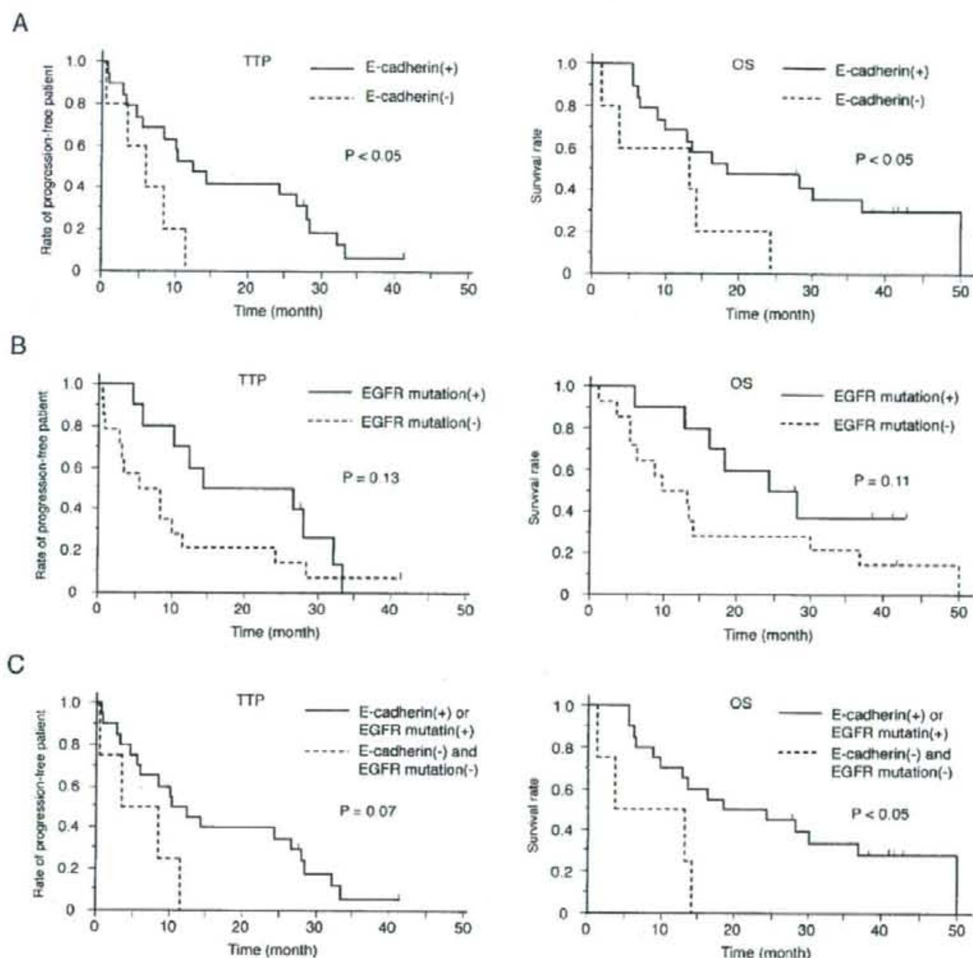

Figure 2. (A) Kaplan-Meier plots of TTP and OS where patients are grouped by the E-cadherin staining of their tumors. (B) Kaplan-Meier plots of TTP and OS where patients are grouped by the EGFR mutation status of their tumors. (C) Kaplan-Meier plots of TTP and OS where the two groups of patients have i) tumors which stain positively for E-cadherin or have an EGFR mutation(s) and ii) tumors which are negative for E-cadherin staining and EGFR mutation.

Table IV. Gefitinib response summarized by the EGFR mutation status and by the staining of p-EGFR, p-Akt or E-cadherin.

	EGFR mutation		p-EGFR		p-Akt		E-cadherin	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
All patients	10	14	3	21	3	21	19	5
Responsive disease (CR + PR)	6	2	1	7	1	7	6	2
P	<0.005		0.23		0.23		0.72	
Controlled disease (CR + PR + SD)	7	7	1	13	1	13	11	3
P	<0.05		0.54		0.54		0.95	

EGFR, epidermal growth factor receptor; p-EGFR, phosphorylated-EGFR; p-Akt, phosphorylated-Akt.

The current study warrants a larger one and presents an important question. We have six panels in Fig. 2, three of which showed significant differences and three of which did not. It is calculated that, if twice as many patients had been enrolled and had shown similar responsiveness and prognoses, all six sets of the two groups compared in Fig. 2 would have shown significant differences. To investigate this, a study should be scheduled where more than twice the number of patients is enrolled. We showed that tumors with a positive E-cadherin staining have a better prognosis after gefitinib therapy. It is, however, not clear whether the E-cadherin expression and EGFR mutation(s) contribute to it independently or synergistically. Basic and clinical researches addressing this issue may provide important information on the role of E-cadherin and EGFR in carcinogenesis.

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Interferon-β augments eosinophil adhesion-inducing activity of endothelial cells

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ABSTRACT: **Viral infections induce exacerbations of asthma. One of the earliest host responses to viral infections is the production of innate cytokines including type I interferons (IFNs), such as IFN-β, which may act to modify airway inflammation. The objective of the present study was to investigate whether IFN-β modifies the eosinophil adhesion-inducing activity of endothelial cells.**

Human umbilical vein endothelial cells (HUVECs) were stimulated with IFN-β for 24 h in the presence or absence of tumour necrosis factor (TNF)-α. Eosinophils were isolated from the peripheral blood of healthy volunteers. The ability of the IFN-β-stimulated HUVEC monolayers to induce eosinophil adhesion was assessed according to the eosinophil peroxidase assay.

Eosinophil adhesion to HUVECs was significantly augmented by IFN-β in the presence of TNF-α but not in its absence. The augmented adhesion was inhibited by anti-α_4 integrin monoclonal antibody (mAb) or anti-β_2 integrin mAb. IFN-β significantly enhanced the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 on HUVECs in the presence of TNF-α.

Interferon-β can augment the adhesiveness of endothelial cells to eosinophils, mainly through the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. This action of interferon-β may contribute to the intensification of airway inflammation in asthma that is associated with exacerbations induced by viral infections.

KEYWORDS: **Asthma, endothelial cells, eosinophilic airway inflammation, viral infection**

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STATEMENT OF INTEREST
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Acute respiratory infections commonly precede asthma exacerbations in both children and adults [1–3]. The majority of episodic exacerbations of asthma are induced by viral respiratory infections, in particular rhinovirus infections [4]. The mechanism by which viral respiratory infections exacerbate asthma is a complex process that may be regulated by the enhanced production of cytokines, chemokines and other classes of inflammatory molecules [4, 5]. An effective antiviral immune response requires the early clearance of viruses and the appropriate termination thereof, to minimise concomitant immunopathology and tissue damage. One of the earliest host responses to viral infections is the production of initial innate cytokines. These cytokines include type I interferons (IFNs) such as IFN-β [6, 7]. WARK *et al.* [7] recently reported that respiratory epithelial cells from asthmatics have a lower IFN-β-producing ability that is associated with a reduced ability to clear viruses. Since IFNs have a variety of pro-inflammatory actions on inflammatory cells, including eosinophils, epithelial cells and endothelial cells [8–12], it is theoretically conceivable that these cytokines may modify and aggravate the inflammatory status of airway diseases, including asthma, during or after viral infection.

Eosinophils are inflammatory cells predominantly found in the airways of asthmatic patients and are likely to contribute to the pathogenesis of asthma through the production of a variety of mediators including cysteinyl (cys) leukotriene (LT) and transforming growth factor-β [11–14]. Although neutrophils play central roles in asthma exacerbations induced by viral respiratory infections, clinical data support the involvement of eosinophils in virus-induced exacerbations and increased airway hyperresponsiveness in asthmatic patients [15–17]. In atopic asthmatics, for example, experimental infections with rhinovirus (RV)16 increased epithelial eosinophil counts; this increase appeared to persist up to convalescence [15]. In asthmatic patients with confirmed viral infection, sputum showed high eosinophilic cationic protein (ECP) levels [16]. In atopic mild asthmatics, increased airway hyperresponsiveness to histamine was correlated significantly with an increase in ECP levels and with changes in eosinophil levels in induced

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sputum after nasal administration of RV16 [17]. For eosinophils, the initial step in their participation in the airway inflammation of asthma is the adhesion of circulating eosinophils to vascular endothelial cells. It is generally accepted that this process is mainly mediated by the interaction between eosinophil integrin adhesion molecules (including α_4 integrins, such as $\alpha_4\beta_1$ (also known as CD49d, CD29 or very late activation antigen-4), and β_2 integrins, such as $\alpha_L\beta_2$ (CD11a/CD18/lymphocyte function-associated antigen-1) and $\alpha_M\beta_2$ (CD11b/CD18/macrophage-1 antigen)) and their counter ligands (vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1) on endothelial cells [18, 19].

The objective of the present study was to evaluate whether IFN-β modifies the adhesive interaction between eosinophils and endothelial cells.

MATERIAL AND METHODS

Reagents

Percoll® was obtained from Pharmacia (Uppsala, Sweden). Anti-CD16 antibody-coated magnetic beads were purchased from Miltenyl Biotec (Auburn, CA, USA). Human umbilical vein endothelial cells (HUVECs) and HuMedia EG were purchased from Kurabo Industries Ltd (Osaka, Japan). Endothelial cell growth medium was purchased from Clonetics Corporation (Palo Alto, CA, USA). Hanks' balanced salt solution (HBSS), PBS and newborn calf serum (NCS) were obtained from Life Technologies (Grand Island, NY, USA). Foetal bovine serum was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). Recombinant human (rh-) IFN-α, IFN-β, IFN-γ and tumour necrosis factor (TNF)-α were purchased from R&D Systems (Minneapolis, MN, USA). Anti-α_4-integrin monoclonal antibody (mAb; clone HP2/1) was purchased from Cosmo Bio Co. Ltd (Tokyo, Japan). Anti-β_2-integrin mAb (clone L130) was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Murine immunoglobulin (Ig)G1 was purchased from ICN Biomedicals, Inc. Anti-P-selectin glycoprotein ligand (PSGL)-1 (CD162) mAb (clone PL-1) was purchased from Immunotech, a Coulter company (Marseille, France). Other reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) unless otherwise stated.

Preparation of HUVECs

HUVECs were prepared as previously described [20]. Briefly, HUVECs were incubated on type IV collagen-coated tissue culture flasks until confluent, transferred to collagen-coated 96-well tissue culture plates and then stimulated either with a combination of IFN-β (30–1,000 pM) and TNF-α (10 pM) or with IFN-β (30–1,000 pM) alone in 5% CO_2 at 37°C for 24 h. After incubation, the incubated mixture was decanted and the HUVECs were washed three times with HBSS. In selected experiments, HUVECs were fixed with 100 μL of 1% paraformaldehyde in PBS at room temperature for 15 min, in order to block the synthesis of mediators. After washing three times in HBSS, 200 μL of 1% glycine in HBSS were added and incubated at ambient temperature for 1 h to quench any residual paraformaldehyde. The plates were then decanted and washed three times in HBSS before use.

Eosinophil isolation

Eosinophil isolation was performed according to the negative immunomagnetic bead selection method, as previously described [21]. To complete all the experiments, eosinophils were isolated from the peripheral blood of 42 healthy volunteers who were aged 20–29 yrs and had an equal sex distribution. Briefly, the heparinised blood was diluted with HBSS without Ca^{2+} and then centrifuged on Percoll® ($1.090\ g\cdot mL^{-1}$; $700\times g$ for 20 min). Plasma, mononuclear cell bands and Percoll® were removed, and red blood cells in the pellets were lysed by hypotonic shock. Granulocytes obtained were washed in 4°C HBSS supplemented with 2% NCS and then incubated with anti-CD16 antibody-coated magnetic beads at 4°C for 40 min. The cells were filtered through the steel wool column in a magnetic field (Miltenyl Biotec) to remove neutrophils bound to magnetic beads. CD16-negative eosinophils (>98% purity and >99% viability) were collected, washed and then resuspended in HBSS supplemented with 0.1% gelatin (HBSS/0.1% gelatin).

Eosinophil adhesion

Eosinophil adhesion to HUVECs was assessed by the residual eosinophil peroxidase activity of adherent eosinophils, as previously described [20–23]. Briefly, eosinophils (100 μL of 1×10^5 cells·mL^{-1} in HBSS/0.1% gelatin) were placed onto the HUVEC monolayers and then incubated at 37°C for 30 min. After five washes in 37°C HBSS, 100 μL of HBSS/0.1% gelatin were added to the reaction wells. As standards, 100 μL of serially diluted cell suspensions (1×10^3, 3×10^3, 1×10^4, 3×10^4 and 1×10^5 cells·mL^{-1}) were added to the empty wells. The o-phenylenediamine (OPD) substrate (1 mM OPD, 1 mM H_2O_2 and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all the wells. After incubation at room temperature for 30 min, 50 μL of 4 M H_2SO_4 were added to stop the reaction and absorbance at 490 nm was determined. The percentage of eosinophil adhesion was calculated from the log dose–response curve. Eosinophil viability after incubation, which was determined by trypan blue dye exclusion, exceeded 98%.

Determination of VCAM-1 and ICAM-1 expression on HUVECs

The expression of VCAM-1 and ICAM-1 was determined by cell ELISA, as previously reported [20, 24]. Briefly, the HUVEC monolayers were incubated in the 96-well tissue culture plates and then stimulated with either a combination of IFN-β (30–1,000 pM) and TNF-α (10 pM) or with IFN-β (30–1,000 pM) alone at 37°C for 24 h [20, 24]. Prior to the evaluation, HUVECs were washed and incubated at 37°C for 30 min with a blocking buffer (PBS containing 5% NCS and 3% nonfat dry milk). Primary antibodies (obtained from R&D Systems), *i.e.* anti-ICAM-1 mAb (clone BBIG-I1), anti-VCAM-1 mAb (clone BBIG-V1) and isotype-matched control murine IgG1, were added to the wells and the incubation was then resumed at 37°C for a further 2 h. HUVECs were washed three times in the blocking buffer and secondary antibody (peroxidase-conjugated sheep anti-murine IgG) was added to the wells. Following a 2-h incubation, cells were washed three times in PBS. The peroxidase conjugate was detected using the OPD substrate in the citrate-urea buffer according to a procedure similar to that used in the eosinophil adhesion assay. The VCAM-1 or ICAM-1 concentration in cells was expressed as absorbance at