Imatinib mesylate inhibits the proliferation-stimulating effect of human lung cancer-associated stromal fibroblasts on lung cancer cells

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Abstract. Platelet-derived growth factor (PDGF) is a significant mediator in the proliferation of cancer-associated stromal fibroblasts (CAFs). The inhibition of CAF proliferation by blocking PDGF signaling could lead to a development of novel cancer therapy. We analyzed whether inhibiting proliferation of lung CAFs by Imatinib mesylate, which has inhibitory activity on PDGF-receptor tyrosine kinase, could suppress the proliferative activity of lung cancer cells which coexisted in the tumor tissue. First, we established primary cultured fibroblasts from human lung cancer tissues. RT-PCR analysis showed that PDGF-receptors (PDGFRα and ß) were more highly expressed in the fibroblasts, whereas PDGFs (PDGF-A, and -B) were more in lung cancer cell lines. Western blotting showed that Imatinib treatment inhibited phosphorylation of PDGFRß, Akt, and Erk1/2 in the fibroblasts. The treatment also significantly inhibited the proliferative activity of the fibroblasts. The inhibitory effects were exerted more definitely in co-administering Imatinib and PDGF-BB, a dimer of the polypeptide chains of B, than in administering Imatinib alone. The conditioned media of the fibroblasts significantly increased the proliferative activity of human lung cancer cell line A549 compared to control culture medium. The proliferation-stimulating effect on A549 cells decreased significantly in the conditioned media of the primary cultured fibroblasts that had been treated with Imatinib. Our results suggest that Imatinib has antitumor activity which is exerted by reducing the proliferation-stimulating effect of CAFs on lung cancer cells, as well as inhibiting the proliferation of CAFs, by way of blocking PDGF signaling.

Introduction

Lung cancer is the most common cause of cancer death with its high incidence and poor prognosis in developed countries; in the United States, it accounted for the deaths of 160,000 patients in 2008 (1). Despite radical resection of the primary tumor and adjuvant chemotherapy, a large number of lung cancer patients suffer from the recurrences (2-4). The poor response to chemotherapy necessitates a development of novel therapeutic strategies to delay recurrences or treat the progressive disease.

Tumor tissue is composed of not only cancer cells but also various stromal cells, such as fibroblasts, vascular cells, and inflammatory cells (5). It has been demonstrated that the behavior of tumor depends not only on genetic and epigenetic alterations in the transformed cells but also on tumor microenvironment (6-8). In many solid tumors, fibroblasts are a major component of tumor stroma, and those in tumor stroma express a myofibroblastic phenotype characterized by constant expression of alpha-smooth muscle actin (α-SMA), and are called cancer-associated stromal fibroblasts (CAFs) (9,10). Tumor microenvironmental factors, including growth factors and proteolytic enzymes produced and secreted by stromal cells such as CAFs, influence various tumorigenic steps: proliferation, invasion, angiogenesis and metastasis (11-14).

Platelet-derived growth factor (PDGF) is shown to be a potent mitogen for fibroblasts and a significant mediator in the development of stroma in various types of malignant tumors (15-17). It plays a role as a dimer of the polypeptide chains of A, B, C, or D (PDGF-AA, -AB, -BB, -CC, and -DD). The PDGF isoforms interact with two tyrosine kinase receptors, PDGFRα and ß; PDGFRα binds all isoforms except PDGF-DD, whereas PDGFRß binds only PDGF-BB and PDGF-DD

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with high affinity (18). PDGFRß is reported to be expressed on stromal cells in different tumor tissues (16,19-21).

Imatinib mesylate, also known as Gleevec, Glivec or STI 571, is a molecular-targeting agent to treat chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST) (22). In CML, the molecular target of Imatinib is c-Abl tyrosine kinase, a Bcr-Abl fusion protein, caused from t(9;22) chromosomal translocation, and in GIST the target is c-Kit tyrosine kinase constitutively activated by gain-of-function mutation (23-25). Furthermore, Imatinib inhibits the tyrosine kinase activity of PDGFR; it is also reported that treatment with Imatinib can suppress the PDGF-mediated growth of cancer cells and CAFs in *in vitro* and *in vivo* experiments (26-30).

In this study, we analyzed the effects of Imatinib on CAFs, which are exerted by blocking the PDGFR tyrosine kinase activity, and examined the possibility of targeting CAFs as a cancer therapy.

Materials and methods

Materials and cell lines. Recombinant human platelet-derived growth factor-BB (PDGF-BB) was purchased from PeproTech EC (London, UK). Imatinib mesylate (registered as Gleevec or Glivec) was purchased from LKT Laboratories (St. Paul, MN, USA) and was reconstituted in dimethyl sulfoxide (DMSO). The stock solution was diluted with DMSO to the appropriate concentrations and immediately administered to the cells for each experiment. The same amount of DMSO (vehicle) was used for negative controls. Human cell lines used for this study were obtained from different institutes; lung carcinoma A549 and adult skin fibroblast TIG-112 were from Health Science Research Resources Bank (Tokyo, Japan), colon carcinoma SW480 from Cell Resource Center for Biomedical Research (Tohoku University), lung carcinomas H23 and H522 from National Cancer Institute, National Institutes of Health (Frederick, MD, USA), lung carcinoma H1299 from American Type Culture Collection (ATCC) (Manassas, VA, USA), and embryonic lung fibroblast WI-38 from Riken Bioresource Center (Tsukuba, Japan). All cell lines were grown in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) (Wako) which contained 10% fetal bovine serum (FBS) (Cambrex, Walkersville, MD, USA). Monoclonal mouse antibody raised against human alpha-smooth muscle actin (α-SMA) was supplied by Sigma-Aldrich (1A4; Saint Louis, MO, USA). Antibodies specific for PDGFRß, phospho-PDGFRß, Akt, phospho-Akt Erk1/2, and phospho-Erk1/2 were obtained from Cell Signaling Technologies (3169, 3166, 9272, 9271,9102, and 9101, respectively; Danvers, MA, USA).

Primary culture of human lung fibroblasts. The samples were obtained from seven patients undergoing lung resection for lung cancer with the approval of the local ethics committee after obtaining written informed consents from the patients. For isolation of primary cultured fibroblasts from normal and cancer tissues of human lung, minced fragments of the lung tissue were incubated in DMEM/F-12 mixed with 3,000 U/ml dispase (Sankojunyaku, Tokyo, Japan) and 500 U/ml collagenase Type IV (Sigma-Aldrich) for 30 min at room temperature. The primary cultured fibroblasts were grown in DMEM/F-12 + 10% FBS which contained 50,000 U/ml penicillin and 50,000 μg/ml streptomycin, and incubated at 37˚C in 5% CO_2-atmosphere. We named the primary cultured fibroblasts from normal lung tissues respectively FK-1, 2, 3 and 4, and those from lung cancer tissues CK-1, 2, 3 and 4. Experiments were performed on those between passages 4 and 7.

Preparation of conditioned media. For obtaining conditioned media, the fibroblasts (TIG-112, WI-38, FK-1, 2, 3 and 4, CK-1, 2, 3 and 4) were respectively seeded in a 10-cm culture dish in DMEM/F-12 + 10% FBS, and were grown to 80-90% confluence. Thereafter, they were incubated in serum-reduced media (DMEM/F-12 + 0.5% FBS) for 4 h, and then in 10 ml DMEM/F-12 + 0.5% FBS for 48 or 96 h after media exchange. Subsequently, the supernatants were collected. They were clarified by centrifugation at 1200 μg for 5 min, and were used fresh as the conditioned media or stored at -80˚C until use.

Immunohistochemistry. Paraffin-embedded serial tissue sections of 5-μm thickness were dewaxed, rehydrated, quenched with 0.3% H_2O_2/methanol, and blocked with 10% goat serum. The primary antibody for α-SMA was applied at a dilution of 1:200 for 60 min at room temperature. Immunohistochemical reactions were visualized with the Histofine Simple Stain MAX-PO (Multi) and DAB substrate Kit (Nichirei, Tokyo, Japan). The sections were counterstained with hematoxylin. Immunostaining of negative controls was carried out by replacing the primary antibody with mouse preimmune IgG (Dako, Glostrup, Denmark).

RNA extraction and RT-PCR analysis. Total RNA was extracted from monolayer cultures of fibroblasts or cancer cell lines with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Total RNA of 1 μg was reverse transcribed by using Random-pdN6 Primer and M-MLV reverse transcriptase (Invitrogen). It was then amplified by using polymerase chain reaction (PCR) as follows: 2 μl template complimentary DNA (cDNA), 0.2 mM dNTPs, 1.25 U/μl Go Taq polymerase (Promega, Madison, WI, USA) and different primer sets (0.5 μM each) in 50 μl water; 30 sec heat denaturing at 95˚C, 30 sec annealing at the melting temperature, and 1-min elongation at 72˚C in 30 cycles. The termination cycle included an extension at 72˚C for 5 min. The primers were purchased from Frontier Science (Ishikari, Japan). The primer sequences and their melting temperatures are presented in Table I. PCR products were electrophoresed in a 2.0% agarose gel and photographed under UV.

Protein extraction, SDS PAGE and Western blotting. For studying the effects of Imatinib and PDGF-BB on protein expression, the primary cultured fibroblasts FK-1 and CK-1 were serum-starved (0.05% FBS) for 48 h, treated with different concentrations of Imatinib (1 or 10 μM) for 4 h, and then either stimulated or not stimulated with 50 ng/ml of PDGF-BB for 10 min. The concentrations of Imatinib were relevant to the clinical concentrations of the drug. Thereafter, the cells were washed with ice-cold PBS and lysed in TNE buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40,

Table I. Sequence of primer sets for RT-PCR.

Target gene	Primer	Sequence	Tm	Product size
α-SMA	Forward	5'-CCAACCGGGAGAAAATGACTCAAATT-3'	55	389
	Reverse	5'-AGGCAACTCGTAACTCTTCTCAAGG-3'		
FAP	Forward	5'-CCAGCAATGATAGCCTCAA-3'	50	472
	Reverse	5'-TAACACACTTCTTGCTTGGA-3'		
PDGF-A	Forward	5'-CCCCTGCCCATTCGGAGGAAGAG-3'	62	227
	Reverse	5'-TTGGCCACCTTGACGCTGCGGTG-3'		
PDGF-B	Forward	5'-CCTTTGATGATCTCCAACGC-3'	59	350
	Reverse	5'-GATCTTTCTCACCTGGACAG-3'		
PDGFRα	Forward	5'-GAAGCTGTCAACCTGCATGA-3'	58	187
	Reverse	5'-CTTCCTTAGCACGGATCAGC-3'		
PDGFRß	Forward	5'-GTGAACGCAGTGCAGACTGT-3'	58	220
	Reverse	5'-AGGTGTAGGTCCCCGAGTCT-3'		
GAPDH	Forward	5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'	60	983
	Reverse	5'-CATGTGGGCCATGAGGTCCACCAC-3'		

Tm, melting temperature.

1 mM EDTA) mixed with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma-Aldrich). The protein in the samples was measured by a modified Lowry method, with the use of DC Protein Assay (Bio-Rad, Hercules, CA, USA), and 10 µg of total protein was separated by SDS-PAGE using 7.5% polyacrylamide gels. Following electroblotting the protein onto PVDF membranes, immunodetection was carried out with a 1:1000 dilution of respective primary antibodies for PDGFRß, phospho-PDGFRß, Akt, phospho-Akt Erk1/2, and phospho-Erk1/2, and a 1:20000 dilution of goat anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (GE Healthcare UK, Buckinghamshire, UK). Antigen-antibody complexes were visualized with chemi-luminescence HRP substrate (Millipore, Billerica, MA, USA) and LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

WST-8 cell proliferation assay. Human lung cancer cell lines A549, H23 and H522 were seeded (2,500 cells/well) in a 96-well plate and maintained for 24 h in DMEM/F-12 + 10% FBS. After pre-incubation in DMEM/F-12 + 0.5% FBS for 4 h, the cancer cells were incubated for 48 or 96 h either in DMEM/F-12 + 0.5% FBS (control) or in the conditioned media that had been generated as described earlier. Cell proliferative activity was assessed by the colorimetric WST-8 assay (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions.

For studying the effects of Imatinib and PDGF-BB on cell proliferation, the primary cultured fibroblasts FK-1 and CK-1 were seeded (2,500 cells/well) in a 96-well plate and maintained for 48 h in DMEM/F-12 + 10% FBS. The fibroblasts were pre-incubated in DMEM/F-12 + 0.5% FBS for 4h, followed by incubation in DMEM/F-12 + 0.5% FBS with different concentrations of Imatinib (1 or 10 µM), with or without PDGF-BB (20 ng/ml), for 72 h. WST-8 assay was performed thereafter. The final concentration of DMSO was 0.1% in each experiment, and any effect on cell proliferation was excluded by appropriate control measurements as indicated in the figures.

Statistical evaluation of data. Data are presented as means ± standard deviation (SD). Differences between groups were compared by Kruskal-Wallis H-test and Mann-Whitney U test. P<0.05 was considered significantly different.

Results

Expression of α-SMA in surgical specimens of human lung carcinoma. We first confirmed the presence of CAFs in lung carcinoma by immunohistochemistry. In 14 human lung carcinoma samples, we analyzed the expression of α-smooth muscle actin (α-SMA), a molecular marker of CAFs. The representative images of α-SMA staining are shown in Fig. 1. Expression of α-SMA was observed in stromal cells of all 14 specimens, whereas the expression was not detected in cancer cells (Fig. 1A-D). In the cases of advanced lung carcinoma, α-SMA was strongly expressed in tumor stroma. The α-SMA-positive fibroblasts were spindle-shaped and complicatedly surrounding cancer cells (Fig. 1A and B). In bronchioloalveolar carcinoma, a layer of α-SMA-positive
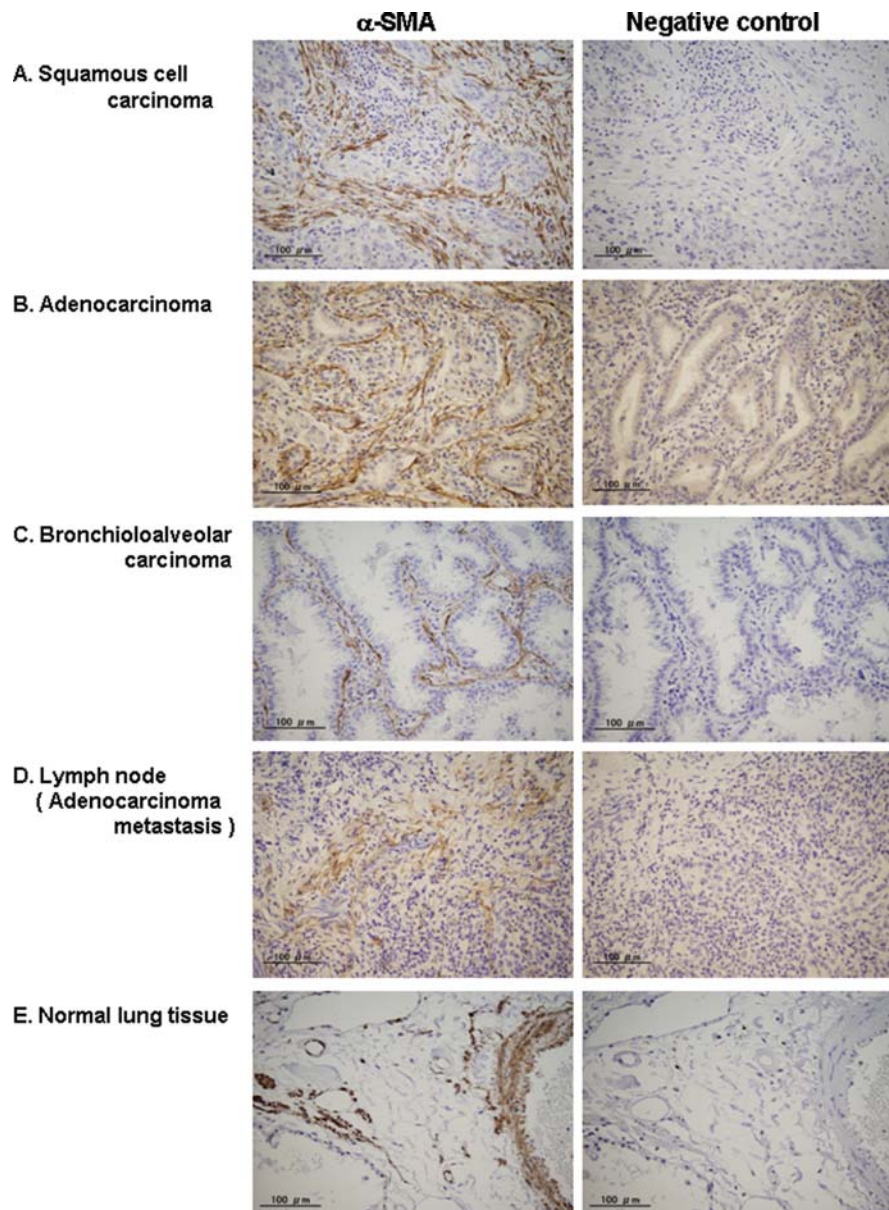
Figure 1. Immunohistochemistry of serial sections of human lung cancer tissues against α-SMA. The immunostaining for α-SMA shows a myofibroblastic phenotype of CAFs that are localized within the stroma. α-SMA was strongly expressed in tumor stroma, but was not detected in cancer cells. Bronchial and vascular smooth muscle cells physiologically expressed α-SMA in normal lung tissue.

fibroblasts was present, lining cancer cells (Fig. 1C). Furthermore, in lymph node metastasis as well, α-SMA-positive fibroblasts were observed in the adjacency of cancer cells (Fig. 1D). In normal lung tissue, however, no immunoreaction to α-SMA was observed in stroma, while it was observed in bronchial and vascular smooth muscle cells, which are known to physiologically express α-SMA (Fig. 1E).

Expressions of PDGF-receptor (PDGFR) and PDGF in fibroblasts and lung cancer cells. In order to investigate the effects of Imatinib on CAFs in lung cancer tissue *in vitro*, we established the primary cultured fibroblasts as described in Materials and methods, FK-1, 2, 3, and 4 derived from four normal lung tissues and CK-1, 2, 3, and 4 from four lung cancer tissues. By using RT-PCR, we analyzed the expressions of α-SMA and fibroblast activation protein (FAP), which are molecular markers of CAFs, in the fibroblasts. As shown in

Fig. 2, both α-SMA and FAP were expressed in all the primary cultured fibroblasts.

We next examined by RT-PCR the expressions of the PDGFRα, ß and PDGF-A, B in the cultured fibroblasts and lung cancer cell lines. As shown in Fig. 2, all the 10 fibroblasts examined expressed both PDGFRα and ß, whereas all the four lung cancer cell lines examined were hardly positive for either transcript. Expression of PDGF-A was observed in all the fibroblasts; however, that of PDGF-B was not in any of them. The expressions of PDGF-A and -B were higher in the lung cancer cell lines than in the fibroblasts.

Imatinib inhibits tyrosine kinase activity of PDGFR and the downstream signal transduction of PDGFR in fibroblasts. Having confirmed that PDGF-BB induced the tyrosine kinase activity of PDGFRß in fibroblasts, we investigated whether Imatinib inhibited the induced kinase activity. After serum-
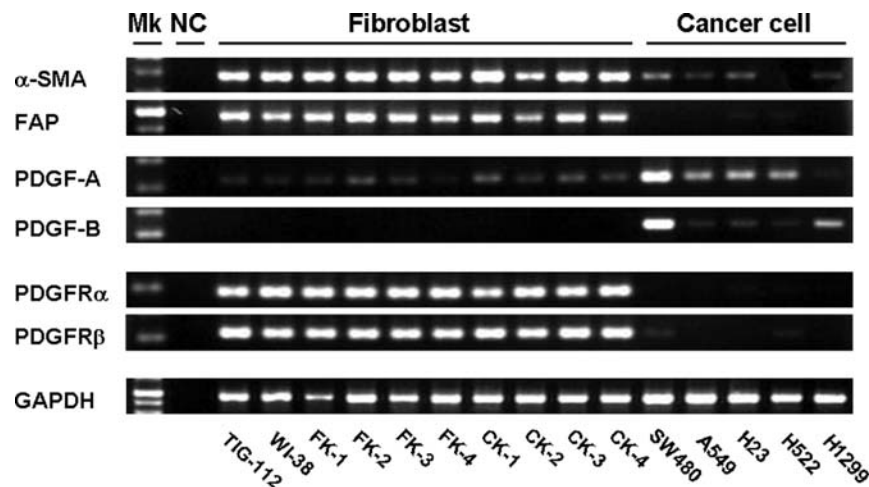
Figure 2. RT-PCR analysis of the expressions of α-SMA, FAP, PDGFs and PDGF-receptors in ten cultured fibroblasts and five cancer cell lines. Three independent experiments were performed on each cultured fibroblast and cancer cell line with similar results. Representative results are shown. SW480, a colon cancer cell line, was used as positive control for PDGFs. The expression of GAPDH was confirmed as internal control. Mk, molecular weight marker; NC, negative control (without template).
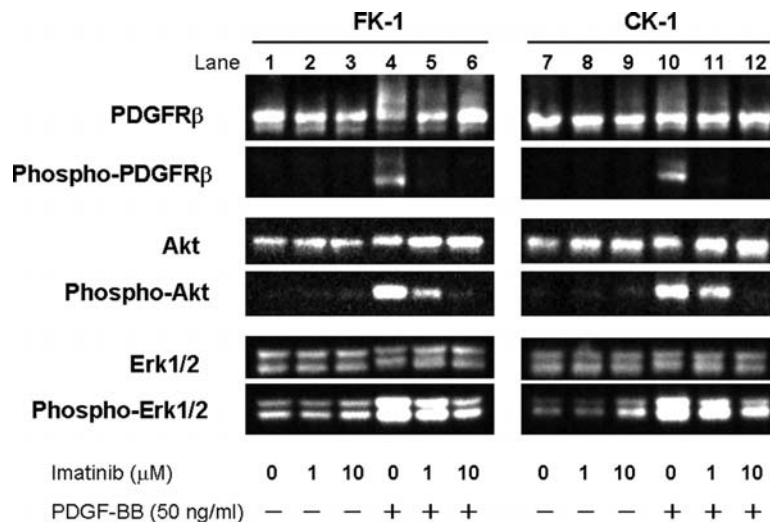

Figure 3. Effects of Imatinib on the activation of PDGFRß, protein kinase Akt and Erk1/2 in fibroblasts. The phosphorylation of protein was evaluated by SDS-PAGE and Western blotting. PDGF-BB induced phosphorylation of PDGFRß in the primary cultured fibroblasts FK-1 and CK-1. The phosphorylation of PDGFRß was almost completely inhibited by 1 μM Imatinib. PDGF-BB-induced phosphorylation of Akt and Erk1/2 was significantly reduced by Imatinib in both the fibroblasts. Three independent experiments were performed on both types of fibroblasts with similar results.

starvation (0.05% FBS, 48 h), the primary cultured fibroblasts FK-1 and CK-1 were treated with 1 or 10 μM Imatinib for 4 h and then stimulated with 50 ng/ml PDGF-BB for 10 min. The protein lysates of the fibroblasts were subjected to Western blotting with anti-PDGFRß or anti-phosphorylated PDGFRß (Tyr751). As clearly shown in Fig. 3, PDGF-BB induced PDGFRß phosphorylation in both types of fibroblasts (Fig. 3, lanes 4 and 10 of PDGFRß). The phosphorylation of PDGFRß was almost completely inhibited by 1 μM Imatinib, while the total amount of PDGFRß protein did not change significantly (Fig. 3, lanes 5 and 11 of PDGFRß).

It is known that activated PDGFRs activate the downstream signaling of PI3K-Akt and Ras-MAPK(Erk1/2) to promote cell growth. Therefore, we examined the activation levels of Akt and Erk1/2 in PDGF-BB-stimulated fibroblasts. PDGF-BB expectedly induced phosphorylation of Akt and

Erk1/2 in both the fibroblasts (Fig. 3, lanes 4 and 10 of Akt and Erk1/2, respectively). We then investigated the effects of Imatinib on the activation of Akt and Erk1/2 by PDGF-BB. The treatment with Imatinib resulted in significant reductions of the phosphorylation of Akt and Erk1/2 in both the fibroblasts in a concentration-dependent manner (Fig. 3, lanes 5, 6, 11 and 12 of Akt and Erk1/2, respectively).

Imatinib inhibits the PDGF-induced proliferation of fibroblasts. We next examined the effects of Imatinib on cell proliferation of the cultured fibroblasts, since we had observed that Imatinib inhibited the kinase activity of PDGFR and the downstream signal transduction. The primary cultured fibroblasts FK-1 and CK-1 were incubated in serum-reduced media (DMEM/F-12 + 0.5% FBS) with two different concentrations of Imatinib (1 or 10 μM), with or without PDGF-BB
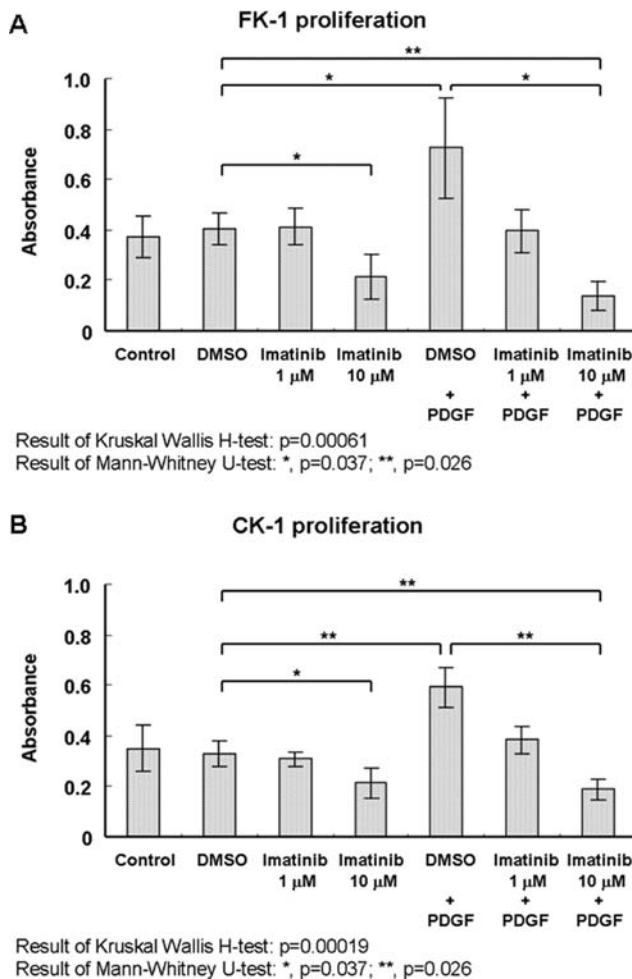
Figure 4. Effects of different concentrations of Imatinib on proliferative activity of fibroblasts. Cell proliferative activity was measured by WST-8 assay. The medium that contained both Imatinib and PDGF-BB exerted more inhibition on the proliferative activities of the primary cultured fibroblasts, (A) FK-1 and (B) CK-1, than the one with Imatinib alone. Three independent experiments were performed on both the fibroblasts with similar results. Values represent the means ± SD of quadruplicate measurements. Results were statistically evaluated by Kruskal-Wallis H-test and Mann-Whitney U test. P<0.05 was considered significantly different.
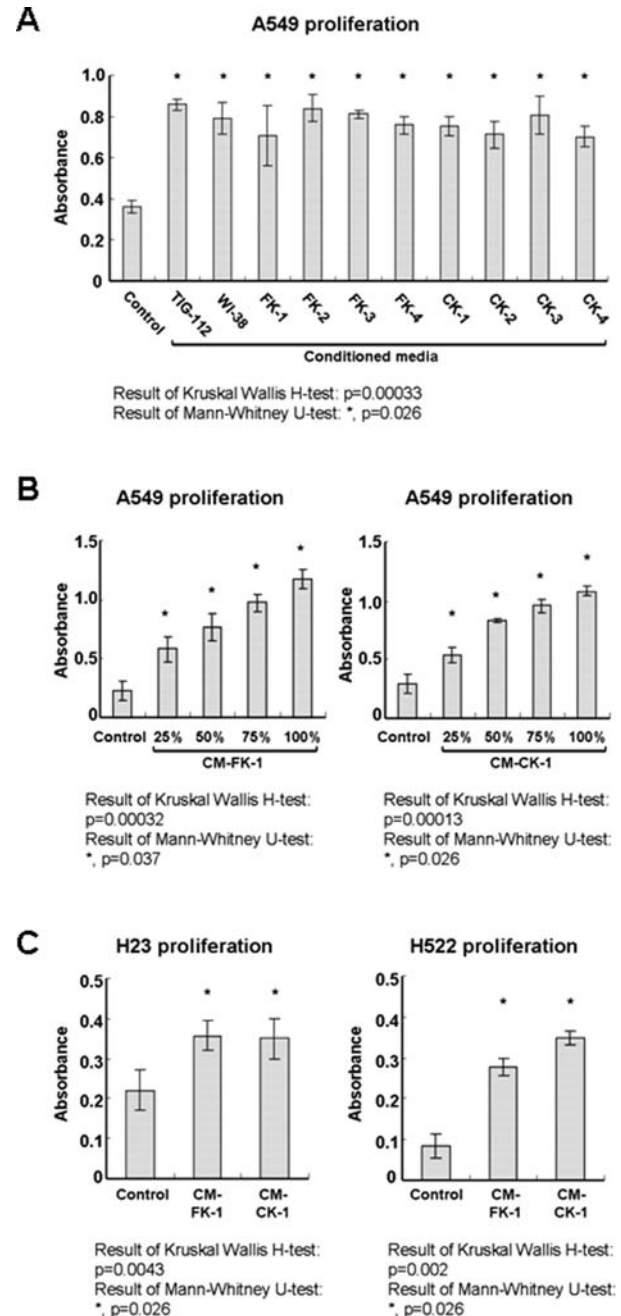

Figure 5. Effects of fibroblast-derived supernatants on proliferative activity of lung cancer cells. Cell proliferative activity was measured by WST-8 assay. (A) The conditioned media (CM) of all the fibroblasts promoted the proliferative activity of A549 cells. (B) The CM of the primary cultured fibroblasts, FK-1 and CK-1, promoted the proliferative activity of A549 cells in a dose-dependent manner. (C) The CM of FK-1 and CK-1, respectively promoted the proliferative activities of H23 and H522 cells. Each experiment was performed at least three times with similar results. Values represent the means ± SD of quadruplicate measurements. Results were statistically evaluated by Kruskal-Wallis H-test and Mann-Whitney U test. P<0.05 versus control was considered significantly different.

(20 ng/ml), for 72 h. The cell proliferation was measured by WST-8 assay thereafter. As shown in Fig. 4, proliferation of both the fibroblasts was significantly decreased to about a half in 10 μM Imatinib treatment compared with that in DMSO (p<0.05), although 1 μM Imatinib did not lead to a significant decrease. Statistically significant 1.5- to 1.7-fold increases of proliferation were observed in PDGF-BB (+DMSO) treatment compared with that in DMSO alone (p<0.05). However, Imatinib significantly inhibited the cell proliferative activity promoted by PDGF-BB in a concentration-dependent manner (p<0.05). There was no significant difference in the cell proliferation between control and DMSO.

Conditioned media of cultured fibroblasts increase the proliferative activity of lung cancer cells. In order to study the functional effects of lung CAFs on the proliferation of lung cancer cells, we examined the proliferation-stimulating effect of the supernatants of cultured fibroblasts on lung cancer cells. We prepared the conditioned media (CM) of the 10

cultured human fibroblasts (TIG112, WI-38, FK-1, 2, 3, 4, and CK-1, 2, 3, 4). Human lung cancer cell line A549 was incubated in the respective CM for 48 h, and their proliferative activities were measured by WST-8 assay. As shown in Fig. 5A, all the CM significantly increased the proliferation of A549 cells to 1.9- to 2.3-fold in comparison to control medium (DMEM + 0.5% FBS) (p<0.05). The CM of FK-1 and
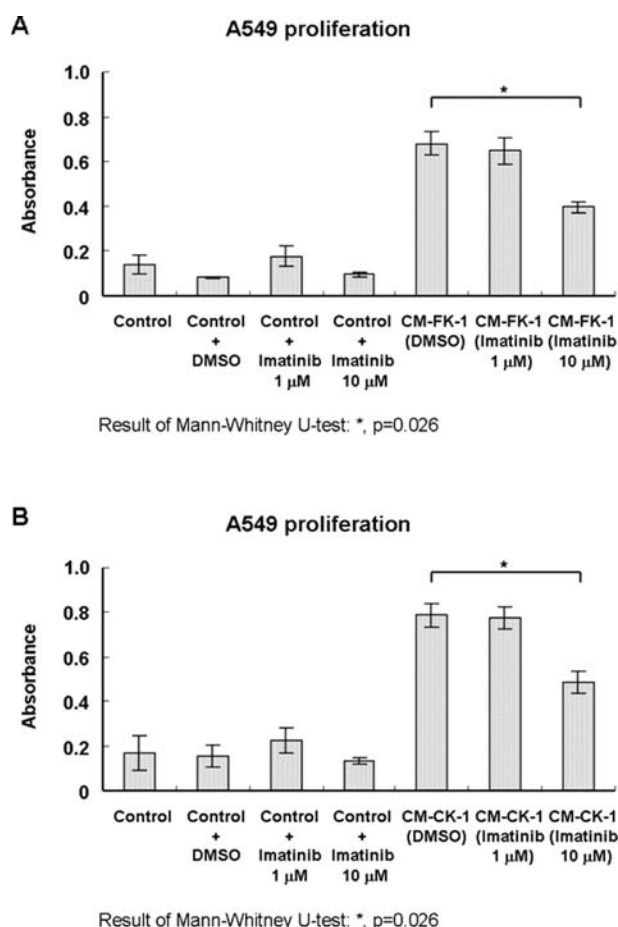
Figure 6. Effects of the conditioned media (CM) of Imatinib-treated fibroblasts on the proliferative activity of A549 cells. The CM were obtained from the fibroblasts, (A) FK-1 and (B) CK-1, which had been treated with different concentrations of Imatinib. Cell proliferative activity was measured by WST-8 assay. The CM of DMSO-treated fibroblasts increased the proliferative activity of A549 cells in comparison to control medium (DMEM/F-12 + 0.5% FBS) with DMSO. The cell proliferation was significantly decreased after 72-h incubation in the CM of 10 μM Imatinib-treated fibroblasts, compared with that in the CM of DMSO-treated fibroblasts. Three independent experiments were performed on both the fibroblasts with similar results. Values represent the means ± SD of quadruplicate measurements. Results were statistically evaluated by Mann-Whitney U test. P<0.05 was considered significantly different.

CK-1 respectively increased the proliferation of A549 cells significantly in a concentration-dependent manner (Fig. 5B). Likewise, the proliferation of two other lung cancer cell lines, H23 and H522, was significantly increased after 96-h incubation in the CM of FK-1 and CK-1 respectively, in comparison to control medium (Fig. 5C) (p<0.05).

Imatinib reduces the proliferation-stimulating effect of fibroblasts on cancer cells. Finally, we investigated whether Imatinib inhibited the proliferation-stimulating effect of lung fibroblasts on lung cancer cells. The primary fibroblasts FK-1 and CK-1 were respectively treated with DMSO, 1 μM Imatinib or 10 μM Imatinib, for 96 h, and then the conditioned media (CM) were obtained as described in the Materials and methods. A549 cells were incubated in the respective CM for 72 h, and then their proliferative activities were measured by WST-8 assay. As shown in Fig. 6, the CM of DMSO-treated

fibroblasts significantly increased the proliferation of A549 cells in comparison to control medium (DMEM/F-12 + 0.5% FBS) with DMSO. However, the cell proliferation was significantly decreased to 58-61% after 72-h incubation in the CM of 10 μM Imatinib-treated fibroblasts compared with that in the CM of DMSO-treated (p<0.05), although there was little decrease after incubation in the CM of 1 μM Imatinib-treated. The proliferation of A549 cells was not significantly inhibited by the incubation in control medium to which 1 or 10 μM Imatinib had been added directly, compared with that in control medium with DMSO.

Discussion

It has been shown that tumorigenesis is influenced and controlled by complicated cellular interactions between cancer cells, stromal cells, and extracellular matrix (ECM) components. There is also growing evidence that tumor stroma plays a significant role in cancer formation (11-14). Fibroblasts are a major component of tumor stroma in many solid tumors. It is suggested therefore that inhibition of fibroblast activity could lead to suppression of tumor development and progression (28-31).

We particularly noted the two reports that Imatinib has anti-fibroblastic effects on bleomycin-induced pulmonary fibrosis in a mouse model (32) and that the effects of Imatinib are exerted by blocking PDGFRs (33). Based on these reports, we suspected that inhibiting the proliferation of lung cancer-associated stromal fibroblasts (CAFs) by Imatinib might indirectly suppress the proliferation of lung cancer cells.

Our results of the cell proliferation assay *in vitro* showed that the conditioned media of lung CAFs (CK-1, 2, 3, and 4) increased the proliferation of lung cancer cells (Fig. 5). The results indicated that the conditioned media of CAFs contained some soluble growth stimulants which promoted the proliferative activity of lung cancer cells. Our results of immunohistochemistry showed that CAFs accounted for most of the lung cancer tissue and were present in the close vicinity of cancer cells, surrounding them (Fig. 1). Taken together, these findings suggested that the growth stimulants produced by CAFs in lung tumor promoted the proliferation of lung cancer cells in a paracrine manner. Furthermore, other reports have shown that growth factors and angiogenic factors, such as HGF, FGFs and VEGF, were induced or up-regulated in CAFs and that the up-regulation promoted proliferation and progression of cancer cells (34-38). Conversely, paracrine stimulation by PDGFs secreted from cancer cells has been shown to induce CAFs in tumor stroma to recruit and proliferate in *in vivo* transplant tumor models of melanoma (15), breast carcinoma (17), colorectal carcinoma (39), and lung carcinoma (40). Other reports of immunohistochemical analysis of human colorectal and breast cancer tissues suggested that stromal fibroblasts responded to PDGFs in a paracrine manner (39,41,42). Those reports showed that cancer cells in tumors, lacking the expression of PDGFRs, expressed PDGFs, whereas stromal fibroblasts expressed PDGFRs. Consistent with these findings, our results of RT-PCR analysis showed that PDGFs were more highly expressed in the lung cancer cell lines, whereas PDGFRs were expressed in all the cultured fibroblasts (Fig. 2). Our results also showed that PDGF-BB stimulated the kinase

activity of PDGFRß (Fig. 3) and increased the proliferative activity of the primary cultured lung fibroblasts *in vitro* (Fig. 4). Namely, these data indicate that the PDGF signaling pathways can play an important role in stromagenesis, and subsequently in cancer progression.

Imatinib mesylate is known to selectively inhibit the tyrosine kinase activity of PDGFR in addition to those of c-Abl and c-Kit (23-25). The present study also demonstrated that Imatinib blocked the kinase activity of PDGFR and suppressed the downstream signaling (Fig. 3), which inhibited the *in vitro* proliferation of CAFs of human lung cancer tissue (Fig. 4). In particular, the inhibitory effect of proliferative activity of CAFs was exerted more definitely in the medium that contained both Imatinib and PDGF-BB than in the medium with Imatinib alone. These findings indicate that Imatinib inhibits cell proliferative activity predominantly by blocking activated (phosphorylated) PDGFR. Furthermore, our results showed that the conditioned medium of the CAFs that had been treated with Imatinib had a decreased effect on the proliferation of lung cancer cells in comparison to that of the CAFs untreated with Imatinib (Fig. 6). The findings indicate that Imatinib treatment decreased the concentration of some soluble growth stimulants secreted by CAFs in the conditioned media, resulting in a decrease of proliferative activity of lung cancer cells. We suspect that the decrease was mainly due to the reduced number of CAFs because of blocking PDGF signaling by Imatinib, based on the findings that Imatinib inhibited DNA synthesis in CAFs (28) and increased the incidence of apoptosis in CAFs (29). However, we have not yet been able to identify the soluble growth stimulants secreted by CAFs and involved in the increase of proliferation of lung cancer cells. As an experimental material, primary cultured fibroblasts have the advantage that they retain the complexity and heterogeneity of mesenchymal cell biology and differentiation *in vivo*, since they contain endothelial and inflammatory cells at the culture establishment. On the other hand, it is difficult to definitely identify the cellular origin of CAFs because of the heterogeneity. Considering the heterogeneity, we could suggest that the phenotypes of the cultured CAFs were not uniform, and that the CAFs with differential phenotypes respectively secreted various growth stimulants; and thus blocking PDGFRs could inhibit only a part of these growth stimulants.

A recent report showed that blocking PDGF signaling in CAFs by Imatinib reduced proliferation and anigiogenesis of cervical cancer in an *in vivo* mouse model, through a mechanism suppressing the expressions of fibroblast growth factor-2 (FGF-2) and fibroblast growth factor-7 (FGF-7) (30). Another report suggested that stromal cell-derived factor-1 (SDF-1) secreted from CAFs played an important role in tumor growth and angiogenesis of invasive human breast carcinoma (13). We performed RT-PCR analysis to examine the expressions of FGF-2, FGF-7, and SDF-1 in the primary cultured lung fibroblatsts FK-1 and CK-1, and found that the expressions of these growth factors and cytokines were not affected by Imatinib treatment (data not shown). We suspect that growth factors different from these are involved in lung carcinoma.

There was no significant difference in our results between the primary cultured fibroblasts CK-1 used as CAFs and

FK-1 used as normal lung fibroblasts (Figs. 2-6). Despite their origin of normal lung tissue, FK-1, 2, 3, and 4 expressed myofibroblastic phenotypes, α-SMA and FAP, and their conditioned medium had the proliferation-stimulating effects on lung cancer cells. A previous *in vitro* study reported that stromal cells isolated from human normal breast tissue expressed α-SMA in culture medium with serum in four or five days (43); the authors discussed that all stromal cells derived from normal human breast tissue retained the potentiality to alter the phenotypes of typical fibroblasts to those of myofibroblasts. They also argued that the phenotypic alteration was induced by external stimuli, such as serum in medium and tumorigenesis, and that the alteration was a general response of stromal cells. In accordance to their explanation, myofibroblastic phenotypes were observed in FK-1, 2, 3, and 4 in our study. The findings suggest that normal fibroblasts of the host could be the origin of CAFs, and that normal fibroblasts could easily convert to CAFs under a culture condition.

CAFs themselves are expected to be a candidate target for a new cancer therapy (44). It has been suggested that interstitial fluid pressure (IFP) of tumor stroma causes difficulty for anticancer drugs in reaching cancer cells effectively, since IFP of tumor stroma is higher due to rich CAFs and ECM than that of normal stroma, which is a typical property of most solid tumors (45). Tumor interstitial hypertension, which high IFP often causes, has been documented clinically in many types of solid tumors, e.g., breast carcinoma, metastatic melanoma, head and neck carcinoma, and liver metastases of colorectal carcinoma (46-48). Other reports suggested that blocking PDGFRs by Imatinib inhibited the growth of tumor stroma and increased drug uptake by decreasing IFP to enhance the transvascular transport of drugs in *in vivo* tumor models (49,50). In these studies, Imatinib treatment in combination with other anticancer drugs decreased the tumor growth, while no effects were observed on the tumor growth in Imatinib treatment alone. Our data also indicate that Imatinib inhibits the proliferation of CAFs, suggesting that chemotherapy combined with Imatinib could improve the therapeutic effects and prognosis of lung cancer.

In conclusion, we have demonstrated that Imatinib has antitumor activity which is exerted by reducing the proliferation-stimulating effect of CAFs on lung cancer cells, as well as inhibiting the proliferation of CAFs themselves, by way of blocking PDGF signaling. We expect that targeting tumor stroma, particularly CAFs, and its mechanisms will offer a new direction and potential for future cancer therapies.

Acknowledgements

References

1. Jemal A, Siegel R, Ward E, *et al*: Cancer statistics, 2008. CA Cancer J Clin 58: 71-96, 2008.
2. Asamura H, Goya T, Koshiishi Y, *et al*: A Japanese Lung Cancer Registry study: prognosis of 13,010 resected lung cancers. J Thorac Oncol 3: 46-52, 2008.
3. Mountain CF: Revisions in the International System for Staging Lung Cancer. Chest 111: 1710-1717, 1997.

4. Tsuboi M, Ohira T, Saji H, et al: The present status of post-operative adjuvant chemotherapy for completely resected non-small cell lung cancer. Ann Thorac Cardiovasc Surg 13: 73-77, 2007.
5. Tremblay G: Stromal aspects of breast carcinoma. Exp Mol Pathol 31: 248-260, 1979.
6. Fidler IJ: Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. Cancer Res 50: 6130-6138, 1990.
7. Tlsty TD: Stromal cells can contribute oncogenic signals. Semin Cancer Biol 11: 97-104, 2001.
8. Bhowmick NA, Neilson EG and Moses HL: Stromal fibroblasts in cancer initiation and progression. Nature 432: 332-337, 2004.
9. Gabbiani G, Ryan GB and Majne G: Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. Experientia 27: 549-550, 1971.
10. Ronnov-Jessen L, Petersen OW, Koteliansky VE and Bissell MJ: The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J Clin Invest 95: 859-873, 1995.
11. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD and Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 59: 5002-5011, 1999.
12. Okusa Y, Ichikura T and Mochizuki H: Prognostic impact of stromal cell-derived urokinase-type plasminogen activator in gastric carcinoma. Cancer 85: 1033-1038, 1999.
13. Orimo A, Gupta PB, Sgroi DC, et al: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121: 335-348, 2005.
14. Hasebe T, Sasaki S, Imoto S and Ochiai A: Highly proliferative fibroblasts forming fibrotic focus govern metastasis of invasive ductal carcinoma of the breast. Mod Pathol 14: 325-337, 2001.
15. Forsberg K, Valyi-Nagy I, Heldin CH, Herlyn M and Westermark B: Platelet-derived growth factor (PDGF) in onco-genesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. Proc Natl Acad Sci USA 90: 393-397, 1993.
16. Lindmark G, Sundberg C, Glimelius B, Pahlman L, Rubin K and Gerdin B: Stromal expression of platelet-derived growth factor beta-receptor and platelet-derived growth factor B-chain in colorectal cancer. Lab Invest 69: 682-689, 1993.
17. Shao ZM, Nguyen M and Barsky SH: Human breast carcinoma desmoplasia is PDGF initiated. Oncogene 19: 4337-4345, 2000.
18. Betsholtz C, Karlsson L and Lindahl P: Developmental roles of platelet-derived growth factors. Bioessays 23: 494-507, 2001.
19. Bhardwaj B, Klassen J, Cossette N, et al: Localization of platelet-derived growth factor beta receptor expression in the periepithelial stroma of human breast carcinoma. Clin Cancer Res 2: 773-782, 1996.
20. Kawai T, Hiroi S and Torikata C: Expression in lung carcinomas of platelet-derived growth factor and its receptors. Lab Invest 77: 431-436, 1997.
21. Ebert M, Yokoyama M, Friess H, Kobrin MS, Buchler MW and Korc M: Induction of platelet-derived growth factor A and B chains and over-expression of their receptors in human pancreatic cancer. Int J Cancer 62: 529-535, 1995.
22. Jones RL and Judson IR: The development and application of imatinib. Expert Opin Drug Saf 4: 183-191, 2005.
23. Druker BJ, Tamura S, Buchdunger E, et al: Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 2: 561-566, 1996.
24. Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA and Zigler AJ: Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. Blood 96: 925-932, 2000.
25. Buchdunger E, Cioffi CL, Law N, et al: Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. J Pharmacol Exp Ther 295: 139-145, 2000.
26. McGary EC, Weber K, Mills L, et al: Inhibition of platelet-derived growth factor-mediated proliferation of osteosarcoma cells by the novel tyrosine kinase inhibitor STI571. Clin Cancer Res 8: 3584-3591, 2002.
27. Matei D, Chang DD and Jeng MH: Imatinib mesylate (Gleevec) inhibits ovarian cancer cell growth through a mechanism dependent on platelet-derived growth factor receptor alpha and Akt inactivation. Clin Cancer Res 10: 681-690, 2004.
28. Mueller L, Goumas FA, Himpel S, Brilloff S, Rogiers X and Broering DC: Imatinib mesylate inhibits proliferation and modulates cytokine expression of human cancer-associated stromal fibroblasts from colorectal metastases. Cancer Lett 250: 329-338, 2007.
29. Gioni V, Karampinas T, Voutsinas G, et al: Imatinib mesylate inhibits proliferation and exerts an antifibrotic effect in human breast stroma fibroblasts. Mol Cancer Res 6: 706-714, 2008.
30. Pietras K, Pahler J, Bergers G and Hanahan D: Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. PLoS Med 5: e19, 2008.
31. Micke P and Ostman A: Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer 45 (Suppl. 2): S163-S175, 2004.
32. Aono Y, Nishioka Y, Inayama M, et al: Imatinib as a novel antifibrotic agent in bleomycin-induced pulmonary fibrosis in mice. Am J Respir Crit Care Med 171: 1279-1285, 2005.
33. Abdollahi A, Li M, Ping G, et al: Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis. J Exp Med 201: 925-935, 2005.
34. Nakamura T, Matsumoto K, Kiritoshi A, Tano Y and Nakamura T: Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: in vitro analysis of tumor-stromal interactions. Cancer Res 57: 3305-3313, 1997.
35. Gmyrek GA, Walburg M, Webb CP, et al: Normal and malignant prostate epithelial cells differ in their response to hepatocyte growth factor/scatter factor. Am J Pathol 159: 579-590, 2001.
36. Giri D, Ropiquet F and Ittmann M: Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. Clin Cancer Res 5: 1063-1071, 1999.
37. Fukumura D, Xavier R, Sugiura T, et al: Tumor induction of VEGF promoter activity in stromal cells. Cell 94: 715-725, 1998.
38. Dong J, Grunstein J, Tejada M, et al: VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. EMBO J 23: 2800-2810, 2004.
39. Kitadai Y, Sasaki T, Kuwai T, et al: Expression of activated platelet-derived growth factor receptor in stromal cells of human colon carcinomas is associated with metastatic potential. Int J Cancer 119: 2567-2574, 2006.
40. Tejada ML, Yu L, Dong J, et al: Tumor-driven paracrine platelet-derived growth factor receptor alpha signaling is a key determinant of stromal cell recruitment in a model of human lung carcinoma. Clin Cancer Res 12: 2676-2688, 2006.
41. Sundberg C, Branting M, Gerdin B and Rubin K: Tumor cell and connective tissue cell interactions in human colorectal adenocarcinoma. Transfer of platelet-derived growth factor-AB/BB to stromal cells. Am J Pathol 151: 479-492, 1997.
42. Coltrera MD, Wang J, Porter PL and Gown AM: Expression of platelet-derived growth factor B-chain and the platelet-derived growth factor receptor beta subunit in human breast tissue and breast carcinoma. Cancer Res 55: 2703-2708, 1995.
43. Ronnov-Jessen L, van Deurs B, Celis JE and Petersen OW: Smooth muscle differentiation in cultured human breast gland stromal cells. Lab Invest 63: 532-543, 1990.
44. Micke P and Ostman A: Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets 9: 1217-1233, 2005.
45. Heldin CH, Rubin K, Pietras K and Ostman A: High interstitial fluid pressure - an obstacle in cancer therapy. Nat Rev Cancer 4: 806-813, 2004.
46. Less JR, Posner MC, Boucher Y, Borochovitz D, Wolmark N and Jain RK: Interstitial hypertension in human breast and colorectal tumors. Cancer Res 52: 6371-6374, 1992.
47. Boucher Y, Kirkwood JM, Opacic D, Desantis M and Jain RK: Interstitial hypertension in superficial metastatic melanomas in humans. Cancer Res 51: 6691-6694, 1991.
48. Gutmann R, Leunig M, Feyh J, et al: Interstitial hypertension in head and neck tumors in patients: correlation with tumor size. Cancer Res 52: 1993-1995, 1992.
49. Pietras K, Rubin K, Sjoblom T, et al: Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. Cancer Res 62: 5476-5484, 2002.
50. Vlahovic G, Ponce AM, Rabbani Z, et al: Treatment with imatinib improves drug delivery and efficacy in NSCLC xenografts. Br J Cancer 97: 735-740, 2007.