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Transforming growth factor-β1 up-regulates the c-met expression in human lung fibroblasts

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Abstract

After lung injury, hepatocyte growth factor (HGF) acts to regenerate epithelial cells, which express the HGF receptor, c-met. However, the roles of HGF with regard to lung fibroblasts expressing the c-met have not been clarified. In this paper, the expression of c-met on human lung fibroblasts was examined after stimulation with transforming growth factor-β1 (TGF-β1) as a mediator of lung injury, such as fibrosis. The human lung fibroblasts stimulated by TGF-β1 expressed a larger amount of c-met mRNA than un-stimulated cells. The amount of c-met protein was also increased by the TGF-β1 treatment, although the amount of c-met on the cell surfaces was comparable to that of the un-stimulated fibroblasts. The proliferation and urokinase-type plasminogen activator (uPA), the protease relating to cell migration, and production of human lung fibroblasts were not stimulated by TGF-β1 and HGF treatment. On the other hand, the expression of Bcl-2, the anti-apoptotic substance, was increased by the addition of HGF after the TGF-β1 treatment. These results suggest that after lung injury, c-met expressed on human lung fibroblasts reduces apoptosis in concert with HGF, and this effect might provide protection from fibrotic processes.

Introduction

Pulmonary fibrosis is thought to be a result of the process of regeneration after a lung injury. When the epithelial basement membrane is destroyed, fibroblasts proliferate and migrate into intra-alveolar spaces, and contribute to the accumulation of extracellular matrix (ECM) molecules[1]. The surface of the intra-alveolar fibrosis is then overlaid by regenerating epithelial cells, and the resultant fibrotic mass is incorporated into the alveolar wall[2-4]. The final stage of lung fibrosis is an extensive structural disorganization of the lung parenchyma with the subsequent progressive loss of alveolar-capillary units. In this stage, fibroblasts exhibit a profibrotic secretory phenotype with a lower growth rate and increased spontaneous apoptosis[1].

Transforming growth factor-β1 (TGF-β1) is a pathogenic mediator in tissue fibrosis in distinct organs[5-7]. TGF-β1 promotes ECM production, suppresses the proteolytic breakdown of ECM proteins, arrests epithelial cell growth, and induces their apoptosis. The inhibition of TGF-β1 production or of its action attenuates progression of fibrosis in several organs[8-10]. An increased expression of TGF-β1 in tissues has been noted in patients with fibrotic diseases, as well as in experimental animals with fibrosis-related disorders.

Hepatocyte growth factor (HGF) is a heparin binding polypeptide originally identified and cloned as a potent

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mitogen for mature hepatocytes[11–13]. Chemically, HGF can be characterized as a heterodimetric (69-kD α-chain and 34-kD β-chain), kringle-containing growth factor with a structural homology to plasminogen[13]. It is secreted by mesenchymal cells as an inactive precursor (pro-HGF). The urokinase-type plasminogen activator (uPA) converts pro-HGF into $\alpha\beta$-HGF, which influences the proliferation, migration, and morphogenesis of epithelial cells and endothelial cells[14–20]. In cell migration, uPA is also known to be one of the serine proteases for the degradation of ECM[21].

In the rat lung, HGF mRNA and HGF activity increased in alveolar type II epithelial cells when injured by an intratracheal administration of hydrochloric acid, and DNA synthesis of alveolar cells increased after the onset of lung injury[22]. In the bleomycin-induced rat lung fibrosis model, exogenous HGF acts as a pulmotrophic factor in vivo and prevents the progression of bleomycin-induced lung injury[23]. In addition, it has been reported that the HGF concentration in sera[22,24] or bronchoalveolar lavage fluids (BALF)[25] of patients with various lung diseases is higher than that of healthy donors. These results suggest that HGF act as a pulmotrophic factor after lung injury[26].

These pulmotrophic actions of HGF are mediated through a high-affinity HGF receptor. This receptor is known to be a c-met proto-oncogene product[27–29]. The c-met protein is expressed in epithelial cells of a variety of organs, melanocytes, and non-epithelial cells, including endothelial cells, microglial cells, neurons, hepatic stellate cells, and muscle cells[30–36].

Most of the information on the role of HGF in lung injuries has been obtained on alveolar type II epithelial cells and endothelial cells[22–26]. However, the roles of HGF with regard to lung fibroblasts expressing c-met have not been determined. In the present study, we tried to explore the c-met expression on human lung fibroblasts and the effect of HGF on these cells after stimulation with TGF-β1, used as a mediator of lung injury.

Methods

Materials

Collagenase, EDTA, sulfuric acid, chloroform, isopropanol, ethanol, Tris, acetic acid, agarose, ethidium bromide, paraformaldehyde, TritonX-100, sodium dodecyl sulphate (SDS), phenylmethyl sulfonyl fluoride (PMSF), acrylamide, sucrose, Tween-20, actinomycin D, cyclohexamide and WST-8 cell proliferation assay reagents were from Wako Pure Chemicals (Tokyo, Japan); newborn calf serum (NCS) was from Mitsubishi Kasei (Tokyo, Japan); medium199 (M199), trypsin, TRIzol, DNA ladder, penicillin-streptomycin mixture, and Fungizone were from GIBCO BRL (Grand Island, NY, USA); human recombinant basic fibroblast growth factor was from Intergen (Purchase, NY, USA); 60-mm-diameter plastic culture dishes and 96 well plastic plates were from Falcon (Naperville, IL, USA); 6-well plastic culture dishes were from Costar (Cambridge, MA, USA); competitive DNA construction kit, competitive RNA transcription kit and one step RNA PCR kit were from TaKaRa Biomedicals (Tokyo, Japan); transforming growth factor-β1 (TGF-β1) was from Genzyme (Cambridge, MA, USA); recombinant human hepatocyte growth factor (HGF) was from R & D Systems (Minneapolis, MN, USA); anti-human hepatocyte growth factor was from Sigma (St. Louis, MO, USA); a polyclonal antibody to human c-met was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); a polyclonal goat anti-rabbit IgG conjugated with horseradish peroxidase was from IBL Laboratories (Gumma, Japan); fluorescein (FITC)-conjugated goat anti-rabbit IgG was from IMMUNOTECH (Marseille, France); non-fat dry milk was from Bio Rad Laboratories (Hercules, CA, USA); nitrocellulose membrane and enhanced chemiluminescence (ECL) detection system were from Amersham Pharmacia Biotech (Bucks, UK); urokinase-type plasminogen activator (uPA) and aprotinin were from Yoshitomi Pharmaceuticals (Osaka, Japan); QuantiTect™ SYBR Green® RT-PCR kit was from QIAGEN K.K. (Tokyo, Japan); human Bcl-2 ELISA kit from Endogen, Inc. (Woburn, MA, USA).

Cell culture

Small sections of human lung tissue, within 1–2 mm of the periphery, were obtained from normal regions of lungs from patients undergoing resection for solitary lung tumors. The surgery was performed by the Surgical Service of Kasumigaura Hospital, Tokyo Medical University (Ibaraki, Japan), and the Surgical Division of Mito-Saiseikai General Hospital (Ibaraki, Japan). Lung fibroblasts, which were non-endothelial, non-epithelial and long-spindle-shaped, were isolated according to the modified method described by Takahashi, et al[21].

In brief, the sections were digested with 0.1% collagenase, and the obtained cells were seeded onto plastic dishes in a growth medium (M199 supplemented with 20% newborn calf serum (NCS), 10 ng/ml of basic fibroblast growth factor, 100 U/ml of penicillin, and 100 μg/ml of streptomycin) and were maintained in a humidified 5% CO_2, 95% air incubator at 37°C. Spindle-shaped cells were separated by a small piece of silicon rubber, and plated onto subsequent dishes. The culture medium was changed twice a week. Cells in younger generations (passages 3–7) were used for each examination.

Cytokine treatment

When the cells became confluent, the medium was

changed to M199 supplemented with 2% NCS for 24 hours, in order to stop the effect of the serum. A medium with TGF-β1 (0, 0.1, 1, 10 ng/ml) was added to the fibroblast cultures. After a 48-hour incubation, total RNA was extracted from the fibroblasts, and c-met mRNA was measured using the method described in the RT-PCR section. Similarly, 1 ng/ml of TGF-β1 was added to the fibroblast cultures. After either 8, 12, 24, 48 hours of incubation, total RNA was extracted from the fibroblasts, and RT-PCR was carried out. In separate experiments, the fibroblasts were pre-treated with 5 μg/ml actinomycin D for 45 min, and were subsequently incubated for one hour after the addition of 1 ng/ml TGF-β1. The cells were also treated with a combination of 1 ng/ml TGF-β1 and 10 μg/ml cyclohexamide (CHX) for one hour. Total RNA was extracted and analyzed for c-met mRNA by RT-PCR.

RT-PCR

To obtain total RNA, cells cultivated in 6-well plates were lysed by the addition of TRIzol. The RNA was extracted with chloroform, isopropanol and ethanol by centrifugation after each TRIzol addition, according to the manufacture's instructions. The extracted RNA was dissolved in distilled water, and the absorbance was measured with a spectrophotometer (UV-1600 ; Shimazu, Kyoto, Japan) at 260 nm to estimate the amount of total RNA.

The set of primers specific for the rat c-met, from which an amplified fragment of 725 bp was obtained, consisted of a forward primer beginning at 2,788 bp with a nucleotide sequence of 5′-CAG TGA TGA TCT CAA TGG GCA AT-3′ and a reverse primer beginning at 3492 bp with a nucleotide sequence of 5′-AAT GCC CTC TTC CTA TGA CTT C-3′. The reason this set of primers for the rat was used in the detection of c-met mRNA in human lung fibroblasts, was that c-met mRNA in human aortic and lung microvascular endothelial cells is expressed by the same methods[37,38]. For standardization of the different RNA samples, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used ; sense primer 5′-TTA GCA CCC CTG GCC AGG-3′ and antisense primer 5′-CTT ACT CCT TGG AGG CCA TG-3′, yielding a PCR product of 540 bp.

Equal amount of total RNA (100 ng) samples from the cultured human lung fibroblasts were added to tubes, and RT-PCR was carried out using a one-step RT-PCR kit, according to the manufacturer's manual, under the following conditions : 1 cycle at 50°C for 30 min ; 1 cycle at 94°C for 2 min ; and 25 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min (TaKaRa Thermal Cycler MP ; Tokyo Japan). The PCR reaction products were separated by electrophoresis on a 2.0% agarose-Tris-acetate-EDTA gel and stained with ethidium bromide, and scanned into a Macintosh G3 computer. An NIH image program (National Technical Information Service, Virginia, USA) was used to calculate the density of each band[39,40].

Western blotting

After the human lung fibroblasts were treated with or without TGF-β1 (1 ng/ml) for 48 hours, the cells were lysed in a lysis buffer containing 1% TritonX-100 and 0.1% SDS, with 1 mM PMSF and 25 μg/ml aprotinin. Equal amounts of protein samples were size-separated on discontinuous 6% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were immersed for 60 minutes in a blocking solution containing 5% non-fat dry milk and 0.1% Tween-20, and then incubated overnight at 4°C with a rabbit anti-c-met antibody. After washing, the membranes were incubated for 1 hour at room temperature with a peroxidase-linked secondary antibody. Blotted proteins were detected using an enhanced chemiluminescence (ECL) detection system. An NIH image program was used to calculate the density of each band.

Flow cytometry

When the cells became confluent, the medium was changed to M199 supplemented with 2% NCS for 24 hours in order to stop the effects of the serum. Medium with or without TGF-β1 (10 ng/ml) was added to the fibroblast cultures. After either 24, 48 or 72 hours of incubation, the cells were dispersed by trypsin/EDTA and washed twice with PBS to remove residual TGF-β1 present in the culture medium. The cells were fixed with 1% paraformaldehyde for 10 minutes and resuspended in PBS with 1% BSA to a final concentration of 2×10^6 cells/ml. Anti-c-met antibody was added to the cell suspension and the cells were incubated over night at 4°C. After the incubation, the cells were washed twice with PBS and fluorescein (FITC)-conjugated goat anti-rabbit IgG was added and the cells were incubated for one hour at 37°C. The cells were washed twice with PBS again and analyzed using a flow cytometer (Epics Elite, Tokyo, Japan). The cells treated without anti-c-met antibodies were used as a negative control.

Cell proliferation

The cells were seeded at numbers of 1×10^4 into a 96-well microtiter plate in a medium containing 20% NCS. Subsequently, the cells were allowed to spread for 24 hours, cultured for an additional 24 hours in a fresh medium containing 2% NCS, and then further incubated for 24 hours in a fresh medium containing 2% NCS with or without TGF-β1 (1 ng/ml). After 24 hours of exposure to TGF-β1, the cells were incubated for 24 hours in a fresh medium with or without recombinant HGF (20 ng/ml) or uPA (10 IU/ml). The cells were incubated for a further 2 hours after the addition of

20 μl/well of WST-8 into each well. The intensity of the color reaction was measured with a spectrophotometer (model 3550-UV, Bio Rad, Tokyo, Japan) at 450 nm.

Real time PCR

The RNA was extracted by the same method described previously. The set of primers specific for human pro-uPA from a transformed human endothelial cell line, from which an amplified fragment of 459 bp was obtained, consisted of a forward primer beginning at 769 bp with a nucleotide sequence of 5′-GCC TTG CTG AAG ATC CGT TCC AAG GAG GGC-3′ and a reverse primer beginning at 1,198 bp with a nucleotide sequence of 5′-CAG GCC ATT CTC TTC CTT GGT GTG ACT GCG-3′.

The 383 bp competitor DNA for uPA, that has the sense and antisense uPA primer sequences at both its ends, was made with a competitive DNA construction kit using λDNA as a template[41]. The competitor RNA was also made with a competitive RNA transcription kit, using the competitor DNA according to the manufacture's manual. Real-time RT-PCR was carried out with an i-Cycler iQ (Bio-Rad, Tokyo, Japan). A master-mix of the following reaction components was prepared to the indicated end concentration: 23 μl RNase-free water, 0.25 μl forward primer (0.5 μM), 0.25 μl reverse primer (0.5 μM), 25 μl of 2×QuantiTect™ SYBR Green® RT-PCR Master Mix, and 0.5 μl QuantiTect™ RT Mix. The plate was filled in with 49 μl of master-mix and a 1 μl in volume solution containing 1 ng total RNA, was added as a PCR template.

Real-time RT-PCR was carried out under the following conditions: Reverse transcription (50°C for 30 min), a PCR initial activation step (95°C for 15 min), amplification and quantification programs repeated 40 times (94°C for 15 sec, 58°C for 30 sec, 72°C for 45 sec with a fluorescence measurement), and a melting curve program (from 50°C to 90°C with a heating rate of 0.5°C per minute and a continuous fluorescence measurement). A standard curve was calculated using the uPA competitor RNA. All samples were run in duplicate. Quantities of uPA in the test samples were normalized to the corresponding input total RNA based on the GAPDH quantity.

Enzyme-linked immunosorbent assay (ELISA) for Bcl-2

When the cells became confluent, the medium was changed to M199 supplemented with 2% NCS for 24 hours in order to stop the effect of the serum. After treatment with or without 1 ng/ml of TGF-β1 for 48 hours, the cells were preincubated in the presence (40 ng/ml) and absence of HGF for 48 hours and then sham-treated with 10 μg/ml of actinomycin D. After 2 hours of exposure to actinomycin D, the cells were washed twice and incubated again in a fresh medium with or without HGF at 37°C for 4 hours. Lysates of cells were finally harvested for the Bcl-2 assays. The amount of Bcl-2, the anti-apoptotic substance, were

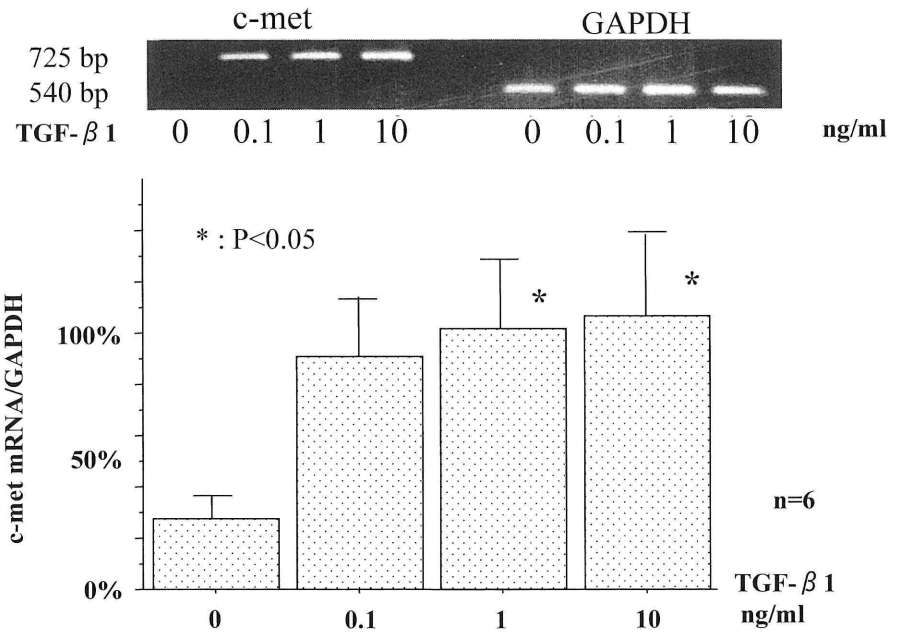

Fig. 1 Effect of TGF-β1 on induction of c-met mRNA in human lung fibroblasts. Cells were treated with medium with or without each concentration (0.1, 1, 10 ng/ml) of TGF-β1. After 48 hours of stimulation, the amount of uPA mRNA was examined by the RT-PCR method. The amount of c-met mRNA was calculated with NIH image and was normalized by the amount of GAPDH mRNA. Values are the means±SD. P values were compared to the amount of c-met mRNA expressed untreated cells.

determined using a Bcl-2 ELISA kit according to the manufacturer's protocol.

Results

Effect of TGF-β1 on the c-met mRNA expression

To investigate whether fibrotic cytokines can upregulate the expression of c-met mRNA, human lung fibroblasts were stimulated with TGF-β1. The amount of c-met mRNA in fibroblasts was significantly increased by concentrations of 1 and 10 ng/ml of TGF-β1 (Fig. 1). The time-course experiments demonstrated a marked upregulation of c-met mRNA by 1 ng/ml of TGF-β1 after 8 hours and the high c-met mRNA level was kept at least 48 hours (Fig. 2). The c-met mRNA upregulation in human lung fibroblasts was reduced by the addition of actinomycin D (Fig. 3). On the other hand, the c-met mRNA accumulated in those cells treated with cyclohexamide (Fig. 3).

Effect of TGF-β1 on c-met protein expression

The effect of TGF-β1 on c-met protein expression was analyzed with a Western blot assay. Compared with the unstimulated cell lysate, TGF-β1 promoted a 190 kD c-met protein expression (Fig. 4). To examine the c-met protein on the surface of human lung fibroblasts, the cells were stained with an anti-c-met antibody after TGF-β1 stimulation and then measured by flow cytometry. Although self-fluorescence in human lung fibroblasts was increased by the TGF-β1 stimulation, the amount of cell-surface c-met protein was mostly equivalent, both with and without the TGF-β1 treatment (Fig. 5).

Cell proliferation assay

From results described above, the transcription and production of c-met were increased in the human lung fibroblasts by the TGF-β1 treatment. To evaluate the effect of HGF/c-met on lung fibroblasts, we examined human lung fibroblast proliferation using WST-8 at first. After stimulation with TGF-β1, the number of lung fibroblasts was not increased by the addition of HGF or uPA, which is expected of a pro-HGF activator (Fig. 6).

uPA mRNA on lung fibroblasts

To evaluate the cell migration, we measured the amount of uPA mRNA, which is one of the proteases related to cell migration, by degradation of the surrounding ECM. TGF-β1 and HGF did not exert any influence on the amount of uPA mRNA (Fig. 7). The amounts of uPA mRNA were not reduced by the anti-HGF antibody either.

Effect of HGF on apoptosis in lung fibroblasts

Although the human lung fibroblasts certainly expressed c-met by the TGF-β1 treatment, the addition of TGF-β1 and HGF was no effects on neither their growth nor the uPA production, which related deeply to cell migration. The anti-apoptotic substance Bcl-2 in human lung fibroblasts was then assayed after the addition of TGF-β1 and HGF, because pretreatment with 40

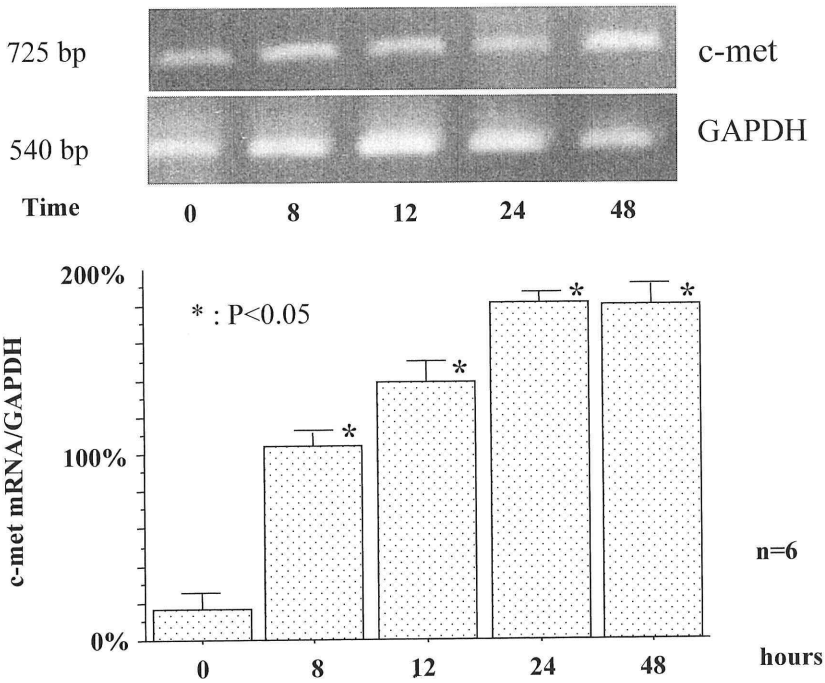

Fig. 2 Time-course of c-met mRNA induction by TGF-β1 in human lung fibroblasts. Cells were treated with the medium containing 1 ng/ml of TGF-β1. The amount of c-met mRNA was examined by RT-PCR at 8, 12, 24, 48 hours after the cytokine addition. The amount of c-met mRNA was calculated with NIH image, and compared to the c-met mRNA value without the cytokine. Values are the means±SD. P values were compared to the amount of c-met mRNA expressed untreated cells.

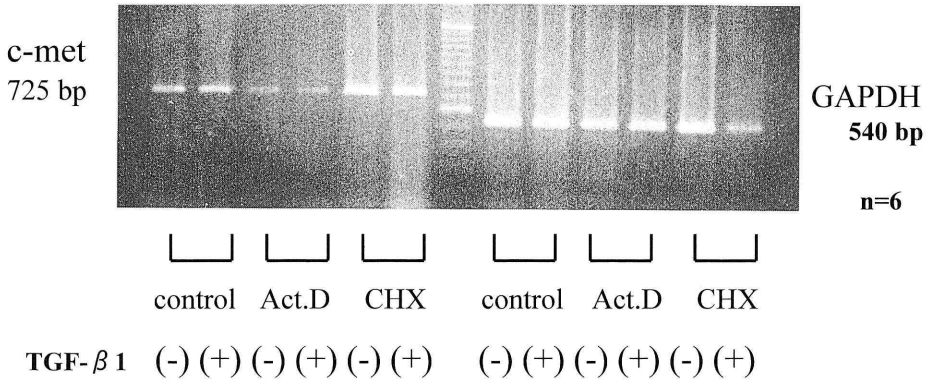

Fig. 3 Stabilization study of the c-met mRNA. Human lung fibroblasts were pre-treated with or without 5 μg/ml actinomycin D for 45 min to block transcription. Cultures were then treated with or without 1 ng/ml TGF-β1 for one hour. Separately, the cells were treated with a combination of 1 ng/ml TGF-β1 and 10 μg/ml cyclohexamide (CHX) for one hour. Total RNA was extracted and analyzed for c-met mRNA by the RT-PCR method.

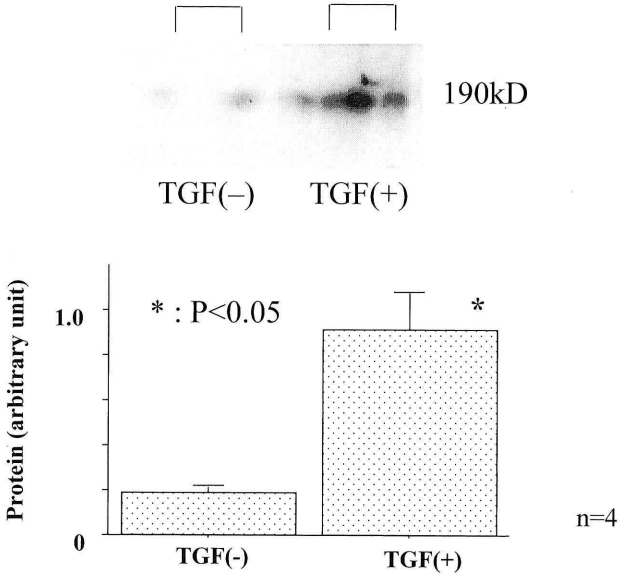

Fig. 4 Upregulation of c-met protein expression in human lung fibroblasts by TGF-β1. The cell lysate were prepared after treatment with TGF-β1 (1 ng/ml) for 48 hours, and was submitted to a Western blotting. An NIH program was used to calculate the density of each band. Values are the means±SD. P values were compared to the amount of c-met protein expressed untreated cells.

ng/ml of HGF for 48 hours effectively protected human lung fibroblasts from an apoptotic cell death induced by actinomycin D (data not shown). To ascertain if the TGF-β1 is associated with cell protection, anti-apoptotic Bcl-2 in human lung fibroblasts were measured after treatments with 1 ng/ml TGF-β1 for 48 hours, subsequent with 40 ng/ml HGF for 48 hours as described in the method section. Although TGF-β1, HGF or even actinomycin D, an apoptotic substance, alone did not express any effect on Bcl-2, both TGF-β1 and HGF treatment induced significantly higher amount of Bcl-2 (Fig. 8). The induction of Bcl-2 was notable when the cells treated with TGF-β1, HGF and actinomycin D.

Discussion

Pulmonary fibrosis is a progressive and usually fatal lung disorder characterized by the occurrence of widely scattered small clusters of fibroblasts, presumably provoked by multiple microscopic lung injuries, and abnormal tissue remodeling[1–4,42]. TGF-β1 was highly noted in fibrosis patients, as well as in experimental animals with fibrosis-related disorders. TGF-β1 down-regulates HGF, which is known as a pulmotrophic anti-fibrotic factor[23,26,43]. In this paper we tried to clarify the roles of HGF/c-met on lung fibroblasts and we examined c-met expression on lung fibroblasts and the effect of HGF on these cells after stimulation with TGF-β1.

The results of this study showed that the amount of c-met mRNA significantly increased after stimulation with TGF-β1 (Fig. 1 and 2). The upregulation of c-met mRNA was inhibited by treatment of the tran-

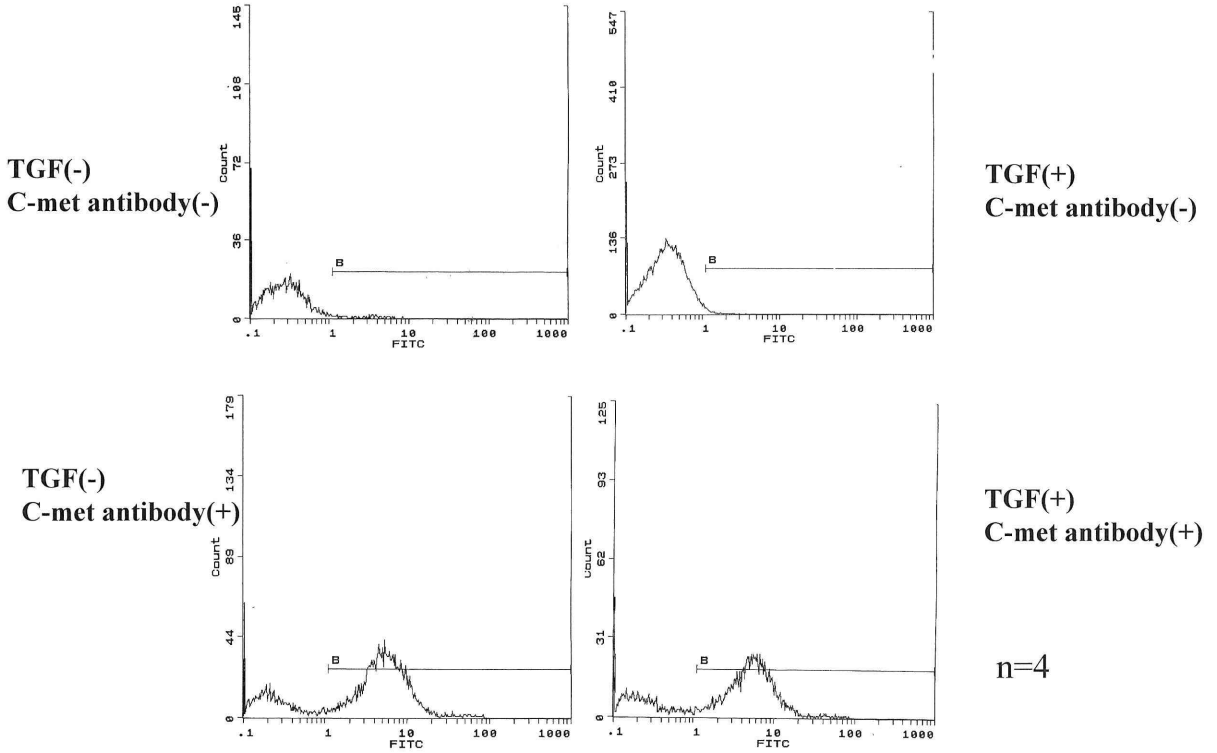

Fig. 5 c-met protein expression on the surface of human lung fibroblasts. After stimulation with TGF-β1 (10 ng/ml) for 48 hours, the cells were fixed and treated with anti-c-met antibody and FITC-conjugated second antibody as described in Material and Methods, and were analyzed using a flow cytometer. The cells treated without anti-c-met antibody cells were used as a negative control. Line B means range of FITC-positive cells.

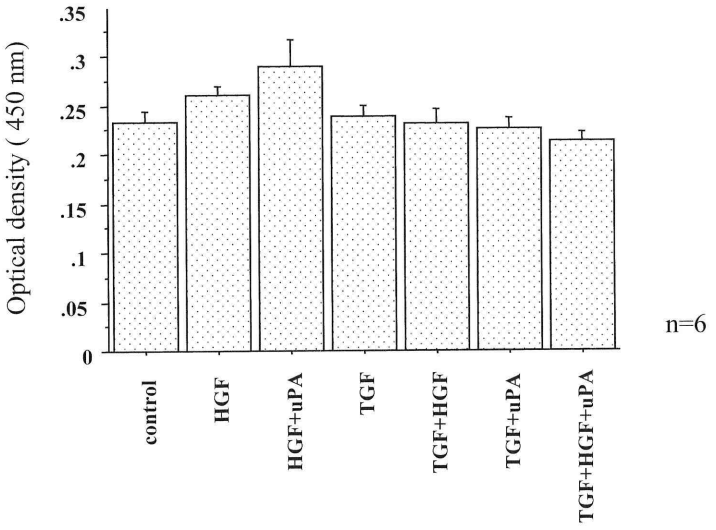

Fig. 6 The proliferation of human lung fibroblasts after stimulation with recombinant HGF, TGF-β1, or uPA. Cell proliferation was measured by WST-8 assay. The cells treated without cytokines were used as a negative control. Values are the means±SD.

scription inhibitor actinomycin D (Fig. 3). Treatment with cyclohexamide, an inhibitor of protein synthesis, resulted in the accumulation of c-met mRNA in the fibroblasts (Fig. 3). These results suggest that TGF-β1 modulates c-met mRNA transcripts. To the best of our knowledge, this is the first report to describe the regulation of c-met mRNA on human lung fibroblasts after stimulation with TGF-β1.

C-met protein also increased after stimulation with TGF-β1 by Western blotting (Fig. 4), but the amount of cell surface c-met protein did not (Fig. 5). These results suggest that the amount of c-met protein increased in the intracellular space, but did not change on the cell surface after stimulation with TGF-β1 for 48 hours, based on

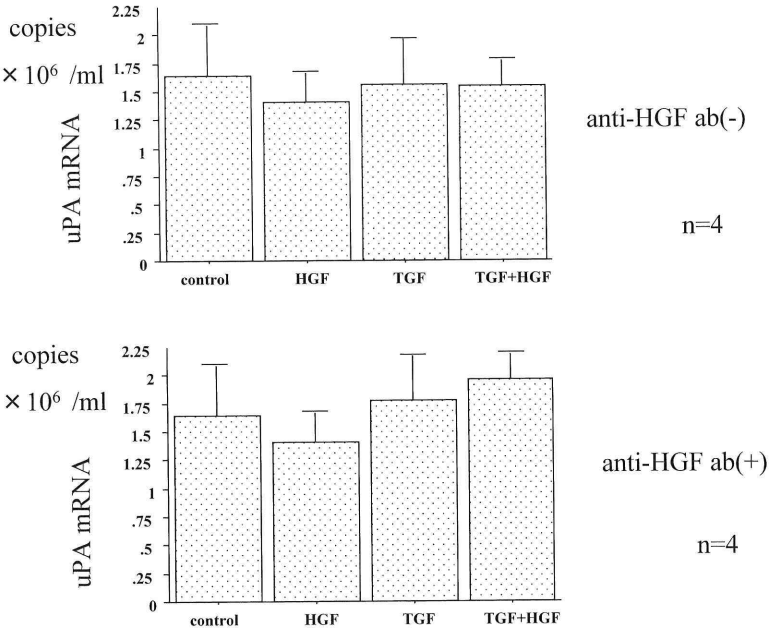

Fig. 7 Effect of TGF-β1 on expression of uPA mRNA in human lung fibroblasts. Cells were treated with medium with 1 ng/ml of TGF-β1 under the condition with or without anti-HGF antibody. The amount of uPA mRNA was examined by real time PCR at 48 hours after the cytokine addition. Values are the means±SD.

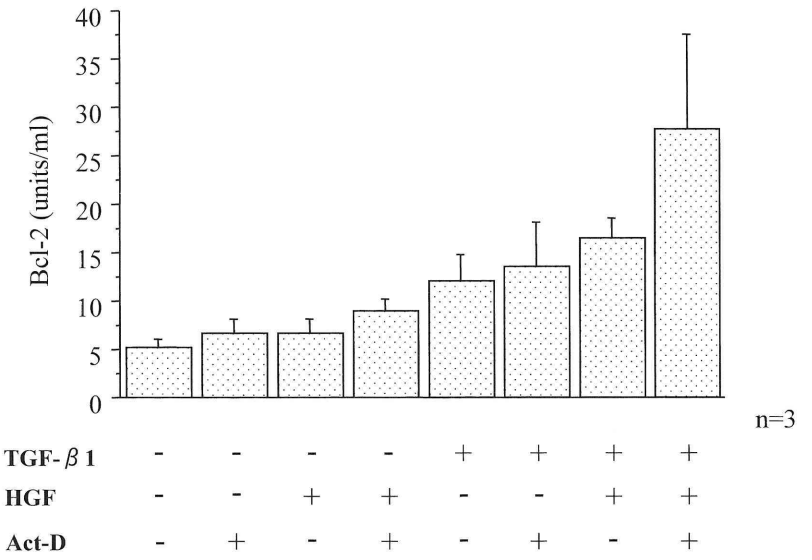

Fig. 8 The effect of TGF-β1 to HGF protection against apoptosis induced by actinomycin D (act D). After treatment with 1 ng/ml of TGF-β1, cells were also treated as described in Material and Methods. Values are the means±SD. P values were compared with actinomycin D-treated cells.

the fact that the c-met mRNA, self-fluorescence and the amount of c-met protein in human lung fibroblasts were increased by TGF-β1 stimulation. These results suggested that some other kind of stimulation might be needed to express c-met on the surface of lung fibroblasts.

Several studies have shown that HGF affects a variety of cell types, especially those of epithelial origin, and elicits responses such as proliferation, migration, and morphogenesis[14–19]. These pleiotropic effects of HGF are all mediated through the c-met receptor. However, few studies have actually targeted the effect of HGF/c-met on fibroblasts. Thus we are tried to explore the effect of HGF/c-met on human lung fibroblasts after stimulation with TGF-β1 as a mediator of lung injury.

First we examined cell proliferation after stimulation with TGF-β1. The number of lung fibroblasts was not increased, even by the addition of HGF and uPA, which was expected as the activator for HGF molecule (Fig. 6). Several studies have shown that after stimulation with

TGF-β1, the growth of fibroblast cells increased more than unstimulated cells and they have also shown that fibroblasts from fibrosis patients proliferated faster than normal fibroblasts[44,45]. Our results differ from those previously reported. One of the reasons for the difference is that in our study, the amount of c-met protein of the cell surface did not change after stimulation with TGF-β1. Ramos and coworkers showed that fibroblasts derived from idiopathic pulmonary fibrosis patients proliferated more slowly than did normal lung fibroblasts[1]. Raghu and coworkers, who obtained fibroblasts from different areas of the same fibrotic lung, found a higher rate of proliferation in cells derived from inflammatory areas and the lowest rate in fibroblasts from dense fibrotic scars[46]. These studies suggest that fibroblasts isolated from a human lung have two phenotypes : fast rate of proliferation type, or slow rate of proliferation type. Akamine and colleagues identified two subpopulations which were isolated on the basis of their differential expression of the receptor C1q[47]. The subgroups differed in rates of proliferation and especially collagen synthesis, both under basal conditions and after stimulation with TGF-β1. In light of these studies, the fibroblasts we used might have been of the slow proliferation type.

To evaluate cell migration, we investigated the amount of uPA mRNA. Shetty and coworkers showed that the binding of urokinase-type plasminogen activator (uPA) to a specific receptor (uPAR) on human lung fibroblasts enables it to regulate cellular proteolysis and remodeling of the extracellular matrix[48]. uPA was mitogenic for normal as well as fibrotic fibroblasts, indicating that receptor binding concurrently localizes cellular proteolytic activity and stimulates mitogenesis. In the present study TGF-β1 and HGF did not exert any influence on the amount of uPA mRNA (Fig. 7).

According to Shetty, TGF-β1 increases uPA binding to its receptor and plasminogen activation at the cell surface, with a greater maximal effect on fibrotic than on normal fibroblasts. It is thought that the cells used in the present study would be normal human lung fibroblasts, because TGF-β1 did not increase uPA mRNA.

After stimulation with TGF-β1, HGF did not have any influence on the proliferation or migration of human lung fibroblasts. We next set up a hypothesis that the effect of HGF/c-met on lung fibrosis is associated with cell apoptosis. Several studies showed that HGF has an influence on apoptosis in a variety of cells[49-52]. To evaluate the effect of HGF in lung fibroblast apoptosis, we examined the expression of Bcl-2 in human lung fibroblasts. This result suggests that HGF is able to reduce the level of apoptosis through the specific receptor, c-met which was up-regulated by the treatment of TGF-β1.

In summary, after stimulation with TGF-β1, the amount of c-met expression in human lung fibroblasts was increased and HGF did not act on the proliferation or migration, but did induce the anti-apoptotic substance in these cells. Although HGF protected human lung fibroblasts from an apoptotic death, HGF did not stimulate the growth of these cells. It would be thought that early passage human lung fibroblasts might be stable in their migration and proliferation after stimulation only with TGF-β1 to the degree used in this study, but this condition might be changed by more stimuli. Studies are currently in progressive in this laboratory to explore the molecular mechanisms involved in the apoptosis of human lung fibroblasts.

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TGF-β1 はヒト肺線維芽細胞における c-met の発現を促進する

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【要旨】 急性肺障害の際、hepatocyte growth factor (HGF) は、その受容体である c-met を持つ上皮細胞の再生を促す。しかしながら、c-met を持つヒト肺線維芽細胞における HGF の役割についてはいままでほとんど論じられて来なかった。今回の研究では、線維化といったような肺障害の際のメディエーター として transforming growth factor-β1 (TGF-β1) を用い、ヒト肺線維芽細胞を刺激し、c-met の発現を調べた。TGF-β1 によって刺激されたヒト肺線維芽細胞では刺激前に比べ c-met mRNA の発現が増加した。また TGF-β1 の刺激によって c-met 蛋白の発現も増加した。しかしながら、細胞表面上の c-met 蛋白の量は刺激していない線維芽細胞上と比較しほぼ変わらなかった。ヒト肺線維芽細胞において TGF-β1 や HGF による刺激は、細胞増殖や uPA (細胞遊走に関連するプロテアーゼ) の発現に変化を与えなかった。しかし、一方で抗アポトーシス酵素である Bcl-2 の活性は HGF の刺激によって増強された。これらの結果より、急性肺障害の際、HGF によってヒト肺線維芽細胞のアポトーシスは減弱され、その結果、肺の線維化から保護されると考えられた。

〈**Key words**〉 肝細胞増殖因子 (HGF), c-met (HGF 受容体), 形質転換因子 (TGF-β1),ヒト肺線維芽細胞, アポトーシス