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RESEARCH ARTICLE

Hepatocyte growth factor both prevents and ameliorates the symptoms of dermal sclerosis in a mouse model of scleroderma

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Systemic sclerosis (SSc) is a connective tissue disorder with an unknown etiology. There are currently no effective therapies for SSc. (In this study, working with a bleomycin(BLM)-induced scleroderma model mice, we performed two transfections of human hepatocyte growth factor (HGF) cDNA into the skeletal muscle and showed that this treatment not only helped to prevent the dermal sclerosis simultaneously injected BLM but also improved the symptoms of dermal sclerosis induced by BLM 4 weeks previously.) RT-PCR, ELISA and an immunohistochemical analysis revealed that both mRNA and protein of human HGF as well as murine HGF were enhanced in the skin, lung, muscle and the serum after two transfections of human HGF cDNA. These analyses also revealed that this treatment significantly reduced both the expression of the TGF-β1 mRNA and the production of TGF-β1 on macrophage-like cells that infiltrated the dermis and the fibroblastic cells in BLM-induced scleroderma. Furthermore, HGF-gene transfection both prevented and ameliorated the symptoms of not only dermal sclerosis but also of lung fibrosis induced by a subcutaneous BLM injection. These results indicated that gene therapy by the transfection of the human HGF cDNA may thus be a useful therapy for SSc and lung fibrosis involved with SSc.
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Introduction

Systemic sclerosis (SSc) is a connective tissue disorder of unknown etiology, which is characterized by an excessive deposition of extracellular matrix protein in the affected skin, as well as in various internal organs.[1,2] Animal models exhibiting all aspects of SSc are currently not available; however, a number of experimental systems that produce some of the pathogenic aspects of SSc have been reported.[3] Bleomycin (BLM) is an antitumor antibiotic frequently used in the therapy of a variety of cancers.[4] Pulmonary fibrosis is one of the well-known side effects of BLM. In addition, the cutaneous side effects of BLM include Raynaud's phenomenon, cutaneous fibrosis, acral keratosis, hyperpigmentation, alopecia, gangrene, and scratch-induced dermatitis.[5–8] BLM-induced pulmonary fibrosis has been established as an experimental animal model for lung fibrosis.[9,10] We recently established a mouse model of scleroderma by repeated local injections of BLM.[11,12] Our mouse model developed dermal sclerosis and lung fibrosis as well as autoantibodies.[11] By using this model, we searched for an effective therapeutical treatment of SSc,[12–14] since, no such satisfactory therapy exists at present.

Hepatocyte growth factor (HGF) was originally identified and cloned as a potent mitogen for hepatocytes.[15,16] It has mitogenic, motogenic and morphogenic effects on various epithelial tissues as well as the liver, kidney, lung and intestine.[17–19] HGF also has an antiapoptotic activity[20] and has been shown to play a role in enhancing hematopoiesis.[21] The intravenous injection of recombinant human HGF (rhHGF) enhanced the regeneration of the liver and kidney in mice. It prevented the acute renal failure induced by a ligation of the renal artery and also suppressed the onset of liver cirrhosis induced by dimethylnitrosamine,[17,22] thus suggesting that HGF plays an important role in the tissue-repair process. HGF may therefore be able to induce tissue-repair in dermal sclerosis associated with scleroderma. Two transfections of human HGF gene into the skeletal muscle of BLM-treated mice not only prevented the development of dermal sclerosis and lung fibrosis, but also seemed to improve the effects of dermal sclerosis in this study. Our results therefore suggest that HGF gene therapy appears to be a potentially useful treatment for both SSc and lung fibrosis involved with SSc in the future.

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Results

Effect of HGF–HVJ-liposomes

In line with the finding of a previous report,[23] two transfections of human HGF gene into skeletal muscle (Figure 1a) resulted in a sustained high serum level of HGF (Figure 2c). Human HGF mRNA was detected not only in the muscle but also in the skin and lung (Figure 2a). Interestingly, human HGF gene transfection enhanced the endogenous murine HGF production in the muscle, skin and lung (Figure 2b, d, e). The total level of HGF in the serum was 1.564±0.566 ng/ml at 2 weeks after the second injection of HGF–HVJ-liposomes and BLM, which was three times higher than the level of endogenous HGF in the sera of the mice that received injections of BLM (482.0±21.55 ng/ml) (Figure 2c); however, BLM itself also significantly induced an enhancement of endogenous HGF in the skin but not in the serum (Figure 2d). The total level of HGF in the muscle, skin and lung extract were 69.457±9.76, 72.03±24.91 and 265.89±18.88 pg/mg tissue in the mice of HGF–HVJ-liposomes injection and BLM (Figure 2b,d,e), which was also much higher than the level of endogenous HGF in the BLM-treated mice. An immuno-

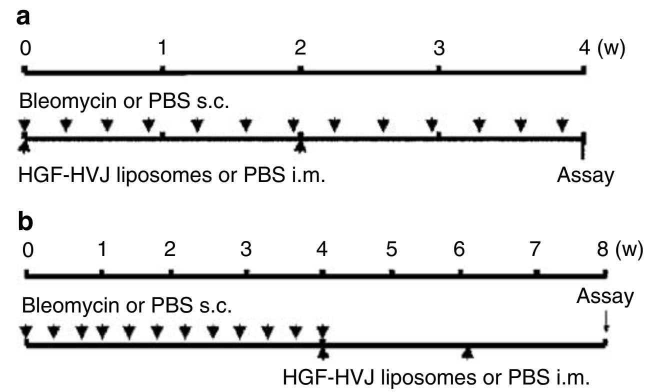

Figure 1 *Experimental protocol for injecting HGF–HVJ-liposomes in C3H/He mice. Schedule for the induction of BLM-induced scleroderma model mouse and the injection of HGF–HVJ-liposomes or PBS. The BLM-induced scleroderma model mouse was induced by the injection of 10 μg/mouse of BLM every 2 days. Control mice received PBS. (a) A measure of 10 μg/mouse of HGF–HVJ-liposomes was injected into the skeletal muscles for a total of twice with a 2 week interval. Normal plasmid vector liposomes were injected twice for the control. (b) A measure of 10 μg/mouse of HGF–HVJ-liposomes was injected into skeletal muscles for a total of twice at 2-week interval 4 weeks after the initial BLM-injection. Normal plasmid liposomes was injected twice for the control.*

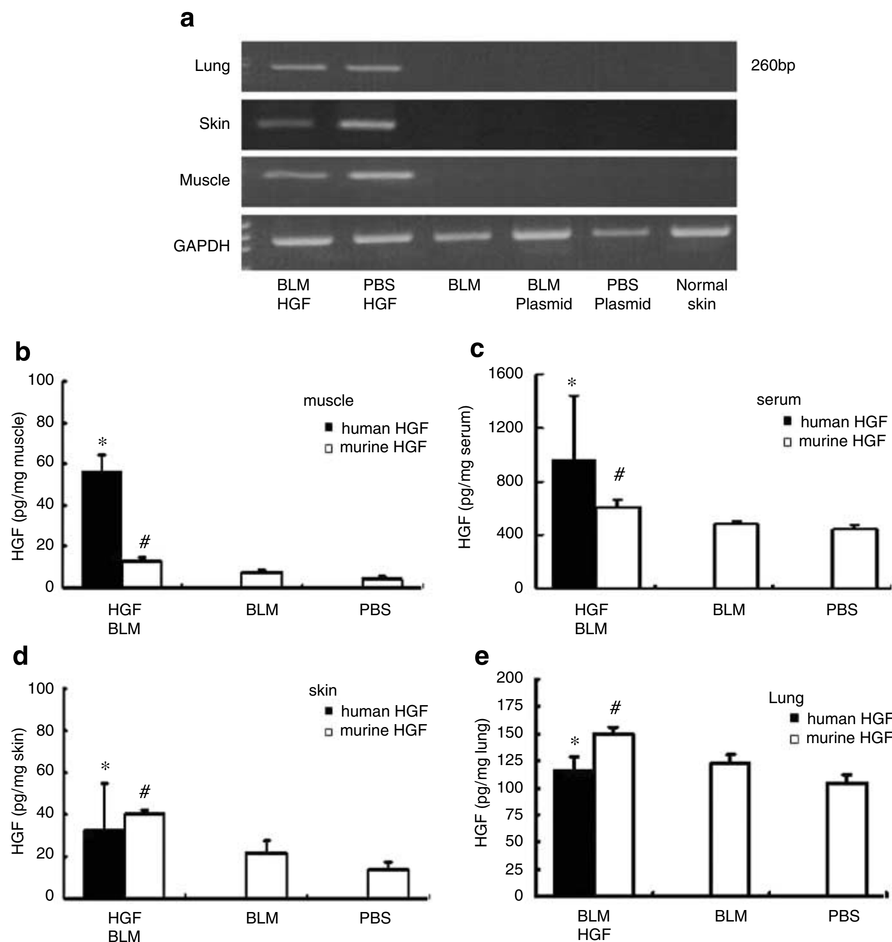

Figure 2 *HGF expression in C3H/He mice injected HGF–HVJ-liposomes. (a) The expression profile of the HGF mRNA in tissue specimens from mice treated with HGF–HVJ-liposomes by RT-PCR. The human and murine HGF concentration in the muscle (b), serum (c), skin (d) and lung tissues (e) of mice treated with HGF–HVJ-liposomes and BLM was determined. The data are the mean±SD of five mice. *P < 0.01 when human HGF level was compared to the mice treated with BLM and/or PBS. ‡P<0.05 when the murine HGF level was compared to that in the mice treated with BLM and/or PBS.*

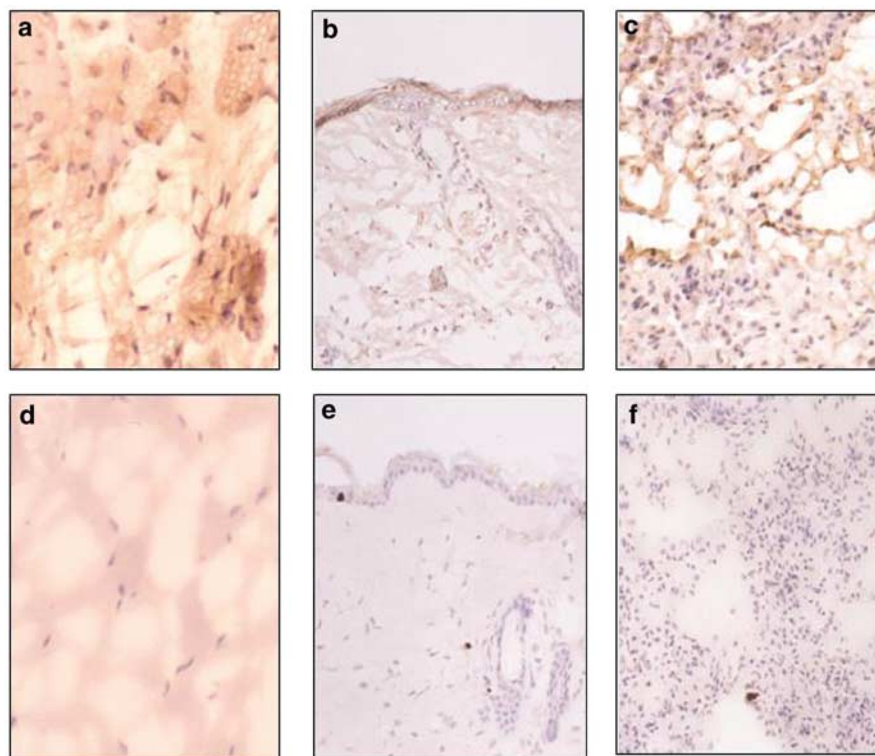

Figure 3 *Immunohistochemical analysis of HGF expression. HGF expression in muscle (a, d), skin (b, e) and lung (c, f) of mice treated with HGF–HVJ-liposome and BLM by using anti-human HGF antibody (a–c). The isotype control antibody used was mouse IgG antibody (d–f).*

histochemical analysis revealed a diffuse expression of HGF in the muscle, skin and lung (Figure 3). These data indicate the transfection of the human HGF gene to mice not only in the skeletal muscle but also in the skin and lung to thus be a successful route to for achieving gene action, while HGF produced in the muscle, skin and lung is then delivered through the serum to other organs.

Prevention of screloderma by HGF-gene transfection

A histological examination revealed that dermal sclerosis induced by BLM was prevented when human HGF-gene transfection was performed simultaneously with a BLM injection (Figure 4a–f). The skin thickness of the mice induced by BLM were 63.2 ± 6.0 μm, while that in the mice injected HGF–HVJ-liposome with BLM was reduced to 44.0 ± 5.6 μm. The skin thickness of the mice receiving BLM and the plasmid vector liposomes was 63.56 ± 4.8 μm (Figure 4g). The accumulation of hyalinized collagen bundles in the dermis was histologically observed in both the BLM-injected mice and the mice receiving both control plasmid vector liposome and BLM (Figure 4a,c); however, no accumulation was found in the mice injected with both HGF–HVJ-liposomes and BLM (Figure 4b). Interestingly, both the degree dermal sclerosis and skin thickness decreased in the HGF-transfected mice; however, HGF-transfected mice showed more intense cellular infiltration than BLM-induced scleroderma mice without HGF-gene transfection (Figure 4b). The collagen content of the skin extract from BLM-induced scleroderma was 908 ± 91.22 μg/mg tissue (Figure 4h), while the collagen content in the skin extracted from HGF-transfected mice was 474.33 ± 58.22 μg/mg tissue. The mRNA expression of collagen I was also suppressed in the HGF-transfected mice (data not shown). These data indicated that HGF transfection to the model mouse decreased not only the skin thickness but also the collagen content in the skin (Figure 4g, h).

Expression of TGF-β1 mRNA and protein in the skin

TGF-β1 is a potent stimulator of collagen synthesis. To clarify whether the modulation of TGF-β1 plays a major role in the prevention of sclerosis induced by HGF transfection in a scleroderma model mouse, we examined the mRNA expression or protein content of TGF-β1 in the skin. The TGF-β1 mRNA expression in the skin from mice treated with both BLM and HGF–HVJ-liposomes (liposomes containing hemagglutinating virus of Japan) decreased in comparison to that from mice treated with BLM alone or that from mice treated with both control plasmid–HVJ-liposomes and BLM (Figure 5a). The TGF-β1 content in the mice treated with both HGF–HVJ-liposome and BLM was 2.4045 ± 1.066 pg/mg skin tissue, while that in the mice treated with BLM were 6.012 ± 0.986 pg/mg skin tissue and that in the mice treated with plasmid–HVJ-liposomes and BLM was also 5.9815 ± 0.933 pg/mg skin tissue (Figure 5b). Immunohistochemically TGF-β1 was positive in both macrophage-like (round) and fibroblastic (spindle) cells in the dermis. The positive rate of TGF-β1 in both the macrophages-like and fibroblastic cells in the HGF-transfected mice decreased significantly in comparison to that in the positive control mice (Figure 5c, d). These data indicate that HGF transfection inhibits the expression and production of TGF-β1 from both macrophage-

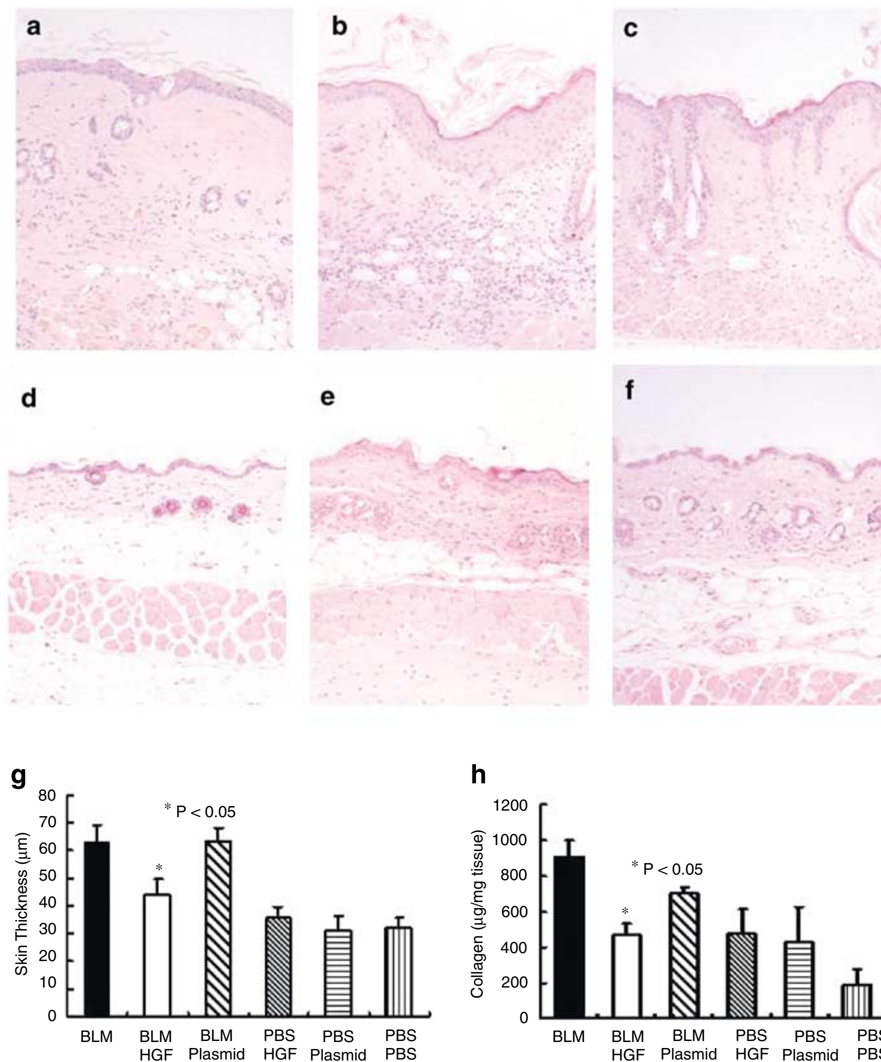

Figure 4 Effect of HGF on preventing BLM-induced scleroderma. Hematoxylin and eosin staining of skin from mice injected BLM with or without HGF-gene transduction with the induction of BLM-induced scleroderma. The mice were treated with (a) BLM for 4 weeks: ×100 (n=5); (b) BLM and HGF–HVJ-liposome simultaneously: ×100 (n=5); (c) BLM and plasmid vector liposome: ×100(n=4); (d) PBS: ×100 (n=3); (e) PBS and HGF–HVJ-liposome: X100 (n=3); (f) PBS and plasmid vector liposome: ×100. (n=3). Dermal thickness and collagen content of the skin in mice treated with bleomycin and/or HGF–HVJ-liposomes were examined. (g) The areas were calculated in arbitrary square units by outlining the dermis on a ×40 view for each microscopic image, in which length (μm) was the average thickness for a broad area of skin (n=4) *P < 0.05 compared to the mice treated with BLM. (h) Collagen content (μg/mg skin tissue) was examined by the SIRCIL collagen assay kit (n=4). *P<0.05 compared to the mice treated with BLM.

like and fibroblastic cells and that this inhibitory effect contributes to prevent the formation of dermal sclerosis.

Treatment of sclerotic skin by HGF-gene transfection

To examine whether HGF-gene transfection effectively prevents not only the development of sclerotic skin but also helps to improve the symptoms of dermal sclerosis, HGF gene was transfected twice with a 2-week interval after sclerotic skin was established by injecting BLM for 4 weeks (Figure 1b). The symptoms of dermal sclerosis induced by BLM injection improved even when human HGF transfection was performed at 4 weeks after the initial BLM injection (Figure 6a–f). The skin thickness of the mice treated with BLM alone was 39.5±3.75 μm and that of the mice injected with the plain plasmid was 39.7±3.0 μm, while that of the mice tranfected with HGF gene after the establishment of the sclerotic skin was reduced to 27.5±3.5 μm (Figure 6g). In line with these data, a histological examination also revealed that an accumulation of hyalinized collagen bundles in the dermis was observed in both the sclerotic skin induced by BLM and sclerotic skin treated with control plasmid liposomes (Figure 6a, c), but not in the sclerotic skin treated with HGF–HVJ-liposomes (Figure 6b). Collagen content of skin extract from BLM-induced sclerotic skin was 926.67±53.3 μg/mg tissue, while that in the skin extracted from the sclerotic skin treated with HGF–HVJ liposomes was 533.3±133 μg/mg tissue (Figure 6h). Although the degree of suppression in the skin thickness and collagen content by HGF treatment was relatively low in comparison to the results obtained regarding the prevention of sclerotic skin development, these data suggest that HGF transfection was able to reduce not only the skin thickness but also the collagen content in the skin even after the establishment of sclerotic skin (Figure 6g, h).

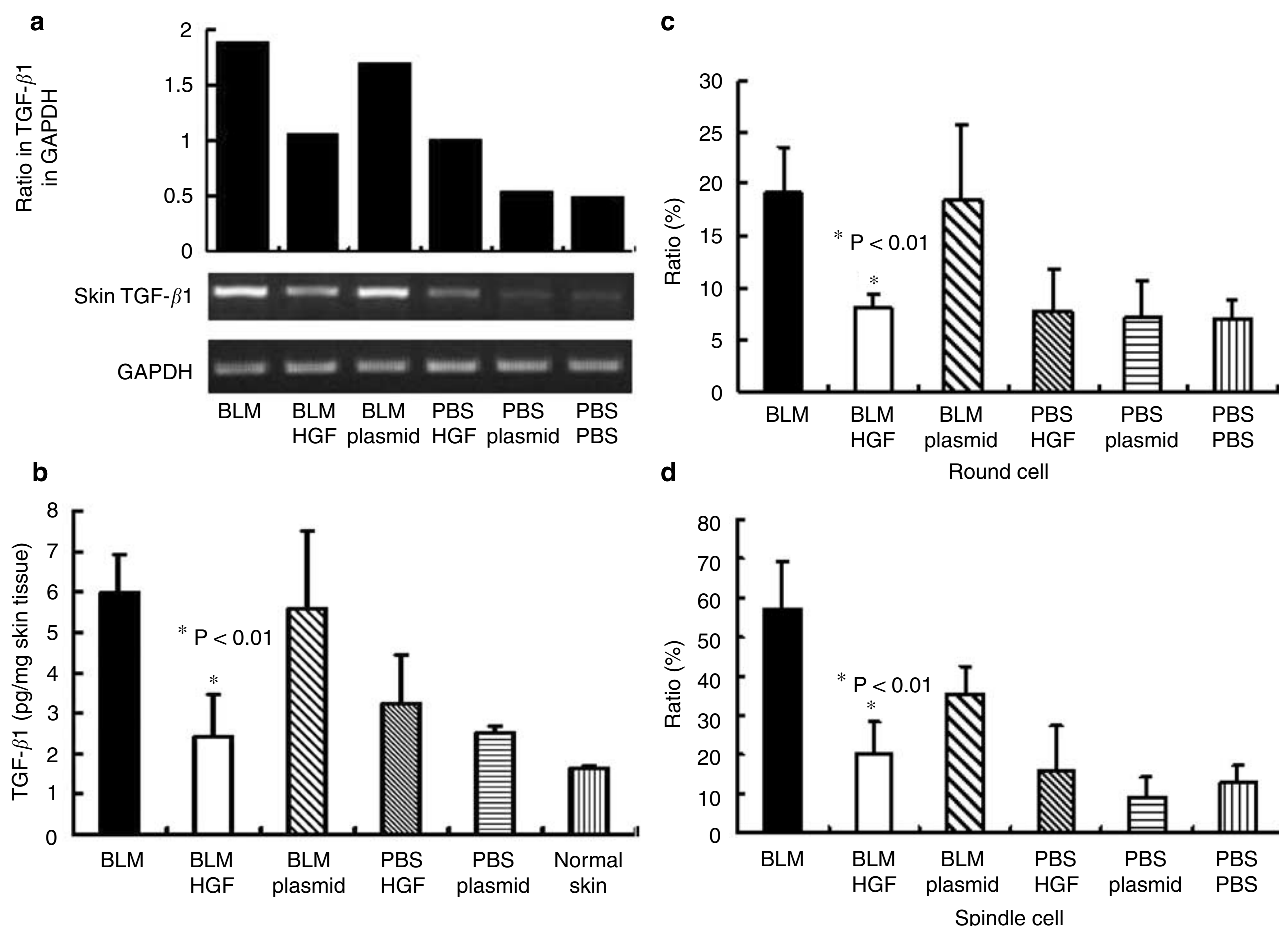

Figure 5 Decrease in TGF-β1 mRNA expression and protein production in mice treated with BLM and HGF–HVJ-liposomes. The mice received a subcutaneous injection of BLM or PBS and an intramuscular injection of HGF–HVJ-liposomes or PBS simultaneously, and 4 weeks after initial injection the back skin was removed. (a) Expression of TGF-β1 mRNA. Total RNA was isolated from the lesional skin, and cDNA was prepared for the detection of TGF-β1. Data were representative of four independent experiments. (b) Protein level of TGF-β1 in skin tissue. Total protein was extracted from the lesional skin, and protein level of TGF-β1 was measured by the ELISA method. The data are the means ±SD from four mice (*$P<0.01$ compared to the mice treated with BLM). The ratio of TGF-β1-positive cells (c: round cells, d: spindle cells) in the dermal tissue treated with BLM or PBS and HGF–HVJ-liposomes or PBS by immunostaining with anti-TGF-β1 antibody was shown. The number of cells was calculated in arbitrary square units by ×400 view microscopic image (×10 per mm^2 skin). *$P<0.01$ compared to the mice treated with BLM.

Expression of TGF-β1 in the established sclerotic skin

To clarify whether the modulation of TGF-β1 plays a major role in the treatment of sclerosis induced by HGF transfection in a scleroderma model mouse, we examined the expression of mRNA or the protein content of TGF-β1 in the skin. The TGF-β1 mRNA expression in the skin treated with both BLM and HGF–HVJ-liposomes decreased in comparison to TGF-β1 in the skin treated with BLM alone or treated with both BLM and control plasmid–HVJ-liposome (Figure 7a). The TGF-β1 protein content in the mice treated with both HGF–HVJ-liposomes and BLM were 1.448±0.463 pg/mg tissue, while that in the mice treated with BLM alone was 4.617±2.251 pg/mg tissue and that in the mice treated with plasmid–HVJ-liposomes and BLM was also 4.002±1.173 pg/mg skin tissue (Figure 7b). Positive immunolabeling for TGF-β1 was immunohistochemically detected in both macrophage-like and fibroblastic cells in the dermis. In the HGF-gene-transfected mice, the TGF-β1-positive ratio of macrophage-like but not fibroblastic cells in the dermis decreased in comparison to that in the dermis from the positive control mice (Figure 7c, d). These data suggest that HGF transfection improved the established sclerotic skin by inhibiting the expression and production of TGF-β1 from macrophage-like cells but not fibroblastic cells.

Prevention and treatment of BLM-induced lung fibrosis by HGF-gene transfection

To examine whether HGF-gene transfection is effective not only for BLM-induced scleroderma but also for BLM-induced lung fibrosis, HGF gene was transfected twice with a 2-week interval at the same time BLM was injected into skin for 4 weeks or twice with a 2-week interval after BLM was injected for 4 weeks (Figure1 a, b). A histological examination revealed that the lung fibrosis normally induced by subcutaneous BLM injection was also prevented and improved the symptoms after human HGF-gene transfection (Figure 8a–h). The numerical fibrotic score was also reduced by HGF-gene transfection regarding both the prevention and treatment of scleroderma (Figure 8i, j). We also determined whether the collagen content of the lung in HGF-transfected mice was also reduced or not. The collagen concentration of

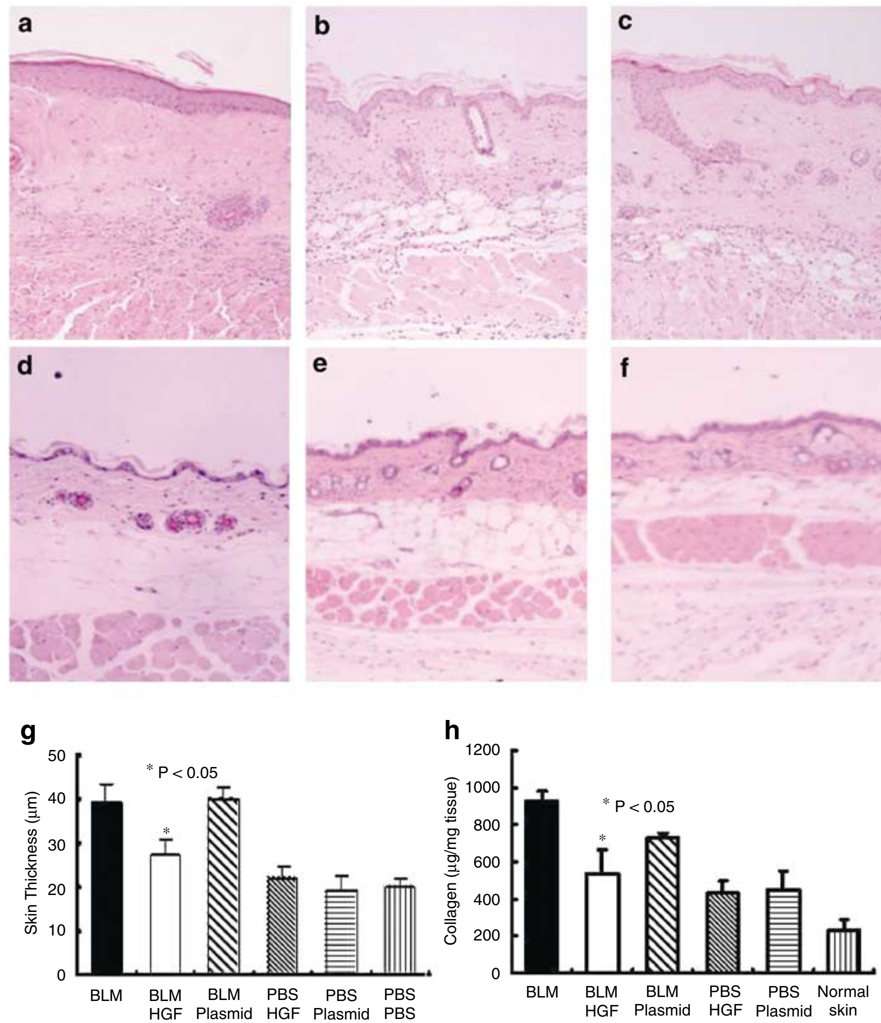

Figure 6 *Effect of HGF on BLM-induced scleroderma 4 weeks before HGF transfection. Hematoxylin and eosin staining of skin from mice injected BLM with or without HGF gene transduction simultaneously after the induction of BLM-induced scleroderma was conducted. (a) The mice were treated with BLM for 4 weeks: ×100 (n=5). (b) The mice were transfected with HGF gene after the the establishment of scleroderma by subcutaneous BLM injection: ×100 (n=5). (c) The mice received plasmid liposomes into skeletal muscle after the establishment of scleroderma by BLM injection subcutaneously: ×100 (n=4). (d). The mice were subcutaneously injected with PBS for 4 weeks: ×100 (n=3). (e) The mice received HGF–HVJ-liposomes into skeletal muscle after the injection of PBS for 4 weeks: ×100 (n=3). (f) The mice received normal plasmid liposomes after the injection of PBS for 4 weeks: ×100 (n=3). These data are representative of four independent experiments. Dermal thickness and collagen content of skin in mice pretreated with BLM and/or HGF–HVJ-liposome were determined. (g) Areas were calculated in arbitrary square units by outlining the dermis on a ×40 view for each microscopic image, in which length (μm) represent the average thickness for a broad area of skin. *P< 0.05 compared to the mice treated with BLM. (h) The collagen content (μg/mg skin tissue) was examined by the SIRCIL collagen assay kit. *P< 0.05 compared to the mice treated with BLM. The data are the means ±SD from four mice.*

lung extract from the HGF-gene-transfected BLM-induced lung fibrosis was lower than that from a positive control regarding both the prevention and treatment (data not shown). To clarify whether or not the modulation of TGF-β1 plays a major role in the prevention of fibrosis induced by HGF transfection in a scleroderma model mouse, we examined the protein content of TGF-β1 in the lung. The protein content of TGF-β1 in the lung treated with both BLM and HGF–HVJ-liposomes decreased in comparison to that of TGF-β1 in the lung treated with BLM or treated with both control plasmid–HVJ-liposome and BLM (data not shown). These data indicated that HGF-gene transfection prevented and improved both BLM-induced scleroderma and lung fibrosis, since HGF-gene transduction inhibited the production of TGF-β1 in the lung.

Discussion

Gene therapy offers great promise as a treatment for a large number of genetic and acquired diseases. The direct injection of DNA into skeletal muscle as a useful tool for delivering a protein to the systemic circulation is considered to be a major medical advance. Furthermore, this approach can be applied to living animals because of its simplicity, safety and lack of toxicity. The technical aspects of this method of gene transfer have now been widely described, but, to date, there have been few, if any, applications for directly treating a disease model.

In this study, HGF-gene transfection not only prevented the development of dermal sclerosis but also ameliorated the symptoms of established dermal sclerosis induced by repeated local injections of BLM for 4

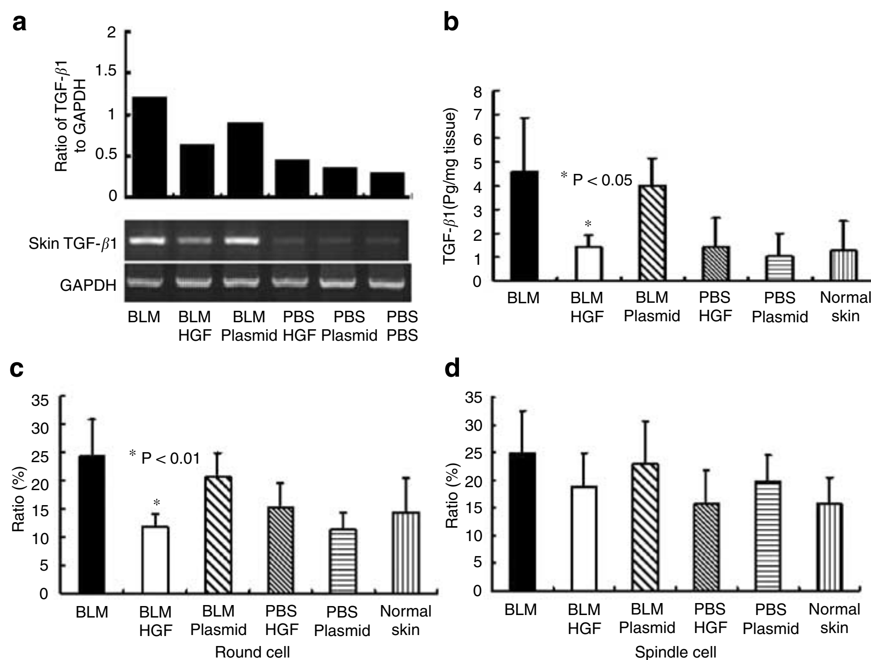

Figure 7 *Decrease of TGF-β1 mRNA expression and production by HGF-gene transfection in an established scleroderma model mice. The mice received an injection of plasmid vector liposomes or HGF–HVJ-liposomes into the skeletal muscle after the subcutaneous injection of PBS or BLM for 4 weeks. (a) The expression of TGF-β1 mRNA. Total RNA was isolated from the lesional skin, and cDNA was prepared for the detection of TGF-β1. The data were representative of four independent experiments. (b) Protein level of TGF-β1 in skin tissue. Total protein was extracted from the lesional skin, and the protein level of TGF-β1 was measured by ELISA method. *P< 0.05 compared to the mice treated with BLM. The data are the means ±SD from four mice(c.d). The ratio of TGF-β1-positive cells (c: round cells, d: spindle cells). The dermal tissue from mice treated with HGF–HVJ-liposomes or plasmid vector liposomes after the injection of PBS or BLM for 4 weeks were immunostained with anti-TGF-β1 antibody. Positive cells were calculated in arbitrary square units by ×400 view microscopic image (×10 per mm² skin). *P< 0.01 compared to the mice treated with BLM. The data are reprentative of four independent experiments.*

weeks. In our preliminary experiment, we demonstrated that recombinant HGF suppressed the induction of dermal sclerosis by BLM (data not shown). However, recombinant protein needs to be injected frequently because of their short half-life; moreover, the pharmacological dose of the recombinant protein required for effective treatment is extremely high. As a result, the administration of high-dose HGF is suspected to induce adverse effects, such as tumorigenesis in other organ. We thus introduced the transgene approach instead of injections of recombinant protein to develop a treatment modality for systemic sclerosis. The mRNA of HGF was expressed in both skeletal muscle, skin and lung tissue. A high level of human HGF protein (0.957 ng/ml) was detected in the serum even 2 weeks after the HGF–HVJ-liposome injections (Figure 2c). In a rat model of lethal liver cirrhosis, repeated transfections of human HGF gene induced a high plasma level of human HGF (0.05–0.1 ng/mg tissue), which was capable of stimulating alveolar epithelial DNA synthesis. They demonstrated that repeated transfection of the human HGF gene induced a high serum level of human as well as endogenous HGF.[24] In line with the finding of this report, endogenous murine HGF was also induced by human HGF-gene transfection in muscle, skin and lung. Our immunohistochemical analysis revealed a diffuse expression of HGF in the skin, lung and skeletal muscle. These data indicated that HGF proteins produced in skeletal muscle, skin and lung were delivered through the serum to other organs. The serum level of HGF detected in our mice (1.564 ng/ml) was thus able to stimulate fibroblasts and macrophages to control dermal sclerosis. Our results show that human HGF-gene transfer into the skeletal muscle, skin and lung of scleroderma model mice demonstrated a marked attenuation of sclerosis. The primary transfection of the human HGF is unknown; however, in line with the finding of another report,[24] the prime transfection site may be in the muscle since HGF–HVJ-liposomes were injected into the muscle. We supposed that both human and murine HGF play a major role in prevention of dermal sclerosis since Nakamura[16] demonstrated that the activity of human HGF is not species-specific in their original paper and Hayashi *et al*[25] also demonstrated that endogenously produced HGF by transfection of human HGF vector can exert autocrine and paracrine stimulatory effects on cell growth *in vitro* system. The increases in skin thickness and extracellular matrix accumulation, especially regarding the amount of collagen, were all reduced by HGF-gene transfection (Figure 4h). A histological examination revealed that HGF-gene transfer prevented not only dermal sclerosis (Figure 4b) but also lung fibrosis (Figure 8c, g). Pulmonary fibrosis should be a serious complication of SSc. If pulmonary fibrosis can be improved while treating SSc by HGF-gene therapy, then it would have a very beneficial effect. The other beneficial effect of HGF-gene transfer in this model cannot be explained only by its effect on disease induction (Figure 4). We found HGF-gene transfer to be equally therapeutic when administered after scleroderma had been established (Figure 6). HGF-gene transfer also reduced sclerosis in scleroderma model mice

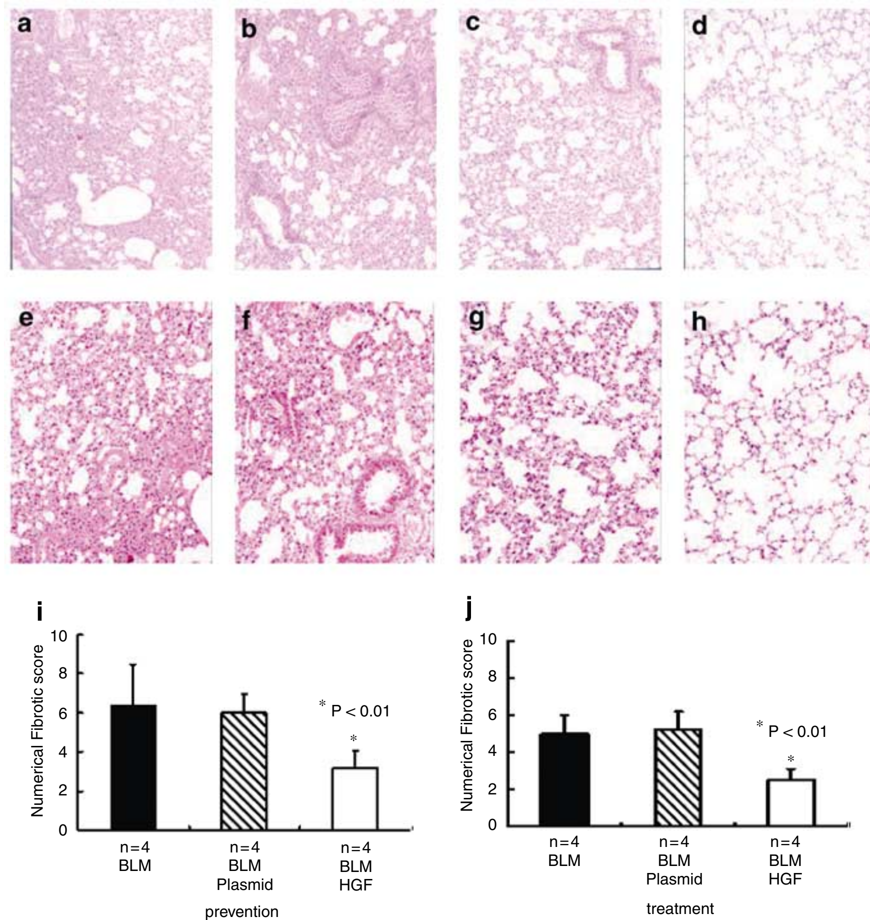

Figure 8 *Effect of HGF on BLM-induced lung fibrosis. H&E and staining of the lung from mice injected BLM with or without simultaneous HGF-gene transduction. The mice were treated with (a) BLM for 4 weeks: ×100 (n=5); (b) BLM and plasmid-HVJ-liposome simultaneously: ×100 (n=5); (c) BLM and HGF–HVJ-liposome simultaneously: ×100 (n=4); (d) PBS for 4 weeks: X100 (n=3); (e) BLM for 4 weeks and then injected PBS into skeletal muscle twice at 2- week interval: ×100 (n=3); (f) BLM for 4 weeks and then plasmid vector liposomes were subcutaneously injected twice at 2-week interval: ×100 (n=3); (g) BLM for 4 weeks and then injected HGF–HVJ-liposomes twice at 2-week interval: ×100 (n=4); (h) PBS for 8 weeks. An evaluation of the fibrotic change in the lung based on the numerical fibrotic score of lung in mice treated with BLM and/or HGF–HVJ-liposomes based on a numerical fibrotic scale (Ashcroft scale) was determined. (i) Prevention: The mice received a subcutaneous injection of BLM or PBS and an intramuscular injection of HGF–HVJ-liposomes or PBS simultaneously, and then 4 weeks after initial injection, the lung was removed and the specimens were fixed and prepared for H&E staining. The data are the means ±SD from four mice. *P< 0.01 compared to the mice treated with BLM. (j) Treatment: The mice received a subcutaneous injection of BLM or PBS for 4 weeks and after the establishment of sclerosis, the mice were injected HGF–HVJ-liposomes or PBS twice at a 2-week interval. Next, the lung was removed, fixed and prepared for H&E staining. The data are the means ±SD from four mice. *P< 0.01 compared to the mice treated with BLM.*

established sclerosis after the BLM treatment for 4 weeks (Figure 6).

To investigate how HGF-gene transfer effectively prevents and improves the symptoms of scleroderma, we examined the production and the expression of TGF-β1, since several report have demonstrated that TGF-β1 plays a major role in the induction of fibrosis and sclerosis.[11,14] In line with previous reports,[11–14,26] HGF-gene transfection inhibited the production of TGF-β1 from macrophage-like cells and fibroblastic cells. Although more infiltrated cells were detected in HGF-gene-transfected mice than in mice transfected with the control vector, the ratio of TGF-β1-expressed cells was less in HGF-gene-transfected mice. Reverse transcriptase-polymerase chain reaction (RT-PCR) and ELISA analyses revealed both mRNA and protein of TGF-β1 to be inhibited by HGF-gene transfection even after the establishment of scleroderma. Interestingly, the ratio of TGF-β1-positive fibroblastic cells was decreased by HGF-gene transfection in the prevention of dermal sclerosis but not in the treatment of dermal sclerosis. These results suggest that there exists another mechanism in the treatment of dermal sclerosis by HGF-gene transfection. The downregulation of TGF-β1 expression by HGF was also noted in rats with liver cirrhosis[24] and in a mouse model of chronic renal disease;[27] however, the precise mechanism of downregulation induced by HGF is not clear. Recently, the possibility that HGF may be involved in the prevention of apoptosis has been reported.[28–29] Matsumoto *et al*. have described HGF as a potent survival factor for pheochromocytoma cell line in culture.[30] As a result, HGF may have a protective effect on damaged cells through an anti-apoptotic mechanism. TGF-β1 is a very potent inducer of tissue fibrosis due to its chemotactic and stimulatory activities for matrix synthesizing cells. We previously reported that TGF-β1 mRNA and protein in the skin increased and peaked from 1 to 2 weeks after the administration of BLM.[11] The

timing of HGF-gene transfer in the prevention of scleroderma coincided with the time course of TGF-β1; however, the timing of HGF-gene transfer in the treatment of scleroderma was later than the peak for TGF-β1 mRNA expression. Another mechanism thus appears to be involved in the treatment of an established scleroderma model mouse by HGF-gene transfer since a large amount of collagen matrix exists in the established scleroderma. One possible mechanism may be metalloproteinase. Recently, HGF has been reported to increase the expression of metalloproteinase-9, and decrease the expression of the tissue inhibitors of matrix metalloproteinase-1 (TIMP-1) and TIMP-2 in chronic renal injury.[31] It is therefore important to analyze proteinase and proteinase inhibitor in the scleroderma model mouse treated by gene transfection in the near future.

Although further studies are needed to examine the effect of HGF on scleroderma, we emphasized the possibility that HGF-gene therapy may be one of the promising candidates for treating SSc and lung fibrosis involved in SSc. In addition, our observations also provide further insight into the cellular and molecular pathogenesis of SSc.

Materials and Methods

Animals

Female C3H/He mice of 8–12 weeks old were purchased from Sankyo Co. Lab. Service (Tokyo, Japan). They were maintained in a pathogen-free mouse facility at Tokyo Medical and Dental University and were cared for in accordance with the guidelines of the Tokyo Medical and Dental University.

BLM-induced scleroderma mice

The mice were treated with subcutaneous injections of 100 μl of 0.1 mg/ml. BLM (Nippon Kayaku Co. Ltd, Tokyo, Japan) every other days for 4 weeks.[11] The mice (n=46) were then killed by cervical dislocation on the day after the final treatment. The treated sites of truncal skin were harvested and divided into two pieces. Phosphate-buffered saline (PBS) was injected every other day subcutaneously into the control mice.

Expression vector and preparation of liposomes containing hemagglutinating virus of Japan (HGF–HVJ-liposome)

The expression vector and preparation of liposomes containing hemagglutinating virus of Japan (HGF–HVJ-liposome). Human HGF cDNA (2.2 kb) was inserted into the *Not*I sites of the pcDNA3.1(−)vector plasmid under the control of the cytomegalovirus (CMV) enhancer–promoter for a high level of expression.[32] HVJ-liposomes containing HGF DNA plasmid vector or control normal pcDNA3.1(−) plasmid vector were prepared as described previously.[23] Phosphatidylcholine, dioleoyophosphatidylethanolamine, sphingomyelin, phosphatidylserine and cholesterol were mixed in a molar ratio of 13.3:13.3:13.3:10:50 for anionic type HVJ-liposome. A dried lipid mixture was prepared and then hydrated in 200 μl of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing 200 μg of plasmid DNA. Liposomes were prepared by vortexing and extrusion. Purified HVJ (Zstrain) was inactivated by UV irradiation (198 mJ/mm^2) just before use. The liposome suspension (2 ml, containing 9.75 mg lipid) was incubated with HVJ (15 000 hemagglutinating units) for 10 min on ice and then for 1 h at 37°C with gentle shaking. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. HVJ-liposome was collected from the top of a 30% sucrose layer, filled up to 1 ml by BSS and stored at 4°C.[23]

Gene transfer into mouse skeletal muscle and the experimental design

HGF–HVJ-liposomes (HVJ-liposomes containing 10 μg of human HGF expression vector) were injected into the skeletal muscle twice with a 2-week interval. For a negative control, HVJ-liposomes containing normal pcDNA3.1 (−) plasmid vectors were injected (n=5).[33–34] These mice were killed by cervical dislocation next day after the final treatment. Thereafter, the serum, lung, skeletal muscle and back skin of the C3H/He mice were harvested. Each tissue specimen was divided into three groups, one group of tissues was fixed in 10% formalin solution and the other was snap-frozen with OCT compound (Miles Inc., Elkhart, IN, USA) in liquid nitrogen and stored at −80°C. The last group of tissues were frozen in liquid nitrogen and stored at −80°C for extraction.

Histopathology and morphometric analysis

The tissue specimens fixed in 10% formalin solution were embedded in paraffin. Sections of 5 μm thickness were prepared and processed for hematoxylin–eosin (H&E) staining. The numbers of infiltrated cells in the dermis were counted in an area of arbitrary square units at a x 400 magnification. A minimum of 20 areas was counted for each tissue specimen.

Immunohistochemical analysis

Cryostat sections of 5 μm thickness were prepared from snap-frozen tissues specimen of skin, lung and skeletal muscle mounted on poly-L-lysin-coated slides and fixed with cold acetone for 5 min. To block the endogenous peroxidase activity, the sections were treated with methanol containing 0.3% hydrogen peroxide for 15 min at room temperature and then were washed in PBS. The sections were stained according to a standard avidin–biotin peroxidase technique by using anti-human HGF antibody (1:100) (Genzyme, Cambridge, MA, USA). Isotype-matched mouse IgG antibody (Biogenesis, UK) was used as a control. To examine the transforming growth factor TGH-β1 activity in the cells, formalin-fixed sections were deparaffinized and stained with anti-TGF-β1 antibody (1:100) (R&D System, Minneapolis, MN, USA) by the alkaline phosphatase method using the DAKO LSAB kit.

Collagen content of sclerotic skin and lung

The 6-mm punch biopsied tissues and lung tissues were homogenized in 400 μl of sample buffer (10 mM Tris-HCl-buffered solution (pH 7.4) containing 2 M NaCl, 1 mM PMSF, 1 mM EDTA, and 0.01% Tween 80). A volume of 100 μl of the extract was utilized to assess the collagen concentration by using a SIRCIL collagen assay kit (Biocolar Ltd, N. Ireland, NI).

RT-PCR of human HGF and murine TGF-β1

Total RNA was extracted from the frozen tissue specimens using an RNAse mini kit (50) (Qiagen Company, Hilden, Germany) and then the specimens were processed according to the manufacturer's instructions. Total RNA was then diluted with sterile diethylpyrocarbonate-treated water and stored at −80°C until use. RT-PCR was performed using the DNA thermocycler (Program Temp control system, PC-700, ASTEC, Tokyo, Japan). Initially, 800 ng of total RNA in 18 μl of diethylpyrocarbonate-treated water was heated at 65°C for 5 min and cooled rapidly. After adding 4 μl of 5 × REV-2 Reverse Transcriptase Buffer (375 mM KCl, 250 mM Tris-HCl buffer, pH 8.3, 40 mM $MgCl_2$ and 50 mM DTT), 4 μl of 2.5 mM dNTP mixture (Takara, Tokyo, Japan), 4 μl of 10 × hexanucleotide mixture (Roche, Mannheim, Germany), and 80 U of Ribonuclease inhibitor (Takara), and 12 U of RAV-2 RT (Takara, Tokyo, Japan) up to 22 μl with DEPC H_2O. The mixture was incubated at 23°C for 10 min, at 42°C for 60 min, at 94°C for 5 min and quickly chilled on ice. The cDNA was amplified by PCR with the use of specific primers for human HGF, mouse TGF-β1, and GAPDH as described.[11,35] The upstream and downstream primers were as follows; HGF sense 5′TCCACAAGCAATCCAGAGGTACGC3′ antisense 5′GAGGGTCAAGAGTATAGCACCATG3′; TGF-β1 sense 5′TGGACCGCAACAACGCCATCTATGAGAAAACC3′, antisense 5′TGGAGCTGAAGCAATAGTTGGTATCCAGGGCT3′; GAPDH sense 5′TGAAGGTCGGAGTCAACGGA3′, antisense 5′GATGGCATGGACTGTGGTCA3′. The PCR conditions were optimized for each set of primers and PCR was performed using different numbers of cycles to ensure that the amplification occurred in a linear range. The PCR reaction mixture contained 5 μl of cDNA, 5 μl of 10 × PCR buffer, 4 μl of 2.5 mM dNTP, 2.5 μl of 20 pM 5′ and 3′ primers, and 1.5 U of *Taq* polymerase, distilled water (RNAse free) added to a total volume of 45 μl. The cycle number was 45 cycles for HGF, 37 cycles for TGF-β1 and 25 cycles for GAPDH, respectively. PCR amplification was performed at 94°C for 45 s, 70°C for 2 min and 72°C for 2 min for HGF primers, at 94°C for 45 s, 60°C for 1 min and 72°C for 2 min for TGF-β1 primers, and at 94°C for 45 s, 58°C for 1 min and 72°C for 2 min for GAPDH primers. After amplification, the PCR products were subjected to electrophoresis in 3% agarose gels for HGF or 1.5% agarose gels for TGF-β1 and GAPDH. The elecrophoresed bands were detected by ethidium bromide under a UV illuminator.

Determination of HGF and TGF-β1 levels by ELISA

Blood was drawn from the cavernous sinus, and centrifuged at 3000 r.p.m. for 15 min. Samples of TGF-β1 and human HGF ELISA for the tissue extract of muscle, skin and lung were prepared as follows. In brief, tissue specimens were excised and homogenized in 400 μl of the sample buffer (10 mM Tris-HCl-buffered solution (pH 7.4) containing 2 M NaCl, 1 mM PMSF, 1 mM EDTA and 0.01% Tween 80), centrifuged at 15 000 r.p.m for 30 min and then quickly frozen in liquid nitrogen. A volume of 100 μl of the sample was used to assess TGF-β1 protein by the ELISA method.[34] Latent TGF-β1 protein in sample was activated by 1 N HCl and 1.2 N NaOH/0.5 M HEPES, and total TGF-β1 protein were conducted using ELISA kits of TGF-β1 (TECHNE Corporation, Minneapolis, MN, USA). A volume of 50 μl of tissue extract was measured using enzyme-linked antibody specific human HGF Analysa immunoassay system kit (TECHNE Corporation, Minneapolis, MN, USA). Human antibody reacts with human HGF but not with mouse or rat HGF. Samples extracted by sample buffer for mouse HGF (Institute of Immunology, Tokyo, Japan) were assayed using the mouse HGF EIA kit (HGF EIA; Institute of Immunology, Tokyo, Japan). It was according to the manufacturer's instructions. In HGF ELISA, mouse or rat HGF is distinguishable from human HGF as the antibody reacts with mouse or rat HGF but not human HGF.[36]

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